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"This is not an Apple"–Yeast Mutualism in Codling Moth

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Abstract The larva of codling moth *Cydia pomonella* (Tortricidae, Lepidoptera) is known as the worm in the apple, mining the fruit for food. We here show that codling moth larvae are closely associated with yeasts of the genus *Metschnikowia*. Yeast is an essential part of the larval diet and further promotes larval survival by reducing the incidence of fungal infestations in the apple. Larval feeding, on the other hand, enables yeast proliferation on unripe fruit. Chemical, physiological and behavioral analyses demonstrate that codling moth senses and responds to yeast aroma. Female moths are attracted to fermenting yeast and lay more eggs on yeast-inoculated than on yeast-free apples. An olfactory response to yeast volatiles strongly suggests a contributing role of yeast in host finding, in addition to plant volatiles. Codling moth is a widely studied insect of worldwide economic importance, and it is noteworthy that its association with yeasts has gone unnoticed. Tripartite relationships between moths, plants, and microorganisms may, accordingly, be more widespread than previously thought. It, therefore, is important to study the impact of microorganisms on host plant ecology and their contribution to the signals that mediate host plant finding and recognition. A better comprehension of host volatile signatures also will facilitate further development of semiochemicals for sustainable insect control.

Keywords Plant-insect-microbe-interaction · Mutualism · Herbivory · Chemical communication · Semiochemicals · Tortricidae · Lepidoptera

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Introduction

"*Ceci n'est pas une pomme*" ("*This is not an apple*"), a painting by René Magritte, shows an apple, which is an incomplete description of an apple, as indicated by its title. It is reminiscent of our past attempts to study host plant interactions in codling moth *Cydia pomonella* (Lepidoptera, Tortricidae) and to identify the chemical cues that mediate codling moth attraction to apple, by studying apple volatiles only (Witzgall et al., 2008). We now show that yeast, found in codling moth larval galleries, is an essential part of the larval diet and that egg-laying females of codling moth sense and respond to yeast volatiles.

Associations between yeasts and insect herbivores are widespread, and these interspecific interactions play a crucial role in yeast and insect evolution (Ganter, 2006; Janson et al., 2008; Klepzig et al., 2009; Herrera et al., 2010). However, mainly bark beetles, ambrosia beetles, drosophilid

flies, and social insects have been studied (Starmer and Fogleman, 1986; Farrell et al., 2001; Mueller et al., 2005; Ganter, 2006; Hofstetter et al., 2006; Davis et al., 2012), and there are but a few records of yeasts that have been found in the frass of lepidopteran larvae (Lachance et al., 1988; Rosa et al., 1992; Molnar and Prillinger, 2005), including codling moth (Listemann, 1988).

The search for yeasts in codling moth larval galleries has been fueled by current studies of *Drosophila melanogaster*, showing that the flies mainly use microbial, and not plant cues, to locate feeding and oviposition sites (Becher et al., 2012). In addition, flies are thought to employ two distinct types of chemosensory receptors to find adult and larval food sources: ionotropic receptors (IRs) and odorant receptors (ORs). Odorant receptors are tuned to plant volatiles and probably evolved during the transition to terrestrial life, while the ancestral IRs sense water-soluble compounds including microbial volatiles (Benton et al., 2009; Croset et al., 2010).

Ionotropic receptors also have been identified recently in the antennal transcriptome of codling moth *C. pomonella* (Bengtsson et al., 2012). A typical IR-encoded odorant is acetic acid (Silbering et al., 2011), which is a key compound of *Drosophila* attraction to yeast (Becher et al., 2010; 2012). In codling moth, acetic acid synergizes attraction to green plant volatiles, which are only weak attractants by themselves (Landolt et al., 2007; Knight et al., 2011). This led us to hypothesize the role of microorganisms in codling moth host finding.

Methods and Materials

Yeast DNA Isolation and Taxonomic Identification Apples (cvs. Aroma, Discovery and Delicious) infested with codling moth larvae were collected in orchards around Alnarp (Scania, Sweden) and Yakima (WA, USA) in July and August of 2011. Larvae (N=25) walked under sterile conditions for 5 min on YPD agar plates (20 g/L peptone, 20 g/L glucose, 10 g/L yeast extract, 20 g/L agar). In addition, larvae (N=12) were surface-sterilized by rinsing in 10 % NaOCl for 10 sec, followed by a rinse in 70 % ethanol solution and in autoclaved distilled water. Larvae then were cut open with a sterile razor blade under a sterile fume hood. A sterilized inoculating loop was used to gather the guts, and this was streaked onto a YPD agar plate. Three typical isolates were selected for taxonomic identification. Apple flowers (May) were dipped directly onto YPD plates (N=10) and undamaged apples (July) were wiped with an inoculating swab (N=10; Alnarp). Plates were incubated at 25 °C for 3 day and emerging colonies of microorganisms were re-isolated up to six times on fresh YPD plates to obtain pure colonies.

Yeast isolates were grown overnight in liquid YPD medium in an incubating shaker at 25 °C. The cells were spun down (10,060 g, 2 min) and washed with water. 200 μl of the lysis buffer (2 % Triton X-100, 1 % SDS, 0.1 M NaCl, 10 mM Tris, 1 mM EDTA, pH 8), 200 μl of a 25:24:1-blend of phenol, chloroform, and isoamyl alcohol, and 100 μL acid-washed glass beads were added to the pellet. The mix was vortexed for 10 min and 200 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) were added. The suspension was centrifuged for 10 min at 10,060 g and 10 μl RNase A (10 mg/ml) were added to the aqueous phase and incubated for 45 min at 37 °C. The DNA was precipitated with 1 ml 96 % ice-cold ethanol and 1/10 volume of 3 M sodium acetate. The mixture was centrifuged for 10 min at 10,060 g at 4 °C. The pellet was washed with ice-cold 70 % ethanol, air-dried and resuspended in 40 μl TE buffer (pH 8). Concentration of genomic DNA was measured on NanoDrop.

The internally transcribed spacer (ITS) region of rDNA and the D1/D2 domains of large ribosomal subunit (LSU) were amplified by using ITS1/ITS4 and NL1/NL4 primer pairs, respectively (White et al., 1990; Kurtzman and Robnett, 2003). The polymerase chain reaction was run under the following conditions: 94 °C, 5 min followed by 30 cycles of 95 °C for 30 s, 50 °C for 45 s, and 72 °C for 45 s with the extension step of 72 °C for 10 min. The PCR products were purified using GeneJET PCR Purification kit (Fermentas, cat. no K0702) and sequenced using PCR primers by MWG Operon (Ebersberg, Germany). The sequences were identified by comparison to GenBank database of non-redundant sequences using BLAST (Altschul et al., 1990). Sequences were determined with an ABI 3730 gene analyzer.

Larval Feeding Assays Unripe apples (cv. Delicious) from an unsprayed orchard (Moxee, WA, USA) were sterilized in 5 % NaOH for 30 min, and then consecutively rinsed and dried, with 70 % EtOH and autoclaved distilled water. *Metschnikowia andauensis* was grown on YPD medium. Half of the sterilized apples were dipped five times into a 500-ml solution of yeast and placed on a paper towel in a fume hood to dry. A gelatin capsule was attached with paraffin on the shoulder of each apple. One end of the capsule was cut-off with a razor blade, and the second part of the capsule was then slid over the cut end. One sterilized black-headed codling moth egg on a <15 mm^2 piece of wax paper was placed inside each gelatin capsule and touching the fruit. Apples were placed inside 350-ml clear plastic cups closed with a lid. Cups were placed in a room maintained at 22 to 25 °C for 35 day. Assays were conducted on five dates with 10 apples per treatment.

After 35 day, each apple was cut in half to expose the feeding galleries, and larvae were scored as alive or dead. A sterilized inoculating metal loop was used to swab the larval gallery and to inoculate a YPD agar plate. Two swipes were made from each gallery. Sub-samples also were collected from the skin of the apple enclosed inside the gelatin capsule, the

apple skin outside the capsule, the calyx of the fruit, and from the seed area of uninfested fruit. Plates were incubated for 2 day, followed by a visual determination of the presence of yeast colonies based on macromorphology.

Oviposition and Upwind Attraction Behavioral Assays Codling moths were reared on an artificial wheat germ-based diet. Adults were kept in 33×33×33-cm Plexiglass cages under a 16:8 L:D photoperiod (65±5 % RH, 22 °C). Females were mated 1 day after eclosion and used for wind tunnel and oviposition experiments on the following day. Adults used for behavioral tests were negative for the presence of *Metschnikowia* yeasts, according to females that were allowed to walk for 5 min on YPD agar plates (N=25).

Single mated females were placed in Plexiglass cages containing 2 apples in the center, spaced at 15 cm. Green apples (cv. Discovery) were sterilized (see above). A hole (ca. 2 mm×3 cm) was punched into the apples (control), and one *M. andauensis* colony from an agar plate was thoroughly spread in this cavity, using a Pasteur pipette (yeast treatment). In a second test, apples infested with codling moth larvae (3rd to 5th instar) were placed side by side with undamaged apples. The number of eggs laid on the apples was counted after 48 h. Presence of yeasts in the larval galleries was verified after the test.

Females were flown in a wind tunnel (180×90×60 cm), illuminated from above at 2–3 lx (Witzgall et al., 2001). Incoming air (30 cm/s, 22 to 24 °C, 50 to 60 % RH) was filtered with active charcoal. Insects were placed in 2.5×12.5-cm glass tubes closed with gauze and kept in the wind tunnel room for 1 h before testing. They were tested individually between 1 and 4 h after onset of the scotophase, in five batches of 10, and the following types of behavior were recorded: flight initiation (take-off from holding tube) and upwind flights over 120 cm towards the odor source. The odor source was liquid minimal growth medium (Merico et al., 2007) inoculated with *M. andauensis* 18 h before onset of the tests. A gentle charcoal-filtered airstream (0.1 L/min) passed through the medium in a wash bottle and was led through a teflon tube (6 mm ID) into the wind tunnel. Control tests were done with growth medium without yeast.

Collection of Fermentation Volatiles and Chemical Analysis Fermentation of *M. andauensis* and *M. pulcherrima*, respectively, in 1 L minimal growth medium (Merico et al., 2007) was done in bioreactors (Multifors/Infors HT 1.4 L, Bottmingen, Switzerland) set to 25 °C, pH 5 and an airflow of 1 L/min, maintaining the dissolved oxygen concentration at >30 % of saturation. Headspace was collected between 24 and 48 h after inoculation with air filters (Super Q, 80/100 mesh; Alltech, Deerfield, IL, USA; Bengtsson et al., 2001). The filters were eluted with 300 μl of hexane, and the filter extracts were stored in glass capillary tubes.

Samples were analyzed on a combined gas chromatograph and mass spectrometer (GC-MS; 6890 GC and 5975 MS, Agilent Technologies, Palo Alto, CA, USA), operated in splitless injection and electron impact (EI) ionization mode at 70 eV. The GC was equipped with fused silica capillary columns (30 m×0.25 mm, df=0.25 μm), DB-Wax (J&W Scientific, Folsom, CA, USA) or HP-5MS (Agilent Technologies). Helium was used as the mobile phase at an average linear flow rate of 35 cm/s. Two μl of each sample were injected (splitless mode, 30 sec, injector temperature 225 °C). The GC oven temperature was programmed from 30 °C (3 min hold) at 8 °C/min to 225 °C (5 min hold). Compounds were identified according to their retention times (Kovat's index) and mass spectra, using a NIST library (Agilent) and with authentic standards.

Electrophysiology Antennal responses of *C. pomonella* females to yeast volatiles were studied by combined GC and electroantennographic detection (GC-EAD), using an Agilent 6890 GC and an electroantennogram (EAG) apparatus (IDAC-2; Syntech, Kirchzarten, Germany) for data acquisition. The GC columns and the temperature programs were the same as for the GC-MS analysis. Hydrogen was used as the mobile phase at an average linear flow of 45 cm/s. At the GC effluent, 4 psi of nitrogen were added and split 1:1 in a Gerstel 3D/2 low dead volume four-way-cross (Gerstel, Mülheim, Germany) between the flame ionization detector (FID) and the EAD. The GC effluent capillary for the EAD passed through a Gerstel ODP-3 transfer line, that tracked GC oven temperature, into a glass tube (30 cm×8 mm), where it was mixed with charcoal-filtered humidified air (18–20 °C, 50 cm/s). The antenna was placed 0.5 cm from the outlet of this tube.

Two- to 3-d-old mated females were used for antennal recordings. The antenna was cut at the base and inserted into the recording glass electrode filled with Beadle-Ephrussi Ringer and connected to a pre-amplifier probe connected to a high impedance DC amplifier interface box (IDAC-2; Syntech). The reference electrode was connected to the antennal tip after cutting the distal segment.

Results

Occurrence of Yeasts in Codling Moth Larval Galleries Figure 1 shows a fresh and a deserted codling moth *C. pomonella* larval gallery. All larvae extracted from field-collected apples in Sweden carried two yeasts, which were identified from ITS and D1/D2 rRNA gene sequence analysis as *Metschnikowia andauensis* and *M. pulcherrima* (Ascomycota, Saccharomycetes). *Metschnikowia pulcherrima* was also found in the guts of codling moth larvae that had been collected in Washington orchards, USA. In Sweden, *M. andauensis* and *M. pulcherrima* were found in

Fig. 1 Two codling moth *Cydia pomonella* galleries, deserted (*left*) and with larva (*right*). The larva does not range the entire apple, but builds voluminous galleries that contain *Metschnikowia* yeasts. Larval galleries, fresh and abandoned, become rarely infested with fungi

all apple flowers samples, and *M. andauensis* also was present in few skin samples of undamaged apples.

Species identification is tentative because there are relatively few nucleotide differences between species of the *M. pulcherrima* clade (Lachance, 2011), and some nucleotides in the divergent regions of ITS and D1/D2 were poorly resolved, suggesting that the isolates may be hybrids.

Occurrence of other microorganisms was not consistent, these were not found on all larvae and there was no overlap between Sweden and USA. These species included the yeast *Cryptococcus tephrensis* (Basidiomycota, Tremellomycetes) and the yeast-like fungus *Aureobasidium pullulans* (Ascomycota, Sordariomycetes) in Washington and the bacterium *Pantoea agglomerans* (Proteobacteria, Enterobacteriales) in Sweden.

In the laboratory, galleries of codling moth larvae feeding on sterilized apples did not contain yeasts. In stark contrast, all feeding galleries in apples that were dipped in yeast prior to larval infestation were positive for yeast ($N=50$). The skin of apples dipped in yeast broth was positive for yeast after 35 day, but the interior cavity of yeast-treated fruits without codling moth larvae was negative for yeasts.

Effect of Yeast on Codling Moth Larval Development Presence of *M. andauensis* yeast in larval galleries strongly synergized codling moth development (Fig. 2a). Larval mortality was significantly lower on sterilized apples that had been dipped into a yeast broth than on sterilized apples without yeast; larval development was accelerated in the presence of yeast and more larvae pupated; and yeast decreased the incidence of mold during a test period of 35 day ($P<0.05$, $P<0.005$ and $P<0.001$, respectively; Fisher's exact test; Fig. 2a). Without yeast treatment, mold was growing inside all apples that contained dead larvae. In the field, even deserted feeding galleries rarely become infested with molds (see also Fig. 1).

Attraction and Egg-Laying Response to Yeast Volatiles Since *M. andauensis* enhanced larval survival on apples, we investigated the behavior of mated codling moth females in response to this yeast. In a dual choice test (Fig. 2b), females laid more eggs on sterilized apples when a mechanically produced cavity was inoculated with *M. andauensis* ($P<0.05$, $t=2.562$, $df=58$; paired t-test). The difference was more pronounced when egg-laying females had the choice between control (mechanical injury alone) and an apple infested with a codling moth larva ($P<0.001$, $t=4.215$, $df=55$; paired t-test; Fig. 2b). Yeast inoculated into mechanically injured apple did not proliferate in the absence of larvae since the injury dried out after a few days.

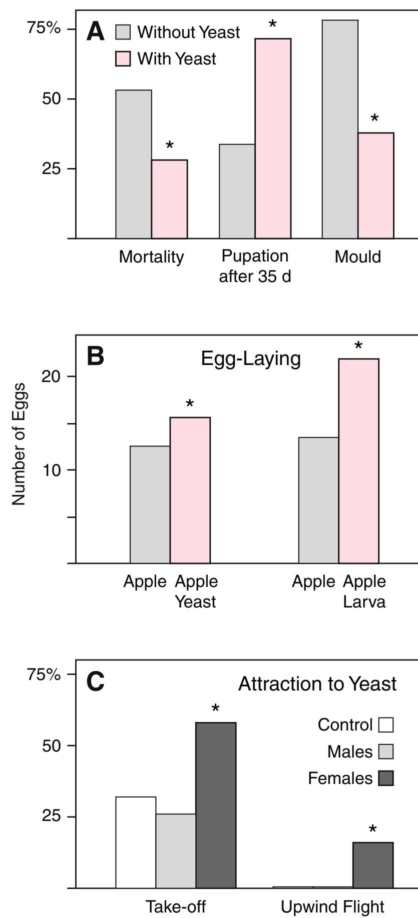

Fig. 2 **a** Codling moth *Cydia pomonella* larval mortality, larval development (number of larvae and pupae) and incidence of mould in larval galleries, during 35 day on sterilized apples, that were inoculated with yeast or left untreated ($N=50$). **b** Number of eggs laid on sterilized apples, with and without yeast inoculation (*left*) and on undamaged apples and apples infested with codling moth larvae ($N=50$). **c** Wind tunnel response of codling moth *C. pomonella* males and females ($N=90$) to *Metschnikowia andauensis* volatiles and to minimal growth medium. *Asterisks* show significant differences at $P<0.05$

The increased oviposition response was obviously due to additional yeast volatiles emanating from the apple. In order to corroborate the behavioral effect of yeast volatiles, we investigated the behavioral effect of *M. andauensis* headspace in a flight tunnel (Fig. 2c). Significantly more females

than males became activated and left the holding cage by flight in response to yeast volatiles ($P<0.05$, $t=3.051$, $df=8$; *t*-test) and only females flew towards the source. However, the females did not closely approach or land on the odor outlet. Minimal medium alone did not elicit an upwind flight response ($P<0.05$; Fisher's exact test; Fig. 2c).

Yeast Volatile Analysis The fermentation headspace of *M. andauensis* and *M. pulcherrima* on minimal medium contained 19 compounds that produced an antennal response in codling moth females. The yeasts produce volatile blends of similar composition, in different proportions (Table 1). The compounds, including phenols and terpenoids, were produced *de novo* from precursors in the minimal growth medium. Most of them have been identified from yeasts and other microorganisms (Swiegers et al., 2005; Schulz and Dickschat, 2007; Hernandez-Orte et al., 2008).

Table 1 Headspace volatiles from *Metschnikowia andauensis* and *M. pulcherrima* yeasts on minimal growth medium, 24 to 48 h after inoculation (mean relative proportion; $N=3$) that elicited an antennal response in codling moth *C. pomonella* female antennae ($N=6$)

Compound [a]	CAS [b]	KI [c]	*M. andauensis* 24–48 h	*M. pulcherrima* 24–48 h
Isobutyl acetate*	110-19-0	1004	tr [d]	– [e]
Butylacetate	123-86-4	1062	0.1±01	0.2±0.1
Isoamyl acetate*	123-92-2	1112	20.6±22.7	4.5±2.9
n-amyl acetate*	628-63-7	1165	0.3±0.2	0.9±0.4
2-heptanone*	110-43-0	1172	1.4±1.6	4.9±0.9
3-methyl-3-butenol*	763-32-6	1238	4.8±3.7	16.1±11.8
2-octanone	111-13-7	1275	–	0.7±0.2
Butyl-isothiocyanate	592-82-5	1306	1.5±2.3	0.1±0.1
Sulcatone*	110-93-0	1326	0.6±0.5	0.4±0.5
2-nonanone*	821-55-6	1380	0.3±0.4	3±0.3
Nonanal*	124-19-6	1385	7.0±5.2	0.8±0.2
Unknown		1620	0.1±0.1	22±0.9
Phenyl acetaldehyde*	122-78-13	1629	0.1±01	0.1±0.1
Unknown		1680	16.3±16.9	44±2.6
Geranial	141-27-5	1722	tr	–
Citronellol*	106-22-9	1755	0.9±0.4	1±0.1
2-phenylethyl acetate*	103-45-7	1804	0.5±0.7	–
Geranylacetone*	3796-70-1	1845	0.5±0.2	–
2-phenylethanol*	60-12-8	1896	43.1±26.7	1.4±1.6

[a] Compounds marked with an asterisk were identified according to retention times on two columns (DB-Wax, HP-5) and mass spectra in comparison with synthetic standards. The other compounds were identified according to mass spectra using a NIST library and published Kovats retention indices (KI). All compounds listed were absent in control fermentations ($N=3$), using minimal growth medium alone

[b] Chemical abstracts service (CAS) registry number

[c] Kovats retention index

[d] Trace amounts

[e] Not detected

Discussion

Codling Moth Larvae are Associated with Metschnikowia Yeasts All larvae of codling moth *C. pomonella* collected in apple orchards carried *Metschnikowia* yeasts, and the results of our study suggest a mutualistic interaction between codling moth and these yeasts. Two species, *M. andauensis* and *M. pulcherrima*, were found in Sweden, and one of them, *M. pulcherrima,* also was found in Washington, USA. Yeasts were present in larval galleries and in the alimentary tract of codling moth larvae.

Larval feeding sustained yeast growth on apple, since yeast inoculated into mechanically injured apple did not proliferate in the absence of larvae. Feeding assays in the laboratory demonstrated that *M. andauensis* had a beneficial effect on codling moth larvae, by accelerating development and by reducing mortality. Yeast not only contributed to the larval diet, it also reduced the incidence of detrimental fungal infestations (Fig. 2a), *Metschnikowia* yeasts are efficient biocontrol agents of fungal fruit diseases (Kurtzman and Droby, 2001; Spadaro et al., 2008; Manso and Nunes, 2011).

An open question is whether codling moth larvae feeding on fruits other than apple also are accompanied by *Metschnikowia* yeasts. Other microorganisms were also found on some codling moth larvae in apple, but their occurrence was less consistent. This compares to bark beetles, which live in association with a consortium of microorganisms, including yeasts and other fungi (Davis et al., 2011).

The association of *M. pulcherrima* and codling moth is not new to literature (Listemann, 1988), and *M. pulcherrima* commonly occurs in apple (Bowen and Beech, 1964; Pelliccia et al., 2011). *Metschnikowia pulcherrima* also forms a symbiosis with the lacewing *Chrysoperla rufilabris*, where it is consistently found in the gut of adult insects (Woolfolk and Inglis, 2004). *Metschnikowia andauensis* and related species also have been found in larval excrements of European corn borer and corn earworm moths (Molnar and Prillinger, 2005). Other *Metschnikowia* yeasts are found with beetles from several families, lacewings, and drosophilid flies (Ganter, 2006; Nguyen et al., 2006; Yaman and Radek, 2008).

Several *Metschnikowia* yeasts associated with flowers are vectored by non-pollinating insects, especially nitidulid beetles and drosophilid flies. Both the formation of needle-shaped ascospores (Lachance et al., 2001, 2005) and the production of volatiles that attract insects (Landolt et al.,

2006; El-Sayed, 2012), which are also perceived by codling moth antennae (Table 1), are indicative of a close evolutionary relationships between *Metschnikowia* yeasts and insects.

Drosophila-yeast interactions are viewed as mutualistic (Starmer and Fogleman, 1986): yeasts provide an essential part of the larval diet (Nasir and Noda, 2003; Becher et al., 2012) and the flies promote long-distance dispersal of the yeast to suitable substrates, which increases the opportunity for outbreeding (Reuter et al., 2007). Mutualistic interspecific interaction eventually gives rise to interspecific communication: the sophistication of yeast-to-insect chemical communication is highlighted by a yeast infesting honey bee pollen, which mimics honey bee alarm pheromone, to attract a hive beetle. The beetle facilitates yeast proliferation inside, and dispersal outside bee colonies (Torto et al., 2007).

Yeast Volatiles Mediate Codling Moth Behavior Yeast fermentation headspace elicited upwind orientation flight behavior in codling moth females, which laid more eggs on apples inoculated with yeast (Fig. 1b, c). This points towards a contributing role of yeast volatiles in finding larval host plants. Both grapevine moth *Lobesia botrana*, a typical herbivore, and the fruit fly *D. melanogaster* show a strong attraction response and oviposition preference for yeast-infested grapes (Tasin et al., 2011; Becher et al., 2012). The flight response of codling females to yeast in the wind tunnel (Fig. 1c) compares to the flight response obtained with apples that provide odor and also visual cues (Reed and Landolt, 2002). In contrast, by using an invisible odor source, we did not manage to observe upwind flights in codling moth females towards apple volatiles in earlier experiments (e.g., Ansebo et al., 2004; Coracini et al., 2004).

Fermentation volatiles may, in addition to larval host plants, also signal adult food sources. Authentic and synthetic yeast odor blends attract a great many moths including codling moth (Dethier, 1947; El-Sayed et al., 2005; Landolt et al., 2011); these moths use many different host plants, and are accordingly not attracted by the same chemical cues for egg-laying. Even floral insect attractants, including 2-phenylethanol or phenyl acetaldehyde (Landolt et al., 2006; El-Sayed et al., 2008) are typical yeast odors (Table 1; Hernandez-Orte et al., 2008).

Another consequence of insect attraction to yeast is yeast transmission. In *C. rufilabris* lacewings, which are associated with *M. pulcherrima*, adults acquire the yeast from the environment, as there is no evidence of vertical transmission (Woolfolk and Inglis, 2004). This explains lacewing attraction to yeast volatiles (Table 1; Toth et al., 2009). *Metschnikowia* yeasts frequently occur in nectar (Pozo et al., 2011), and we found *M. andauensis* and *M. pulcherrima* in apple flowers. The onset of the first codling moth flight is tightly correlated with apple flowering (Bradley et al., 1979), which would provide a source of yeast inoculum.

Plant-derived volatiles known to attract codling moth females, such as pear ester, β-farnesene, or (*E*)-4,8-dimethyl-1,3,7-nonatriene are synergized by acetic acid (Landolt et al., 2007; Knight et al., 2011), which is a typical yeast metabolite. Acetic acid was not produced by *Metschnikowia* growing on minimal medium (Table 1), but a range of other *Metschnikowia* volatiles were perceived by codling moth female antenna, including compounds that are behaviorally active in other insects, for example nonanal, phenyl acetaldehyde, or 2-phenylethanol (Table 1; El-Sayed, 2012). It is still unclear which plant and yeast signals mediate codling moth host finding and oviposition, and which of the bioactive compounds are perceived via the OR and IR olfactory subsystems (Silbering et al., 2011; Bengtsson et al., 2012).

Attractant signals from yeasts are a resource for the behavioral manipulation of egg-laying female moths, since this communication channel is chemically distinct from the pervading background of green plant volatiles. This background odor interferes with field attraction of moths to plant volatiles (Knudsen et al., 2008), which is an obstacle for their widespread practical use (Coracini et al., 2004; Loeb et al., 2011; Knight and Light, 2012). Codling moth is controlled by pheromone-mediated mating disruption on 200.000 ha worldwide, but insecticide sprays are still a necessity at high population densities. Development of powerful attractants for reliable monitoring or for mass-trapping of codling moth females would greatly promote areawide use of pheromone-based control (Witzgall et al., 2010).

Microbial Mutualism and Evolutionary Diversification of Insect Herbivores The diversification of several phytophagous insect lineages has been linked with associated microorganisms and their effect on host plant ecology (Janson et al., 2008). *Metschnikowia* yeast clearly enhanced codling moth larval development, by providing nutritional services and by reducing adverse fungal infestations (Fig. 2a). Yeast may accordingly facilitate feeding on apple of different phenological stages, from unripe to fully grown mature apples, and may possibly contribute to colonization of other, taxonomically unrelated hosts, such as walnut.

Another important effect of microorganisms on insect-plant interactions is that they contribute with behaviorally active compounds to the bouquet of plants. Host shifts, which essentially contribute to the evolutionary diversification of insect herbivores (Fordyce, 2010), are probably to a large extent mediated by the chemical similarity between old and new hosts (Dres and Mallet, 2002; Smadja and Butlin, 2009). A showcase example of such a chemosensory-mediated host plant shift is the apple maggot *Rhagoletis pomonella* (Diptera: Tephritidae) sibling species complex, where the compounds that encode recognition and host finding have been identified by chemical analysis and behavioral studies (Linn et al., 2003; 2005a, b). Interestingly, several of the compounds that mediate

attraction of *Rhagoletis* fruit flies to fruit of their respective hosts apple, hawthorn, and flowering dogwood are known to be produced by fungi and yeasts, such as isoamyl acetate, ethyl acetate, 3-methylbutan-1-ol, and 1-octen-3-ol (e.g., Swiegers et al., 2005; Schulz and Dickschat, 2007; Hernandez-Orte et al., 2008). Yeasts or other microorganisms may facilitate host shifts by increasing the chemical and nutritional similarity between plants.

Our discovery that yeast metabolites contribute to host finding behavior in a typically herbivorous moth has consequences for ongoing research on how moths recognize and find their hosts. The interaction between yeast and plant volatiles in host finding behavior, including the coding of IR and OR ligands by the insect olfactory system and the identification of behaviorally active compounds, is a current research challenge. The knowledge generated by this work will facilitate the further development of environmentally safe semiochemicals for population monitoring and control of codling moth and other insects.

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Synergistic Inhibition of the Lethal Fungal Pathogen *Batrachochytrium dendrobatidis*: The Combined Effect of Symbiotic Bacterial Metabolites and Antimicrobial Peptides of the Frog *Rana muscosa*

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Abstract A powerful mechanism for protection against disease in animals is synergy between metabolites present in the natural microbiota of the host and antimicrobial peptides (AMPs) produced by the host. We studied this method of protection in amphibians in regard to the lethal disease chytridiomycosis, which is caused by *Batrachochytrium dendrobatidis* (*Bd*). In this study, we show that the AMPs of *Rana muscosa*, as well as the metabolite 2,4-diacetylphloroglucinol (2,4-DAPG) from *Pseudomonas fluorescens*, a bacterial species normally found on the skin of *R. muscosa*, were inhibitory to the growth of *Bd in vitro*. When both AMPs and 2,4-DAPG were used in growth inhibition assays, they worked synergistically to inhibit the growth of *Bd*. This synergy resulted in reduced minimum concentrations necessary for inhibition by either 2,4-DAPG or AMPs. This inhibitory concentration of AMPs did not inhibit the growth of a *P. fluorescens* strain that produced 2,4-DAPG *in vitro*, although its growth was inhibited at higher peptide concentrations. These data suggest that the AMPs secreted onto frog skin and the metabolites secreted by the resident beneficial bacteria may work synergistically to enhance protection against *Bd* infection on amphibian skin. These results may aid conservation efforts to augment amphibian skins' resistance to chytridiomycosis by introducing anti-*Bd* bacterial species that work synergistically with amphibian AMPs.

Keywords *Batrachochytrium dendrobatidis* · Antimicrobial peptides · Metabolites · *Pseudomonas fluorescens* · Synergy · 2,4-diacetylphloroglucinol · Chytridiomycosis · Amphibian conservation · Probiotic

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Introduction

Amphibian species around the world are undergoing dramatic population declines and local extinctions, and it is estimated that 40 % of amphibian species are vulnerable to extinctions (Simon et al., 2004; Hoffman et al., 2010). Although habitat loss and fragmentation is one critical cause of these declines (Collins and Storfer, 2003), population losses in pristine habitats in Australia, Central and South America, and the western United States have been linked to the emergence of a lethal fungal skin pathogen, *Batrachochytrium dendrobatidis* (*Bd*) (Berger et al., 1998; Weldon et al., 2004; Lips et al., 2006). *Batrachochytrium dendrobatidis* is spread by its waterborne zoospores and colonizes the keratinized layer of juvenile and adult amphibian skins, where it matures into a zoosporangium that is capable of producing new zoospores that can either infect a second host or re-infect the same host (Berger et al., 2005). The resulting disease, chytridiomycosis, is fatal to many, but not all, amphibian species (Berger et al., 1998; Woodhams et al., 2005, 2006, 2007a; Lips et al., 2006). The

factors that determine resistance to chytridiomycosis in certain amphibian species are not fully understood. Chytridiomycosis is currently regarded as the greatest disease threat to biodiversity (Kilpatrick et al., 2010), and understanding what makes certain species resistant to lethal *Bd* infections is crucial for amphibian conservation efforts.

The skin of amphibians is protected by both innate and adaptive immune responses. Innate responses in amphibians include phagocytic cells, complement proteins, lysozyme, and the production and secretion of antimicrobial peptides (AMPs) via granular glands onto the surface of the skin to fight off invading pathogens such as *Bd* (Daly, 1995; Simmaco et al., 1998; Zasloff, 2002; Conlon et al., 2004). Previous work has shown that the AMP secretions, as well as purified individual peptides, from numerous amphibian species can inhibit the growth of *Bd in vitro* (Rollins-Smith et al., 2002, 2006; Woodhams et al., 2005, 2006, 2007a; Rollins-Smith and Conlon, 2005; Conlon et al., 2007; Rollins-Smith, 2009). The production of peptide secretions that are effective inhibitors of *Bd* growth is correlated with both survival of amphibian species in the wild (Woodhams et al., 2005, 2006) and with the overall resistance of certain species when experimentally infected with *Bd* (Woodhams et al., 2007a). The adaptive immune system also appears to play a role in protection against chytridiomycosis, although the exact mode of protection is not understood (Ramsey et al., 2010). A deficiency of either of these systems could increase the overall susceptibility of a species to chytridiomycosis, leading to fatal infections.

Another defense against *Bd* infection is the bacterial microbiota resident on amphibian skin. Inhibition of fungal growth has been associated with over 50 bacterial strains in over 20 genera found on the skins of amphibian embryos and adults (Harris et al., 2006; Lauer et al., 2007, 2008; Woodhams et al., 2007b, c). The mutualistic bacterial species, *Janthinobacterium lividum*, isolated from the yellow-legged mountain frog (*Rana muscosa*), the four-toed salamander (*Hemidactylium scutatum*), and the red-backed salamander (*Plethodon cinereus*), produces anti-*Bd* metabolites at concentrations lethal to the fungus (Brucker et al., 2008a). Three anti-*Bd* metabolites secreted by *J. lividum* and another skin bacterial species, *Lysobacter gummosus* have been identified: 2,4-diacetylphloroglucinol (2,4-DAPG), indole-3-carboxaldehyde (I3C), and violacein (Brucker et al., 2008a, b). 2,4-DAPG inhibited *Bd* growth *in vitro* with an IC_{50} of 8.7 μM and a minimum inhibitory concentration (MIC) of 136 μM (Brucker et al., 2008b). I3C and violacein also inhibited *Bd* significantly with MICs of 68.9 μM and 1.8 μM, respectively (Brucker et al., 2008a).

Rana muscosa, a frog species native to the Sierra Nevada Mountains of California, has AMPs that effectively inhibit *Bd* growth *in vitro* (Rollins-Smith et al., 2006). Although populations of *R. muscosa* vary in their AMP compositions, many of the peptides are shared among populations (Woodhams et al., 2007c). Populations that have no experience with *Bd* usually experience dramatic population declines once *Bd* arrives. However, there are populations that coexist with *Bd*. There was a positive relationship between the proportion of individuals with anti-*Bd* bacteria on the skins of two species of *Rana* found in the Sierra Nevada and their resistance to *Bd* (Woodhams et al., 2007c). A second survey conducted after the initial *R. muscosa* decline showed that the *Bd*-naive frogs that populated lakes had higher proportion of anti-*Bd* bacteria on the skin and were able to survive and persist after the emergence of *Bd* (Lam et al., 2010). It is possible that both AMP defenses and the microbiota present on the skin play a role in dictating whether a population of an amphibian species is resistant to *Bd*. However, other stressors, such as pesticide or environmental contamination, increased UV radiation, or increases in ambient temperature, could lead to alterations of either AMP defenses or the skin microbiota and affect resistance to *Bd*.

The potential for synergy between bacterially-produced metabolites and the AMPs secreted onto amphibian skin has not been explored in amphibians, but synergies between bacterial metabolites and AMPs have been found in other systems (Cassone and Otvos, 2010). We hypothesized that metabolites and AMPs present on the skin of amphibians will synergize to more effectively inhibit the growth of *Bd* as compared to either compound alone. We tested this hypothesis using AMPs from *R. muscosa*, a species undergoing documented population declines linked to *Bd* (Briggs et al., 2005; Woodhams et al., 2007c), as well as 2,4-DAPG, one of the metabolites secreted by bacterial species resident on the skin of *R. muscosa* (Brucker et al., 2008a,b, Lam et al., 2010).

Methods and Materials

Maintenance of Batrachochytrium dendrobatidis Cultures Stocks of *Bd* isolate 423, obtained from Dr. Joyce Longcore (University of Maine), were serially transferred monthly with antibiotics to inhibit bacterial contamination. Stocks contained 50 ml 1 % tryptone broth, 1 ml previous stock, 50 μl penicillin (100 mg/ml), and 50 μl streptomycin sulfate (200 mg/ml). All monthly stocks were incubated at 23 °C for 7–10 day without illumination before storage at 4 °C for approximately 1 month. For experiments, 1 ml aliquots of monthly stocks were transferred to 50 ml 1 % tryptone broth and incubated at 23 °C. Experimental stocks were serially transferred weekly by adding 1 ml stock to fresh broth.

Collection of Batrachochytrium dendrobatidis Zoospores Bd plates were prepared by adding 1 ml weekly experimental stock onto 1 % tryptone agar plates. *Bd* zoospores were harvested after a 1 week incubation from plates, and

separated from mature sporangia (Harris et al., 2009a). Briefly, plates were flooded with 4 ml tryptone broth for 20 min. The liquid was removed via pipette and passed though a sterile coffee filter to remove mature sporangia. An additional 1 ml tryptone broth was added to wash the plate, removed via pipette, and filtered. Zoospores were counted using a hemocytometer and diluted to 1×10^6 zoospores/ml using tryptone broth.

Amphibian Antimicrobial Peptides Antimicrobial peptides were obtained from Dr. Louise Rollins-Smith (Vanderbilt University). Natural mixtures of antimicrobial peptides were originally collected from two *R. muscosa* frogs in 2005 by norepinephrine injection and collection of gland secretions. Although the exact peptide composition of these samples is unknown, all *R. muscosa* individuals secrete similar peptide profiles and include peptides such as temporin-1M and ranatuerin-2Ma and -2Mb (Rollins-Smith et al., 2006). These natural mixtures were enriched by removing non-peptide components then stored at −20 °C as in Ramsey et al. 2010. After purification, the peptide concentrations were determined by MicroBCA assay (Pierce, Rockford, IL, USA) using bradykinin (Sigma Chemicals, St. Louis, MO, USA) as a standard.

Organic Extraction of Liquid Broth Cultures and Preparation for Analysis A *P. fluorescens* strain isolated from a *Rana muscosa* individual was co-cultured with *Bd*, then re-isolated from co-culture and incubated in a 1 % solution of tryptone broth at room temperature for 4 days without illumination. The bacterial species was identified by sequencing a portion of its 16 S rRNA gene and comparing the sequence to the GenBank database (Lauer et al. 2007). A10 ml sample of this culture was extracted in triplicate with an equal volume of ethyl acetate (EtOAc). The combined organic layers were dried over Na_2SO_4, filtered, and evaporated *in vacuo*. This uncharacterized sample (5.1 mg) was dissolved in high-performance liquid chromatography (HPLC)-grade methanol (1 ml) for analysis by reverse phase (RP)-HPLC (Agilent Technologies, 1200 series, Wilmington, DE, USA). The chromatogram of the crude extract was compared to that of a 2,4-DAPG standard (10 ppm; Sigma Chemicals, St. Louis, MO, USA) with diode array detection at 270 nm, the λ_{max} for 2,4-DAPG (Brucker et al., 2008b). The samples were injected (100 µl) into the HPLC equipped with a C18 reverse phase column (5 µm; 4.6×150 mm; Agilent Technologies, Wilmington, DE, USA) and eluted at 1 ml/min. The solvent program was isocratic for 2 min in 10 % acetonitrile/water (v/v) containing 0.1 % acetic acid followed by a linear gradient to 100 % acetonitrile containing 0.1 % acetic acid over 18 min, and a final 3 min isocratic period before returning to the initial conditions.

LC/MS Analysis of Extract A sample of 10 µl of the resuspended *P. fluorescens* culture extract was diluted 100-fold in HPLC grade methanol before analysis by LC/MS (Agilent Technologies; 6530 Accurate-Mass Q-TOF). The diluted culture extract or 10 ppb DAPG standard (5 µl) was injected into the LC equipped with an Eclipse Plus C18 column (2.1×100 mm) at a temperature of 50 °C, and eluted at 0.3 ml/min. The solvent program was isocratic for 0.25 min in 5 % acetonitrile/water (v/v) containing 0.1 % formic acid, followed by a 3.75 min linear gradient to 95 % acetonitrile containing 0.1 % formic acid and a final 0.5 min isocratic period before returning to the initial conditions. The samples were detected with a quadrupole, time-of-flight [Q-TOF] mass spectrometer using an ESI ion source run in negative mode.

Batrachochytrium dendrobatidis Growth Inhibition Assay Before each assay, *Bd* zoospores were harvested as described above. Zoospores were plated (5×10^4 zoospores/50 µl, five replicates) in tryptone broth in 96-well flat-bottom microtiter plates with serial dilutions (50 µl total) of: 1) AMP mixture diluted in sterile water (1.56 µg/ml to 200 µg/ml); 2) purified 2,4-DAPG (Sigma, St. Louis, MO) diluted in sterile water (1.1 µM to 136 µM); 3) a combination of both AMPs and 2,4-DAPG diluted in sterile water or with sterile water as the positive control. Negative control wells contained zoospores that were heat-killed at 60 °C for 1 hr. Plates were incubated for 1 week at 23 °C before assessing growth by measuring optical density at 490 nm ($O.D._{490}$) with a microplate reader. Percent growth was calculated as: (1 - [(X-Y)/X])*100, where X is the positive control optical density value, and Y is an experimental well optical density value. This assay was conducted twice, with ten total replicates per sample.

Pseudomonas fluorescens Growth and Challenge Assay We used *P. fluorescens* as it entered log phase for challenge assays with AMPs to maximize bacterial growth. The growth curve of *P. fluorescens* was determined using a standard colony forming unit (CFU) plating technique, with CFUs plotted against optical density at each hour. Logarithmic phase occurred after approximately 5 hr.

The challenge assay was conducted with 50 µl *P. fluorescens* (1×10^3 CFU/ml, five replicates) plated in tryptone broth in 96-well flat-bottom microtiter plates with or without serial dilutions (50 µl total) of antimicrobial peptides diluted in sterile water or sterile water as the positive control. Negative control wells received 5 µl streptomycin sulfate (200 mg/ml) plus 45 µl sterile water. Plates were incubated for 18 hr at 26 °C before measuring $O.D._{490}$. This assay was conducted twice with ten total replicates per sample.

Data Analysis and Statistics We defined MIC as the lowest concentration that yielded percent growth not significantly different from the negative control (Rollins-Smith et al., 2006). We used a two-tailed *t*- test with Bonferroni correction for multiple comparisons and an *alpha* level of 0.05. We determined the separate effects of AMP concentration and 2,4-DAPG concentration and interaction effects using a two-way ANOVA. All analyses were performed using the SAS statistical software.

Results

Confirmation of the Production of 2,4-DAPG by P. fluorescens Strain BOH3 A peak with the same retention time and spectral features as 2,4-DAPG was found in the *P. fluorescens* co-culture with Bd (Fig. 1). The LC/MS yielded a peak that corresponded to $C_{10}H_{10}O_5$ (*m/z*=209.04497, M – H, −0.14 ppm error) at 3.921 min; this compared favorably to that of the 2,4-DAPG standard (3.925 min, *m/z*=209.04561, M – H, +2.92 ppm error).

Effect of AMPs on B. dendrobatidis Growth Enriched skin peptide mixtures from the mountain yellow-legged frog, *R. muscosa,* strongly inhibited the growth ($P<0.01$) of *Bd* zoospores *in vitro* (Fig. 2a). Concentrations of AMPs≥ 50 μg/ml were inhibitory, as overall zoospore growth was significantly reduced compared to the positive growth control. The MIC was 100 μg/ml. These data show that the AMPs from *R. muscosa* are effective inhibitors of *Bd* growth.

Effect of 2,4-DAPG on B. dendrobatidis Growth Purified 2,4-DAPG, produced and released by *P. fluorescens* and other bacteria, also strongly inhibited the growth ($P<0.01$) of *Bd in vitro* (Fig. 2b). All concentrations≥4.4 μM were inhibitory, and the MIC was 17 μM. These data show that 2,4-DAPG is an effective inhibitor of *Bd* growth.

Combined Effects of AMPs and 2,4-DAPG on B. dendrobatidis Growth When *Bd* cultures are exposed to mixtures of AMPs and 2,4-DAPG, inhibition occurred at significantly lower concentrations than observed for either compound alone (Fig. 3; Supplemental Fig. 1). When used in combination, inhibition was detectable at 12.5 μg/ml AMP and at 1.1 μM 2,4 DAPG, levels that were 4-fold lower than the lowest concentration necessary for inhibition for either compound alone (Fig. 3, Supplemental Fig. 1). A statistically significant interaction on percent growth was noted across all combinations tested by ANOVA ($F=6.13$; $df=8,63$; $P<0.001$). This is consistent with a non-additive, synergistic effect between AMPs and 2,4-DAPG.

Effects of AMPs on the Growth of P. fluorescens To determine whether AMPs are inhibitory to *P. fluorescens*, the purified natural mixtures of *R. muscosa* AMPs were used in a bacterial challenge assay. After 18 hr of growth in the presence of AMPs, there was strong inhibition of *P. fluorescens* growth ($P<0.01$) at the highest concentrations tested (≥ 50 μg/ml); however, the MIC was not reached. At the lower AMP concentrations necessary for synergistic interactions with 2,4-DAPG, there was little to no inhibition of *P. fluorescens* growth (Fig. 4).

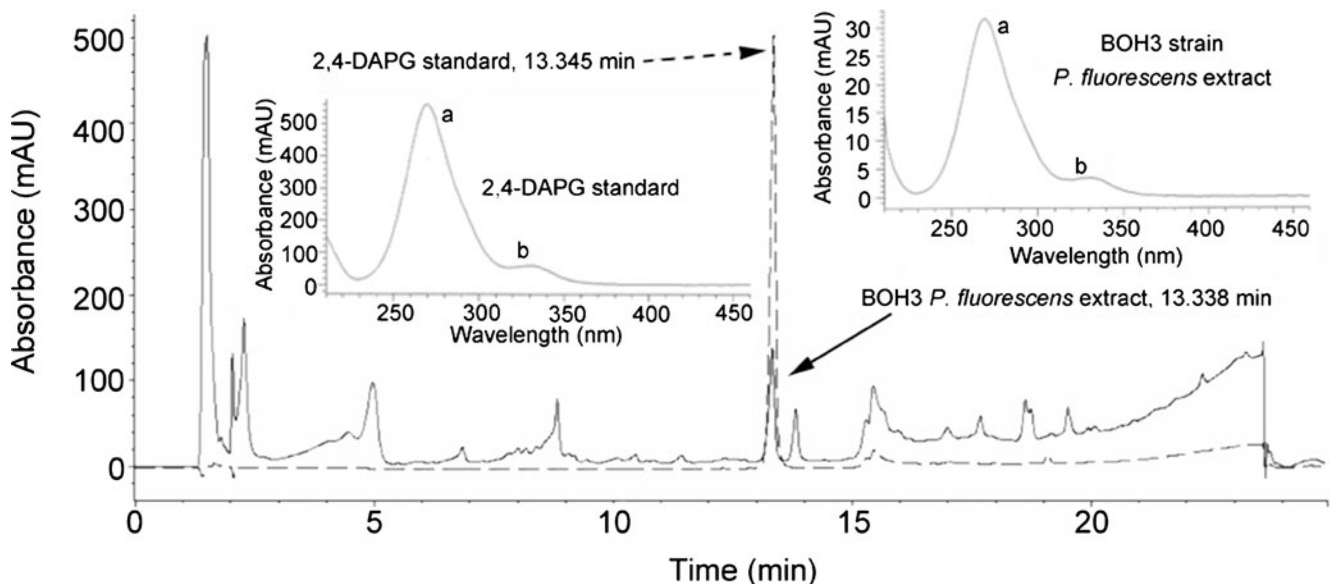

Fig. 1 Chromatographic comparison of the 2,4-DAPG standard (*dashed line*) with the extract of BOH3 strain of *Pseudomonas fluorescens* exposed to *Batrachochytrium dendrobatidis* (*solid line*). Insets are UV–VIS spectra of the 13.3 min peak from 2,4-DAPG standard (*left*) and the extract (*right*)

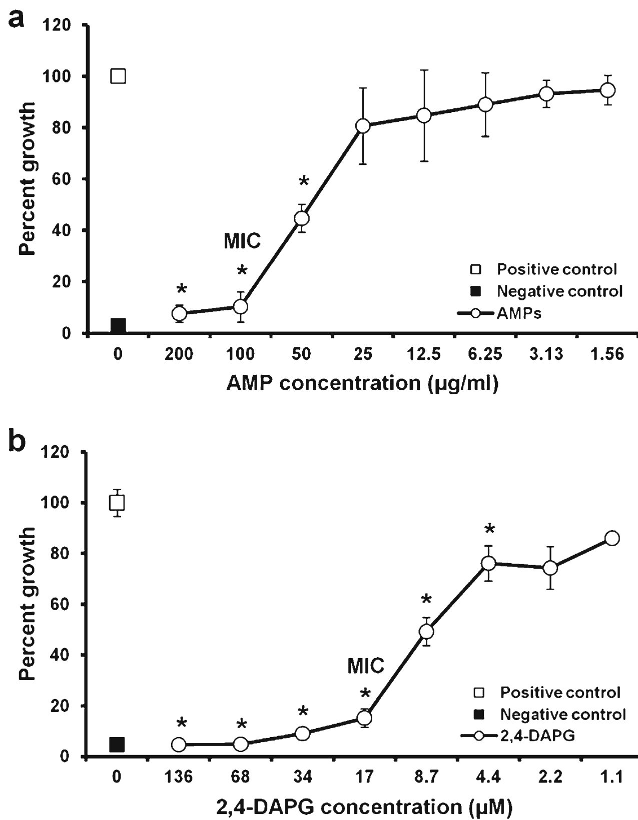

Fig. 2 Inhibition of *Batrachochytrium dendrobatidis* growth *in vitro* by antimicrobial peptides from *Rana muscosa* or the metabolite 2,4-DAPG from *Pseudomonas fluorescens*. *Bd* zoospores were cultured with or without dilutions of **a** natural mixture of skin peptides from *R. muscosa* (*squares*) or **b** 2,4-DAPG from *P. fluorescens* strain BOH3 (*open circles*). The positive growth control (open square) was *Bd* zoospores in broth plus water. The negative growth control (*closed square*) was heat-killed *Bd* zoospores (60 °C, 1 hr) in broth plus water. Each point represents the mean ± standard error for *N*=5. * Significantly reduced growth ($P<0.01$) compared to the positive growth control. *MIC* minimum inhibitory concentration

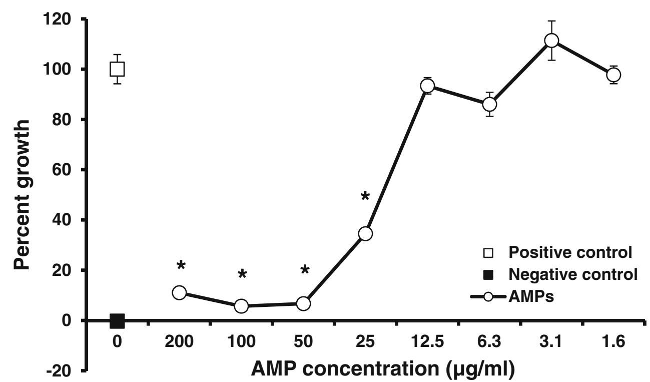

Fig. 4 Inhibition of *Pseudomonas fluorescens* growth *in vitro* by antimicrobial peptides from *Rana mucosca. P. fluorescens* was cultured with or without dilutions of a natural mixture of skin peptides from *R. muscosa* (*open circles*). The positive growth control (*open square*) was *P. fluorescens* in broth plus water. The negative growth control (*closed square*) was *P. fluorescens* in broth plus water containing 5 µl streptomycin (200 mg/ml). * Significantly reduced growth ($P<0.01$) compared to the positive growth control. Data from one of two independent experiments

Discussion

We provide evidence for a synergistic interaction between AMPs secreted by the frog *R. muscosa* and the bacterially-produced metabolite 2,4-DAPG released by *P. fluorescens*, a bacterial species present in the natural skin microbiota of *R. muscosa* (Woodhams et al., 2007c, Lam et al., 2010). Each treatment inhibited *B. dendrobatidis* when tested alone. When combined together, the concentration of both AMPs and 2,4-DAPG needed to inhibit *Bd in vitro* was reduced four-fold. These finding support our hypothesis that a mutualistic relationship exists between *R. muscosa* and *P. fluorescens*. This study evaluated the potential synergy between

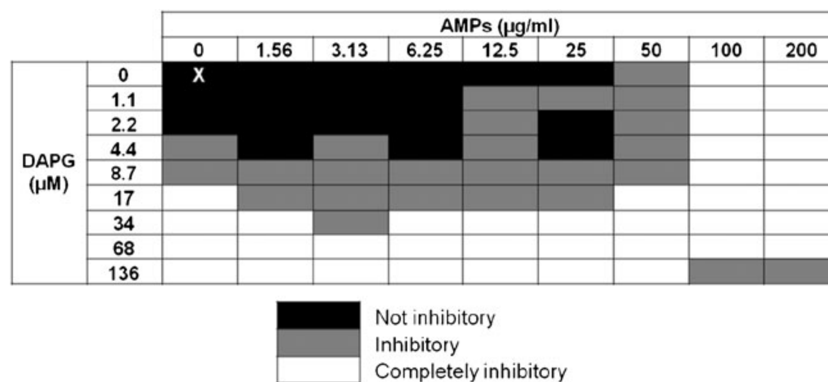

Fig. 3 Inhibition of *Batrachochytrium dendrobatidis* growth *in vitro* by combinations of antimicrobial peptides from *R. mucosa* and the metabolite 2,4-DAPG from *Pseudomonas fluorescens*. *Bd* zoospores were cultured with or without dilutions of mixtures of skin peptides from *R. muscosa* (AMPs) or 2,4-DAPG from *P. fluorescens* strain BOH3. Zoospores grown in the absence of either treatment were the positive growth control, whereas heat-killed zoospores (60 °C, 1 hr) were the negative growth control. *Black squares* denote combinations that were not inhibitory (no significant difference when compared to the positive growth control). *Gray squares* denote combinations that were inhibitory (significantly less growth than the positive growth control and significantly more growth than the negative control). *White squares* denote combinations that were completely inhibitory (not significantly different from the negative growth control)

one metabolite and the AMPs of one amphibian species. Future studies need to be conducted to establish whether synergy exists between other metabolic products or groups of metabolites, and AMPs from other species or individual AMPs.

The concentration of AMPs that occur naturally on the skin of *R. muscosa* are not known. The South African clawed frog, *Xenopus laevis*, secretes concentrations of AMPs both in a resting state (3.3 mg/ml in mucus) and after stress (20 mg/ml in mucus) that are inhibitory to *Bd* (≥ 125 μg/ml) (Ramsey et al., 2010). The northern leopard frog (*Rana pipiens*), also secretes inhibitory levels of AMPs while resting and after a mock predator attack, and the secretions inhibited *Bd* growth *in vitro* (Pask et al., 2012). Experiments to determine whether populations of *R. muscosa* can produce sufficient AMPs to inhibit *Bd* or beneficial bacteria in the wild should be conducted. Although previous studies have estimated the concentrations of the bacterial metabolites violacein and indole-3-carboxaldehyde (both produced by *J. lividum*) on the skin of the amphibian *Plethodon cinereus* (Brucker et al. 2008b), there are no published results that establish the skin concentrations of 2,4-DAPG in amphibians.

In order to more fully explore the potential mutualism between *R. muscosa* and *P. fluorescens*, we evaluated whether *P. fluorescens* could survive and grow in the presence of AMPs at concentrations that are necessary for synergism. At the lower concentrations that led to synergy with 2,4-DAPG, the AMPs did not inhibit *P. fluorescens* growth. These data suggest that *P. fluorescens* may survive on the skin of *R. muscosa* even when low levels of AMPs are being secreted to defend against pathogens. In this case, a potential mutualism could minimize the cost to the amphibian of AMP secretion, while the bacteria benefit by surviving in an environment with limited competition from other microbes. However, the presence of *Bd* or the addition of bacteria to the skin may lead to upregulation of AMPs to levels that could disturb the skin microbiota and potentially impair this synergy, although there is no report in the literature of AMP upregulation after microbe introduction. The results of this study suggest that a mutualistic relationship has developed between *R. muscosa* and *P. fluorescens*. Future studies are necessary to further test this hypothesis, including determining the concentration of AMPs naturally produced by *R. muscosa* in the presence or absence of *P. fluorescens*, as well as evaluating the concentration of 2,4-DAPG released by naturally-occurring *P. fluorescens* on the skin of *R. muscosa*. The combined results of this and future studies have implications for our understanding of symbiosis, amphibian immunology, and the disease dynamics of a lethal amphibian pathogen. For example, the effectiveness of AMPs as a mechanism of innate immunity may depend on synergies with bacterial metabolites. Since microbial community structure and, therefore, metabolites are likely to be variable, the ability of skin pathogens to evolve resistance may be difficult.

These results also provide preliminary support for use of a bioaugmentation or probiotic approach to treat susceptible amphibians in the field. We suggest that a critical criterion of an effective probiotic is that the bacterium works synergistically with the host species AMPs. Probiotic treatments using anti-*Bd* bacterial species on the skin of amphibians increased the overall protection of those amphibians to *Bd* infections (Harris et al. 2009a, b), highlighting the importance of this approach. An ongoing field study in the Sierra Nevada of California also showed that probiotic treatment increased resistance to *Bd* in *Bd*-endemic ponds (Vredenburg et al., 2011 and personal communication). Given the success of probiotic trials in amphibian conservation, we urge continued research in this area in order to optimize efficacy.

Bioaugmentation approaches to combating chytridiomycosis in the wild present an ecological dilemma. It is possible that the probiotic could spread to nontarget species in the wild or alter ecosystem dynamics when introduced. One way to minimize nontarget effects is to use a probiotic species found in the local environment. Previous experiments have determined that the addition of bacteria to control diseases in agricultural contexts are effective and have no adverse effects on nontarget species (Berg et al., 2007; Scherwinski et al., 2008). The decision on whether bioaugmentation would be worthwhile will benefit from using an ecological ethics framework (Minteer and Collins, 2008), which balances the risks between amphibians, ecosystems, and the general public. As humans alter their environments, antifungal microbes may be eliminated from waters and soils. As a result, amphibians may be losing their protective skin microbiota, and the restoring of this microbiota may be critical to an amphibian species' survival in the future. The addition of bacterial species that produce beneficial metabolites to the skin of amphibians that are threatened by *Bd* would allow for synergy to occur between metabolites and amphibian AMPs, making the skin a more hostile environment for *Bd* and thus preventing fatal chytridiomycosis.

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Induction of Chlamydospore Formation in *Fusarium* by Cyclic Lipopeptide Antibiotics from *Bacillus subtilis* C2

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Abstract The culture filtrate of *Bacillus subtilis* strain C2 showed strong activity against the pathogenic fungus *Fusarium solani* f. sp. *radicicola*. A partially purified fraction (PPF) from the extract induced chlamydospore formation in *Fusarium*. Reverse-phase high performance liquid chromatography yielded 8 different fractions, six of which had chlamydospore-inducing activity. Mass spectrometry and nuclear magnetic resonance analyses identified the main active constituent as C_{17} fengycin A (FA17), a cyclic lipopeptide. The effect of FA17 on morphology and physiology of two *Fusarium* species was dependent on the lipopeptide concentration. When challenged with FA17 at concentrations (0.5, 8, 64 μg ml^{-1}) below the minimum inhibitory concentration (MIC) (128 μg ml^{-1}), two species of *Fusarium* formed chlamydospores from hyphae, germ tubes, or inside the conidia within 2 days. At concentrations close to the MIC, FA17 caused *Fusarium* to form sparse and swollen hyphae or lysed conidia. The other five fractions were identified as fengycin A homologues. The homologues could also induce chlamydospore-like structures in 17 species of filamentous fungi including some specimens that do not normally produce chlamydospores, according to their taxonomic descriptions. Like other chlamydospores, these structures contained nuclei and lipid bodies as revealed by DAPI and Nile Red staining, and could germinate. This is the first study to demonstrate that under laboratory conditions fengycin, an antifungal lipopeptide produced by *B. subtilis*, can induce chlamydospore formation in *Fusarium* and chlamydospore-like structures in many filamentous fungi.

Keywords Bacteria-fungal interaction · Rizosphere bacteria · *Fusarium* · Fengycin · Chlamydospore · *Bacillus subtilis*

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Introduction

Fusarium, a genus of plant pathogenic fungi, causes diseases that affect most species of cultivated plants, including root and stem rots, blights, and wilts (Nelson et al., 1994). Chlamydospores are the principal means of long-term survival for *Fusarium* species during unfavorable periods in the soil and play an important role as the primary inocula that infect plants (Couteaudier and Alabouvette, 1990). Chlamydospore formation and phenotypic traits vary among plant pathogenic *Fusarium* species and have been widely used as a key taxonomic feature for this group of organisms (Leslie and Summerbell, 2006). Some *Fusarium* species can form chlamydospores in large numbers when macroconidia are added to soil (Nash et al., 1961). It has been hypothesized that this form of chlamydospore formation in *Fusarium* can be initiated in response to soil substances originating from bacteria (Goh et al., 2009). One study (Harish et al., 1998) suggests that formation of chalmydospore-like structures by *F. udum* is associated with

tolerance to the mycolytic activity of chintinase produced by *Bacillus subtilis*.

Fungi and bacteria co-inhabit a wide variety of environments, from soils and food products to plants and mammals. These microorganisms influence each other's physiology and metabolism as well as the health of the plants that they might colonize (de Boer et al., 2005). *Bacillus subtilis*, which is ubiquitous in soil, has been categorized as a plant growth-promoting rhizobacterium (PGPR) by Kloepper et al. (1989), and it is reported to be effective for the biocontrol of plant diseases caused by soilborne pathogens (Asaka and Shoda, 1996) including *Fusarium* species (Jensen et al., 2002; Chan et al., 2003). *Bacillus subtilis* produces a variety of antifungal cyclic lipopeptides (CLPs) including members of the surfactin, iturin, and fengycin families, and it has been suggested that these CLPs are responsible for plant disease reduction following treatment with *Bacillus* strains (Ongena and Jacques, 2008). One study shows that iturin A can induce chlamydospore formation in *Trichoderma harzianum* and *Glioladium roseum* at low concentration (Li et al., 2005). We proposed to study the effect of *B. subtilis* lipopeptides on morphogenesis of *Fusarium* and other filamentous fungi, and to isolate compounds that may stimulate chlamydospore formation.

Methods and Materials

Microorganisms and Growth Conditions Strains of bacteria and fungi used in this study are listed in Table 1. The bacterial strain C2, which manifested strong *in vitro* antifungal activity against *F. solani* f. sp. *Radicicola* (*Fsr*), was isolated from the rhizosphere of the *Panax notoginseng*-growing areas in the Wenshan country, Yunnan Province, southwest China. *Fsr* causes a serious root rot in *Panax notoginseng*, a well-known medicinal plant indigenous to the mountains of Yunnan province (Luo et al., 1997). Strain C2 was cultured in Luria Bertani (LB) agar at 37±2°C. This strain was permanently stored in the China General Microbiological Culture Collection Center (CGMCC 3586, Beijing, China).

Nematode-trapping fungal isolates were grown at 28±2°C on corn meal agar (CMA) supplemented with 2 g K_2HPO_4. Other fungal isolates were routinely grown on potato dextrose agar (PDA).

Identification of Bacterium C2 Bacterial phenotypic characterization by physiological and biochemical tests was performed according to standard methods (Krieg and Holt, 1984).

To confirm the identification of the selected bacterial isolate, 16S rDNA sequence homology analysis was undertaken. DNA extraction was conducted with the Promega wizard genomic DNA purification kit for Gram-positive bacteria (Promega, Madison, WI, USA). The 16S rDNA gene of bacterium C2 was amplified using the forward primer 27 F and the reverse primer 1492R (Lane, 1991). The polymerase chain reaction (PCR) temperature program was 94°C for 4 min followed by 94°C for 1 min, 54°C for 40 sec and 72°C for 2 min for 30 cycles, and with a final 30 min extension at 72°C. Following amplification, the PCR product was purified and sequenced with an ABI PRISM model 3770 DNA sequencer. Sequence comparison to standard databases was performed using BLAST through the NCBI sever (Altschul et al., 1997).

Bioassay for Inducing Chlamydospore Formation in Fusarium Fsr and *F. moniliforme* (*Fm*) were used in the bioassay. Spore suspensions were harvested from 8–12-d-old cultures with 5 ml sterile water containing 0.01 % Triton X-100. The conidial suspensions were passed through four

Table 1 Microorganisms used in this study

Microorganism	Source[a]
Bacteria	
Bacillus subtilis C2	This study
Fungi	
Fusarium solani f. sp. *radicicola*	YNU
Fusarium moniliforme (CA)	YNU
Fusarium graminearum (CA)	YNU
Trichoderma viride	YNU
Trichoderma hamatum (1.329)	YNU
T. hamatum (1.319)	YNU
Paecilomyces lilacinus (CA)	CGMCC
Rhizopus japonicus	YNU
Coprinus comatus	YNU
Stropharia rugoso-annulata	YNU
Stereum sp.	YNU
Rhizoctonia solani (CA)	YNU
Sclerotinia sclerotiorum (CA)	YNU
Botrytis cinerea (chlamydospores rare)	YNU
Nematode-trapping fungi	
Arthrobotrys oliospora	YNU
Arthrobotrys superba	YNU
Monacrosporium ellipsosporum (CA)	YNU
Duddingtonia flagrans	YNU
Endophytic fungi	
Cladosporium sp. (isolated from *Arenaria serpyllifolia*) (CA)	YNU

[a] YNU, Laboratory for Conservation and Utilization of Bio-Resources & Key Laboratory for Microbial Resources of the Ministry of Education, Yunnan University (Kunming, China); CGMCC, China General Microbiological Culture Collection Centre (Beijing, China); CA, chlamydospores absent

layers of cheesecloth to remove hyphal fragments, vortexed for 10 sec to break up clumps of cells, and the number of spores was counted by using a hemocytometer. The concentration of each suspension was adjusted to 10^5 conidia ml^{-1} with sterile water.

The following bioassay was used to guide the purification of compounds capable of inducing chlamydospore formation in fungi. A 200 μl fungal inoculum as described above was spread on the surface of a PDA plate with a sterilized glass spreader. A filter paper (8 mm diam) was wet with 50 μl of the sample, dried and placed on the surface of the inoculated plate. The plates were returned to the incubator at 28±2°C for approximately 24 h, and the treated areas were then examined microscopically for the presence of chlamydospores. All samples were tested in triplicate.

Production and Partial Purification of Compounds Inducing Chlamydospore Formation A loop of *B. subtilis* strain C2 cells from a slant culture of fresh nutrient agar was inoculated to a 300 ml flask containing 150 ml LB broth (pH 7.0). The flask was incubated on a rotary shaker at 180 rpm for 24 h at 36°C. Aliquots of this fresh culture (3 ml) were inoculated to other flasks containing the same medium. The flasks were incubated under the same condition as described above for 36 h. A total of 40 l of C2 cultures was obtained for further purification of active compounds. The C2 cells were removed from the culture broth (40 l) by centrifugation at 30,000 g, and the supernatant was reduced to 4 l *in vacuo* at 50°C before extracting three times with an equal volume of *n*-butanol. The concentrated *n*-butanol layers were pooled and evaporated to yield a gum (180 g). The gum was redissolved in ethyl acetate/MeOH (1:1) and chromatographed on 100–200-mesh silica gel, eluted with ethyl acetate/MeOH (1:1) followed by MeOH. Fractions eluted with methanol were combined (28 g). The methanol fraction was redissolved in 50 % H_2O and subjected to medium pressure chromatography (MPLC) using a C18 reverse phase (RP) column eluted with MeOH/H_2O (50, 60, 70, 80, 80, 90, and 100 %). The active fractions, which eluted with 70–100 % MeOH/H_2O, were combined (812 mg) and further applied to Sephadex LH-20 with MeOH to afford the partially purified fraction (PPF) (56 mg). The active PPF was analyzed by thin-layer chromatography (TLC) on silica plates with chloroform/methanol/water/formic acid mixture (65:25:4:0.5, vol/vol/vol/vol) as the mobile phase. Spots were visualized by charring with H_2SO_4 or with ninhydrin. The R_f value of the active PPF was 0.36.

HPLC and LC-MS The active PPF collected from Sephadex LH-20 was subsampled for analysis by HPLC (Agilent 1200, USA). The remainder of the material was fractioned by semi-preparative RP-HPLC (Agilent 1200, USA) with UV detection. All HPLC separations were carried out in a ZORBAX StableBond C18 column (length, 250 mm; particle size, 5 μm, Agilent, USA) at 30°C. For analytical or semi-preparative separations, 4.6-mm-diam or 9.4-mm-diam columns were used, respectively. Eluting solution A was water with 0.1 % trifluoroacetic acid, and eluting solution B was MeCN with 0.1 % trifluoroacetic acid. For the analytical separation, a linear gradient from 48 to 53 % B over 40 min was used for elution at a flow rate of 1 ml min^{-1}. For the semi-preparative separation, a linear gradient from 48 to 53 % B over 40 min was used for elution at a flow rate of 4.0 ml min^{-1}. All chromatograms were monitored at 220 nm.

Qualitative analyses of the active PPF by LC-MS were carried out using a Waters series HPLC 2695 with a Thermo Finnigan LCQ Advantage (Thermo Finnigan) electrospray ionization mass detector (ion trap). The partially purified extract was introduced into the source at a flow rate of 0.5 ml min^{-1} using gradient elution on an Xterra MS column (C18, 3.0×150 mm, 3.5 μm, Waters Corporation) at 25°C. Mobile phase A was water with 0.5 % formic acid, and mobile phase B was MeOH, and the elution gradient was 0 to 65 % B over 30 min. For off-line MS studies, pure lipopeptide in MeOH was introduced into the ESI interface by continuous infusion using a syringe pump at a flow rate of 3 μl min^{-1}. In MS^2 experiments, helium was used as the collision gas. Only a single parent ion was kept in resonance (isolation width, *m/z* 1 to 3) and all other ions were ejected from the trap without mass analysis. The ion then was agitated and allowed to fragment by collision-induced dissociation. The sample concentration was in the pmol ml^{-1} range. Data acquisition was in the positive mode.

NMR Spectroscopy The pure lipopeptide was dissolved in DMSO-d_6 and NMR spectra were recorded at 19°C on a Bruker Avance DRX 500 spectrometer using a 3 mm (^1H, ^{13}C) triple-resonance probe head equipped with a supplementary self shielded *z*-gradient coil. Spectra were processed using Bruker XWINNMR or MestRe-C software (Cobas et al., 2000). Double quantum filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY) (Hartmann-Hann spectroscopy) and nuclear Overhauser enhancement spectroscopy (NOESY) experiments were recorded with a 1.5 sec recovery delay in the phase-sensitive mode using the States-TPPI method (Volpon et al., 2000). Proton chemical shifts were referenced to the solvent chemical shift ($\delta(^1H)=2.50$ ppm).

Minimum Inhibitory Concentrations (MICs) The MIC was defined as the minimum concentration of a compound resulting in no visible growth of *Fsr* or *Fm* on the agar. The MICs of the purified lipopeptides were evaluated using the agar dilution method (Radford et al., 1997). The surface

of each petri plate was inoculated with a 25 μl of conidial suspension of *Fsr* or *Fm* (about 1×10^4 conidia ml^{-1}) and spread evenly with a sterilized glass spreader. The MIC experiments were repeated twice.

Chlamydospore Formation in Fusarium Induction of chlamydospore formation in strains of *Fsr* and *Fm* by the purified lipopeptide was studied using agar cultures. The purified lipopeptide was dissolved at a concentration of 1000 μg ml^{-1} in 2.1 ml of dimethyl sulphoxide (DMSO). A series of concentrations (0.75, 1.5, 24, 192, 300 μg ml^{-1}) then were prepared using 0.5 ml of sterile water to achieve each concentration. An aliquot of molten, cooled PDA (2.5 ml) was added to each concentration of lipopeptide, and the contents were mixed, poured into petri dishes (3.5 cm diam), and allowed to solidify. Two control plates were included in each test run. One control plate contained the highest concentration of DMSO, and the other contained sterile water, in addition to the agar. The surface of each petri plate was inoculated with a 25 μl (about 1×10^4 conidia ml^{-1}) of conidial suspension of *Fsr* or *Fm* and spread evenly with a sterilized glass spreader. The plates were incubated for 2 d at 28±2°C, and the chlamydospore formation was observed microscopically (Olympus BX51TF, JAPAN) from three uniform cork-borer disks of the media cut randomly from the plates. Each fungus was tested twice.

Antagonistic Activity and Morphogenesis in Other Fungi by Cyclic Lipopeptide Homologues Some species of filamentous fungi including some specimens that do not routinely produce chlamydospores, based on their taxonomic descriptions (Table 1) were chosen to examine the effect of fengycin A homologues (FAH) on hyphal growth inhibition and chlamydospore formation. The paper disc assay was used to detect antifungal activity and observe morphogenesis in fungi after challenged with antibiotics (Nielsen et al., 2000). Paper discs loaded with about 10 μg of fengycin homologues were placed equally distant on the opposite sides of the mycelial plug. The plates were incubated for 3–8 d at 28±2°C. Antifungal activity was measured by the diameters of the halos around the paper disks, and fungal morphogenesis was examined microscopically from uniform cork borer disks of the media cut in the center of the colony surrounding the point of fungal inoculation. Each fungus was tested twice.

Vitality and Fluorescence Staining of Chlamydospore-like Structures The fungal colonies were covered with 5 ml of sterile potato dextrose broth (PDB), and suspensions were made by gently probing the surface with the tip of a Pasteur pipette, generating a mixture of hyphal fragments and in some cases conidia. An aliquot of these suspensions (each 1 ml) and 2 μg of FAH were transferred to microfuge tubes (1.5 ml) and mixed. The tubes were incubated for 2 d at 28±2°C before centrifuging at 3000 rpm for 3 min, discarding the supernatants, and rinsing the pellets with sterile water × 3.

DAPI staining was carried out by adding 40 μl of deionized H_2O and 20 μl 10 g l^{-1} DAPI in 0.1 M phosphate-buffered saline to the pellets. The thick-walled spores had low permeability to DAPI. To improve straining we treated the chlamydospore-like structures by heating them at 90°C for 1 min in a heat block, and we returned them to room temperature until examined. Lipid particles of the pellets were strained using 10 μg ml^{-1} Nile Red for 5 to 10 min. Stained chlamydospore-like structures were examined with a Nikon 800 Eclipse microscope (Nikon Corporation, Japan) equipped for epifluorescence with a mercury lamp.

Pellets described above were suspended in 50 μl sterile water and transferred to petri plates with PDA, then spread evenly with a sterilized glass spreader. The vitality of chlamydospore-like structures was observed microscopically (Olympus BX51TF, JAPAN).

Results and Discussion

Biochemical tests indicated that strain C2 was *B. subtilis* (Krieg and Holt, 1984), and this conclusion was confirmed by 16S rDNA sequence analysis (GenBank accession number AY867792).

The active PPF showed chlamydospore-inducing and antifungal activity. LC/MS technology was used to characterize the PPF, and confirm its identiy as a family of *Bacillus* CLPs (Bie et al., 2009). The ESI mass spectra of the active PPF showed a group of molecular ion peaks $[M + H]^+$ at m/z 1422, 1436, 1450, 1464, 1478, 1492, and 1506, respectively. The molecular weights of the seven homologues differed from one another by a 14 Da difference, implying the presence of fengycin or plipastatin (Bie et al., 2009). Distinguishing between these two lipopeptides, which differ only in the stereochemistry of the Tyr residues (L vs. D diastereoisomers), was possible only by comparison of their respective ^1H chemical shifts in DMSO (Umezawa et al., 1986; Vanittanakom and Loeffler, 1986; Volpon et al., 2000).

The active PPF was fractioned by semi-preparative RP-HPLC, and eight fractions were collected. All fractions, except fractions 1 and 2, induced chlamydospores and had antifungal activities. Fraction 6 was the only fraction in sufficient quantity to allow us to isolate a significant amount of the lipopeptide (5.5 mg).

The major peaks of fraction 6 in the mass spectrum were observed at *m/z* 1478.8 $[M + H]^+$ and 1500.8 $[M + Na]^+$, consistent with fengycin A or B (Bie et al., 2009). Collision-

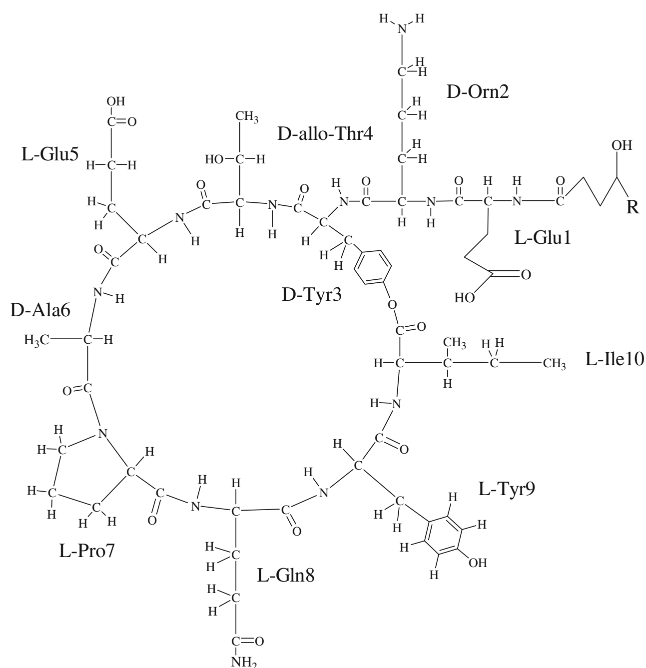

Fig. 1 Chemical structure of fraction 6 obtained from cell culture supernatant of *Bacillus subtilis* C2 ($R=C_{13}H_{27}$)

Table 2 Inhibitory activity of fengycin homologues (10 μg) against different filamentous fungi

Filamentous fungi	Inhibiton zones (mm)
Fusarium solani f. sp. *radicicola*	2±1
Fusarium moniliforme	–
Fusarium graminearum	2±1
Trichoderma viride	12±2
Trichoderma hamatum (1.329)	13±2
T. hamatum (1.319)	13±2
Paecilomyces lilacinus	17±3
Rhizopus japonicus	2±1
Coprinus comatus	15±2
Stropharia rugoso-annulata	12±2
Stereum sp.	11±2
Rhizoctonia solani	14±3
Sclerotinia sclerotiorum	11±1
Botrytis cinerea	17±3
Arthrobotrys oliospora	19±4
Arthrobotrys superb	24±3
Monacrosporium ellipsosporum	23±4
Duddingtonia flagrans	23±3
Cladosporium sp.	7±1

Values are means ± S.D. (mm) of the separate experiments. –, No inhibiton zone

induced dissociation of the $[M + H]^+$ ion signal produced fragments at *m/z* 1080 and 966. The ^{1}H-NMR chemical shifts (Supplemental Table S1) are identical with fengycin in the literature, and their spin systems were easily assigned (Vanittanakom and Loeffler, 1986). Sequential assignments (Wüthrich, 1986) were achieved by the NOESY spectra (data not shown), and we used the unique Ala6 and Ile 10

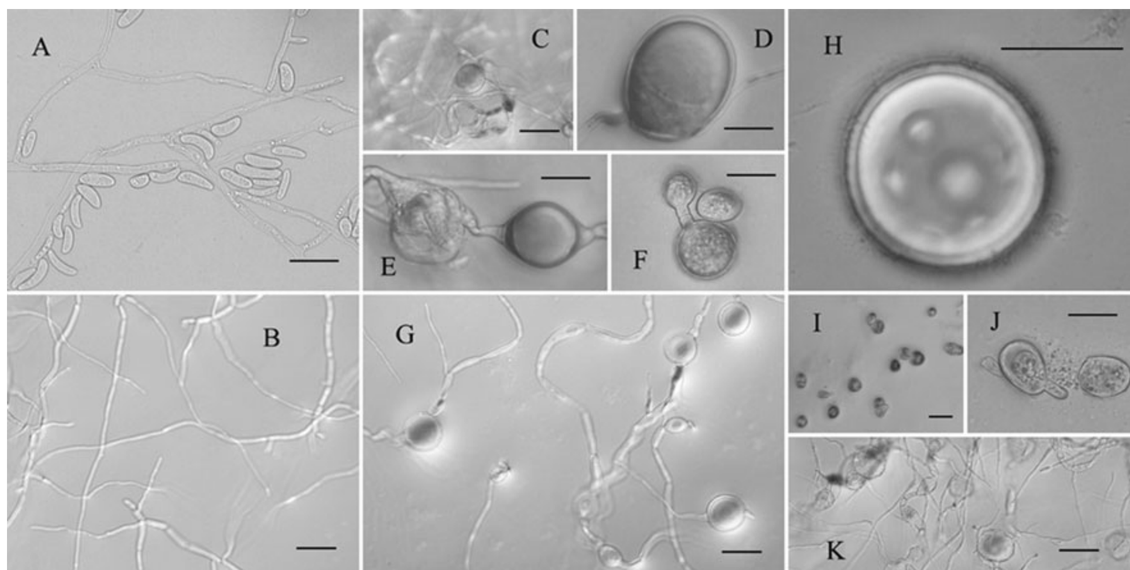

Fig. 2 Effects of the different concentrations of C_{17} fengycin A (FA17) on the morphology of *Fsr*. (**a**) Conidia of *Fsr*; (**b**) Normal mycelia of *Fsr* cultured on the PDA without or with 0.25 μg ml^{-1} FA17; (**c**) Hyphae chlamydospore of *Fsr* in the presence of 0.5 μg ml^{-1} FA17; (**d**, **e**) Hyphae chlamydospores of *Fsr* in the presence of 8 μg ml^{-1} FA17; (**f**, **g**) *Fsr* formed chlamydospores from conidial, germ tube or hyphae in the presence of 64 μg ml^{-1} FA17; (**h**) Conidial chlamydospore of *Fsr* with typical thickened walls in the presence of 64 μg ml^{-1} FA17; (**i**) Conidial chlamydospores of *Fsr* in the presence of 100 μg ml^{-1} FA17; (**j**) Swollen conidia of *Fsr* lysed in the presence of 100 μg ml^{-1} FA17; (**k**) Sparse and swelling hyphae of *Fsr* in the presence of 64 μg ml^{-1} FA17. The scale bar is 20 μm. *Fsr*: *Fusarium solani* f. sp. *Radicicola*

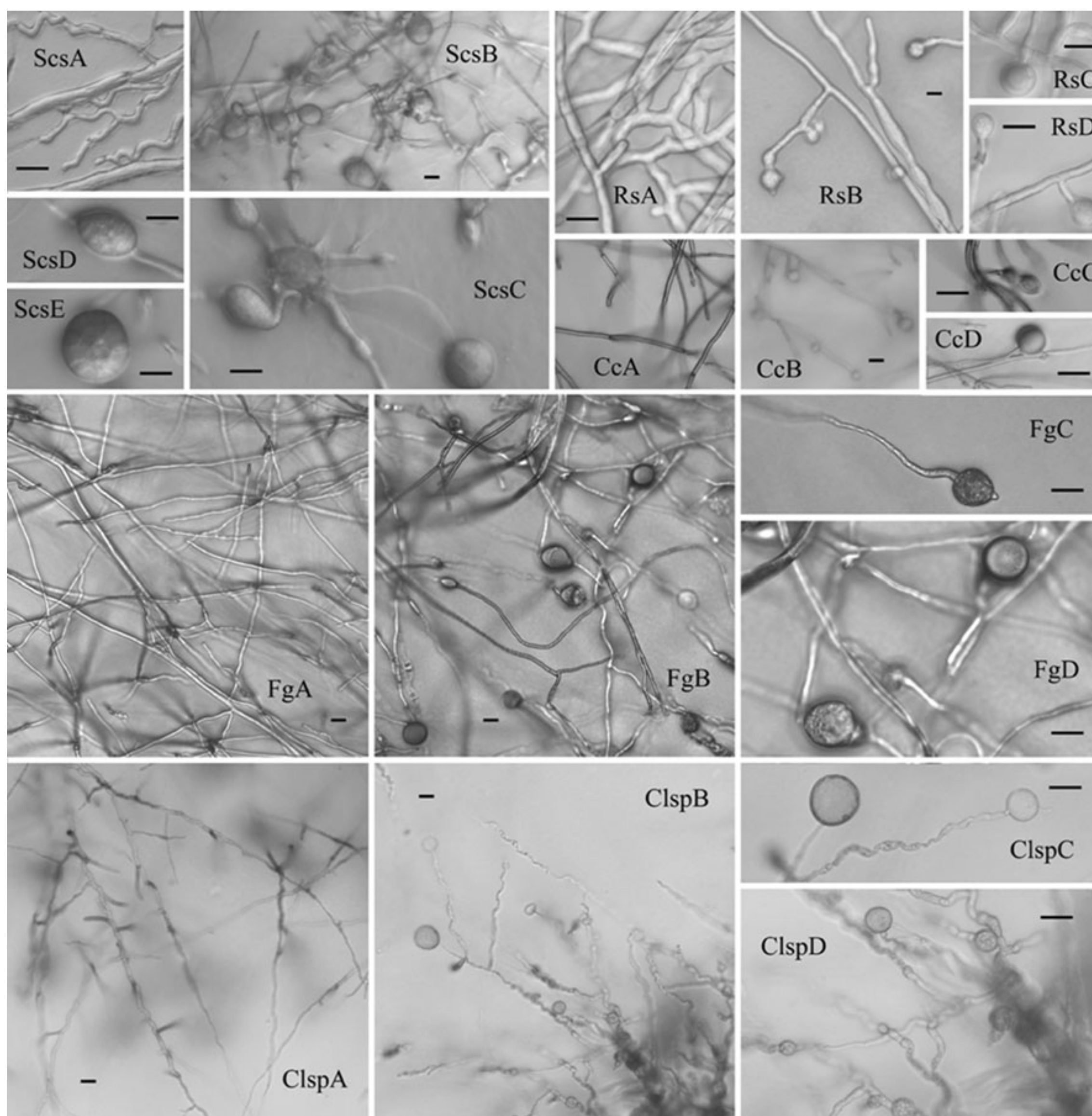

Fig. 3 Photographs of *Sclerotinia sclerotiorum* (Scs), *Rhizoctonia solani* (Rs), *Coprinus comatus* (Cc), *Fusarium graminearum* (Fg), and *Cladosporium* sp. (Clsp) grown on PDA plates at 28°C, as challenged without (**a**) or with purified fengycin A homolog added to a sterile paper disc (10 μg per disc) (**b**, **c**, **d**). The scale bar is 20 μm

spin systems as starting points for the sequential assignments of the compound. The greatest chemical shift difference between the fengycin A and B isoforms concerns the Hα of residue 6 (Ala or Val). These data indicated that fraction 6 was C_{17} fengycin A, a cyclic lipopeptide containing ten amino acids and a 17-carbon hydroxyl fatty acid (Fig. 1).

The quantities of the other active fractions purified by HPLC were insufficient to determine their structures by NMR. However, on-line LC-ESI-MS in positive modes showed that all of the compounds were closely related to fraction 6, with molecular masses ranging from 1435 to 1463, 1491, and 1505 Da. The other five compounds were most likely C_{14}–C_{16}, C_{18} and C_{19} fengycin homologues, as described by Vanittanakom and Loeffler (1986).

The MIC of C_{17} fengycin A (FA17) for both *Fsr* and *Fm* was 128 μg ml^{-1}. The effect of FA17 on the morphology of *Fsr* and *Fm* varied with its concentration. As compared with control group, the conidia of *Fsr* (Fig. 2a, b) and *Fm* (data not shown) formed hyphal and conidial chlamydospores when they were challenged with FA17 at concentrations below the MIC (Fig. 2c, d, e, f, g, i). When challenged with lower concentrations of FA17, 0.5 μg ml^{-1} to 64 μg ml^{-1}, the conidia of *Fsr* formed more hyphal chlamydospores

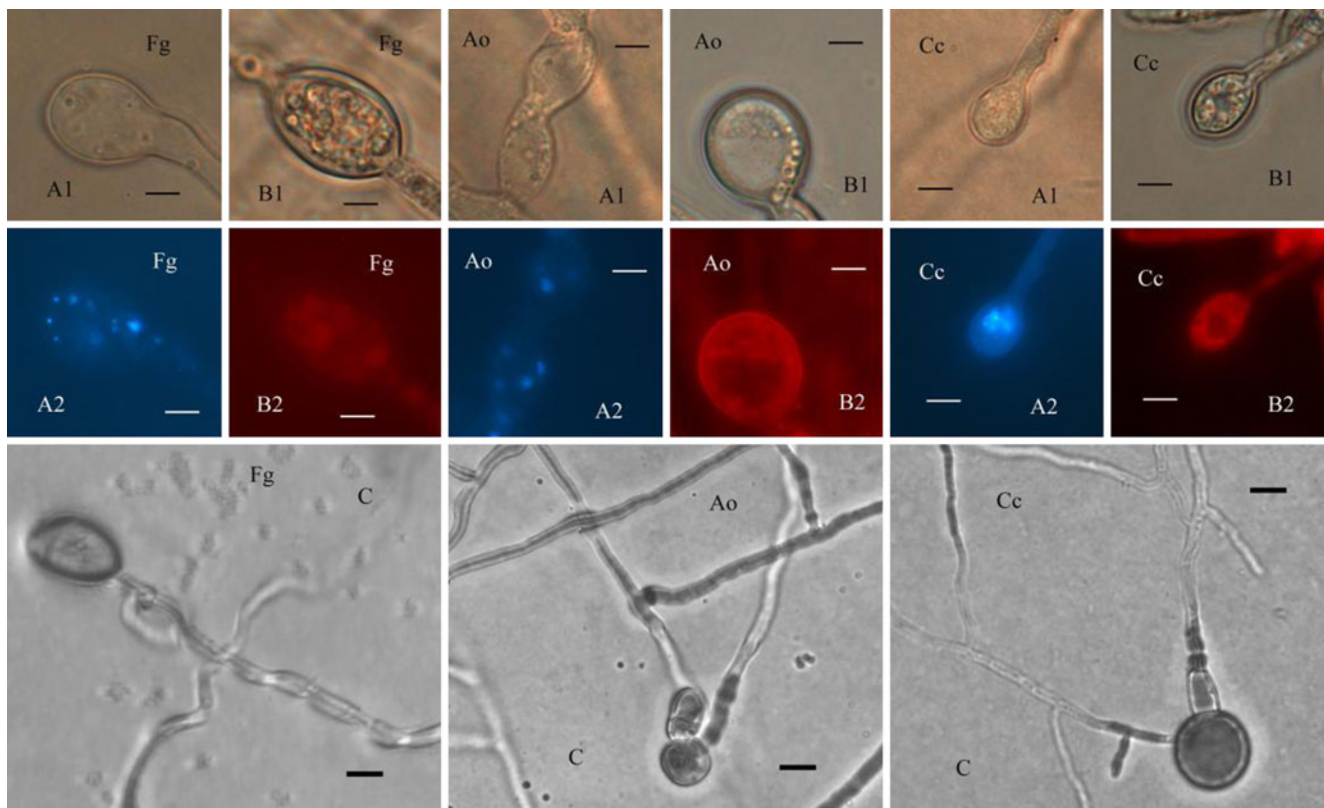

Fig. 4 Some fungal chlamydospore-like structures induced by fengycin A homolog are metabolically active and could be identified as chlamydospores. (**a1**) DAPI staining reveals nuclei. DIC. (**a2**) Fluorescent images of the same field; (**b1**) Nile Red staining reveals lipid bodies. DIC. (**b2**) Fluorescent images of the same field; (**c**) Chlamydospore-like structures can germinate new branches. *Fusarium graminearum* (Fg); *Arthrobotrys oliospora* (Ao); *Coprinus comatus* (Cc). Scale bars=20 μm. DIC: differential interference contrast

(Fig. 2c, d, e, g), and a few conidial chlamydospores (Fig. 2f) for 2 days. With increase of FA17 concentration, more conidia of *Fsr* differentiated into conidial chlamydospores (Fig. 2i). FA17 also caused sparse and swollen hyphae or conidia to form when *Fsr* or *Fm* was exposed to 64 μg ml^{-1} FA17 and higher concentrations up to the MIC (Fig. 2j, k). The chlamydospores of *Fsr* showed typical thickened walls (Fig. 2h). Most chlamydospores were globular or sub-globular, with thick warty or smooth walls, ranging in size from 11.8× 10 μm to 22.0×21.8 μm for *Fsr* and from 16.8×17 μm to 21.4×23.5 μm for *Fm*. The diameters of a few chlamydospores of *Fsr* could even reach 40×60 μm.

Filamentous fungi showed different sensitivities to FAH as revealed by the sizes of the inhibition zones that formed when the fungi were challenged with the same concentration of FAH (Table 2). The fungal sensitivity ranged from the most sensitive nematode-trapping fungi such as *M. ellipsosporum*, *A. superba*, or *D. flagrans* to the insensitive ones such as *Rhizopus japonicus*or *Fusarium* (very small inhibition zone or not inhibited). Although filamentous fungi differed in sensitivities to FAH, the exposure of filamentous fungi to FAH led to chlamydospore-like structures in all tested fungal species. Five representative species of fungi are shown in Fig. 3. These chlamydospore-like structures appeared to be formed by the conversion of hyphal compartments themselves, consistent with observations of chlamydospore generation in other fungi (Lin and Heitman, 2005).

To find out whether these chlamydospore-like structures were chlamydospores, we strained these structures with DAPI and found that they contained nuclei, raising the possibility that they could be independently surviving cells (Fig. 4A2). Interestingly, with prolonged incubation in PDB containing FAH, these intercalary and terminal structures could be released from the hypae after hyphal lysis (data not shown), again supporting the idea that these structures could be independent entities and might survive longer than their producing hyphae. In many fungi, chlamydospores are long-term survival structures produced in response to harsh environments. Lipid particles are essential for the chlamydospores to conduct their survival function (Stevenson and Becker, 1972). We stained chlamydospore-like structures with the lipid body dye Nile Red and found that these structures were enriched in lipid (Fig. 4B2). Furthermore, they could germinate new branches

(Fig. 4c), indicating that they were viable and capable of reproduction. These experiments suggested that these structures were not likely to be swollen hyphal cells but were typical chlamydospores.

In this study, FAH could also induce chlamydospore formation in *Fusarium* and many filamentous fungi at concentrations lower than MIC under laboratory conditions. Members of the *Bacillus* genus produce three families of antimicrobial CLPs including surfactin, iturin, and fengycin (Ongena and Jacques, 2008). Iturin production seems to be restricted to *B. subtilis* and *B. amyloliquefaciens*, while fengycin production is more widespread among multiple species, including *B. coagulans*, *B. pumilus*, *B. licheniformis*, *B. cereus*, *B. thuringiensis*, *B. mojavensis*, *B. megaterium*, *B. polyfermenticus*, and *Brevibacillus brevis* (Raaijmakers et al., 2010). *In situ* CLP production has been demonstrated for *Bacillus* populations growing on roots, leaves, fruits and soil (Asaka and Shoda, 1996; Ongena et al., 2007; Romero et al., 2007; Kinsella et al., 2009). Lipopeptides may be produced in the rhizosphere (or soil) by *Bacillus* isolates at concentrations lower than threshold concentration for inhibitory activity but may still be able to stimulate chlamydospore formation by the filamentous fungi. Given the predominance of *Bacillus* species in various natural habitats and the widespread fengycin production by *Bacillus*, its contribution to fungal chlamydospore formation may be important. *Bacillus* and its lipopeptides could have an important impact on microbial interactions in soil. Purification and identification of such substances could make it possible to conduct a detailed experimental study of the mechanism of chlamydospore formation in fungi.

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Responses of Soil Microbial Communities in the Rhizosphere of Cucumber (*Cucumis sativus* L.) to Exogenously Applied *p*-Hydroxybenzoic Acid

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Abstract Changes in soil biological properties have been implicated as one of the causes of soil sickness, a phenomenon that occurs in continuous monocropping systems. However, the causes for these changes are not yet clear. The aim of this work was to elucidate the role of *p*-hydroxybenzoic acid (PHBA), an autotoxin of cucumber (*Cucumis sativus* L.), in changing soil microbial communities. *p*-Hydroxybenzoic acid was applied to soil every other day for 10 days in cucumber pot assays. Then, the structures and sizes of bacterial and fungal communities, dehydrogenase activity, and microbial carbon biomass (MCB) were assessed in the rhizosphere soil. Structures and sizes of rhizosphere bacterial and fungal communities were analyzed by polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) and real-time PCR, respectively. *p*-Hydroxybenzoic acid inhibited cucumber seedling growth and stimulated rhizosphere dehydrogenase activity, MBC content, and bacterial and fungal community sizes. Rhizosphere bacterial and fungal communities responded differently to exogenously applied PHBA. The PHBA decreased the Shannon-Wiener index for the rhizosphere bacterial community but increased that for the rhizosphere fungal community. In addition, the response of the rhizosphere fungal community structure to PHBA acid was concentration dependent, but was not for the rhizosphere bacterial community structure. Our results indicate that PHBA plays a significant role in the chemical interactions between cucumber and soil microorganisms and could account for the changes in soil microbial communities in the continuously monocropped cucumber system.

Keywords Autotoxin · *Cucumis sativus* L. · Negative plant-soil feedback · *p*-Hydroxybenzoic acid · Soil microbial communities

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Introduction

Through root exudation, root deposition, and susceptibility to enemies and symbionts, plants can change soil biology, chemistry, and structure in ways that alter subsequent plant growth, which is referred to as plant-soil feedback (Kulmatiski et al., 2008). In agriculture, a well-known phenomenon of negative plant-soil feedback is soil sickness, a reduction in both crop yield and quality caused by continuous monocropping in the same land (Bonanomi et al., 2005). Although the underling mechanisms are still unclear, several mechanisms have been proposed to explain the source of negative feedback including soil nutrient depletion, the build-up of soil-borne pathogens and parasite populations, changes in soil microbial communities, and the release of allelopathic compounds (Wardle et al., 2004; Bonanomi et al., 2005; Huang et al., 2006; Kulmatiski et al., 2008). In the latter case, negative feedback has also been defined as autotoxicity (Singh et al., 1999). Studies have shown that soil microbial communities are obviously changed in continuous monocropping systems of many plant species (Nayyar et al., 2009; Li et al., 2010). Changes in soil microbial communities may influence plant growth because soil microorganisms are involved in many processes crucial to plant survival and performance (Reinhart and Callaway, 2006; Hoshino and Matsumoto, 2007; Nijjer et al., 2007). However, the causes for the alterations in soil microbial communities still are not well-clarified.

Most plants produce various classes of secondary metabolites, including phenolics, which may have a role in allelopathy and plant-soil feedbacks (Bais et al., 2004; Kulmatiski et al., 2008). It is established that, at proper concentrations and conditions, some phenolic compounds are active allelochemicals, and can accumulate in the soil with detrimental effects on the growth of crop plants (Inderjit and Duke, 2003). Increasing evidence shows that soil microorganisms can be the targets and mediators of allelopathy in plants, with either positive or negative effects (Cipollini et al., 2012). In negative plant-soil feedback, allelochemicals released by plants might alter soil microbial communities and promote soil-borne pathogens (Kong et al., 2008; Zhang et al., 2010; Pollock et al., 2011). Inderjit et al. (2009) even suggested that in some soils and for some plant species, (±)-catechin, a phytotoxin secreted by *Centaurea maculosa*, may need to interact with soil microbial communities to cause plant growth inhibition. However, how allelochemicals influence soil microorganism communities is still incompletely understood.

Cucumber (*Cucumis sativus* L.) is a crop of much economic importance in many countries. Cucumber root exudates and plant debris have been shown to have autotoxicity potential (Pramanik et al., 2000). Autotoxins, including *p*-hydroxybenzoic acid (PHBA), have been identified in the substrates used in cucumber cultivation (Politycka et al., 1984), cucumber root exudates (Pramanik et al., 2000), and cucumber rhizosphere soil (Zhou et al., 2012). Previous studies also have demonstrated that PHBA is toxic to cucumber (Pramanik et al., 2000), and can influence the development and growth of soil-borne pathogens *in vitro* and the number of culturable soil microorganisms (Wu and Wang, 2006; Wu et al., 2009b).

We hypothesized that PHBA could negatively affect the growth of its plant producer and change rhizosphere microbial communities. In a previous greenhouse experiment, we found that rhizosphere microbial communities changed in continuously monocropped cucumber system (Zhou and Wu, 2012). Therefore, we also investigated if the changes in soil microbial communities caused by PHBA are linked to the changes in the continuous monocropping system. In this work, cucumber seedlings were treated with different concentrations of PHBA (0.1, 0.25, 0.5, 1.0 μmol g^{-1} soil). Then, the structure and size of microbial communities, soil dehydrogenase activity, and microbial biomass carbon (MBC) were estimated in the rhizosphere soil. Rhizosphere bacterial and fungal community structures and sizes were analyzed by polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE), and real-time PCR, respectively.

Methods and Materials

Greenhouse Experiment Cucumber seeds (*C. sativus* L. cv. Jinlv 3) were sterilized in 2.5 % NaClO and germinated in perlite. After emergence, seedlings were planted in cups (10 cm diam, 10 cm high) containing 150 g soil. The soil was collected directly from the upper soil layer (0–15 cm) of an open field in the experimental station of Northeast Agricultural University, Harbin, China (45°41’N, 126° 37’E), which was covered with grass and undisturbed for more than 15 year. The soil was a black soil (Mollisol) with sandy loam texture, contained organic matter, 3.67 %; available nitrogen, 89.02 mg kg^{-1}; available phosphorus, 63.36 mg kg^{-1}; available potassium, 119.15 mg kg^{-1}; EC (1:2.5, w/v), 0.33 mS cm^{-1}; and pH (1:2.5, w/v), 7.78. No fertilizer was added during the experiment. There was one cucumber seedling per cup. Cucumber seedlings were maintained in a greenhouse (32 °C day/22 °C night, relative humidity of 60–80 %, 16 h L/8 h D).

Previous studies found that phenolics could be depleted rapidly after being added into the soil due to microbial utilization of the compounds (Shafer and Blum, 1991; Souto et al., 2000). So, in this study, PHBA was applied into the soil periodically to maintain the desired levels as described before (Shafer and Blum, 1991). Our previous study found that PHBA in soils repeatedly cultivated with cucumber ranged from 0.29 to 0.46 μmol g^{-1} soil DW (data not shown). Cucumber seedlings at the one-leaf stage were treated with different concentrations of PHBA every other day (once every 48 h) to achieve final concentrations of 0.1, 0.25, 0.5, 1.0 μmol g^{-1} soil. The solution pH was adjusted to 7.0 with 0.1 M NaOH solution because soil pH is considered as a dominant factor that regulates soil microbial communities (Fierer and Jackson, 2006). Soil treated with distilled water (pH 7.0) was used as the control. Soil water content was adjusted every 2 d with distilled water to maintain a constant weight of cups. Each treatment had 5 plants and was done in triplicate.

Cucumber Seedling Growth Parameters Ten days after treatment, cucumber seedling leaf area, plant height, and plant dry weight were measured. Cucumber leaves were scanned by a Microtek ScanMaker i800 plus system (WSeen, Hangzhou, China), and leaf area was calculated by a LA-S Leaf Area Analysis software (WSeen, Hangzhou, China). Plant dry weight was measured after oven drying at 70 °C to constant weight.

Rhizosphere Soil Sampling Cucumber rhizosphere soil samples were collected from 5 plants in each replicate as described before (Zhou and Wu, 2012): only the soil adhering to the roots was considered as rhizosphere soil, which was collected by shaking it off from the roots in the air. After sieved (2 mm), the fresh soil samples were immediately transferred to the laboratory. Part of these samples was used for soil dehydrogenase activity and MBC content estimation, and the other part was stored at -70 °C before DNA extraction.

Soil Dehydrogenase Activity and MBC Content Estimation Soil dehydrogenase activity was determined by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) (Tabatabai, 1994). Fresh rhizosphere soil samples (5 g) were incubated at 37 °C for 24 h in the dark, and the concentration of triphenylformazan (TPF) in the extracts was spectrophotometrically measured at 485 nm with methanol as the blank. Dehydrogenase activity was expressed as μg TPF g^{-1} soil 24 h^{-1} on an oven-dried soil basis.

Soil MBC content was determined on a 15-g oven-dry equivalent field-moist soil sample (<5 mm) by the chloroform-fumigation-extraction method (Vance et al., 1987). Fumigated and non-fumigated soil samples were extracted with 0.5 M K_2SO_4 for 30 min (soil:0.5 M K_2SO_4 ratio of 1:5, w/v), and filtered. Extractable organic carbon in soil extracts was analyzed by the dichromate digestion method. An extractability factor of 0.38 was used to calculate MBC (Vance et al., 1987).

DNA Extraction and PCR-DGGE Rhizosphere bacterial and fungal community structures were analyzed with the PCR-DGGE method. Total soil DNA was extracted with an E.Z.N.A. Soil DNA Kit (Omega Bio-Tek, Inc., GA, USA). PCR amplification of partial bacterial 16S rRNA gene was performed with the primer set of GC-338f/518r (Muyzer et al., 1993). A nested PCR protocol was used to amplify fungal internal transcribed spacer (ITS) regions of the rRNA gene with primer sets of ITS1F/ITS4 (White et al., 1990; Gardes and Bruns, 1993) and GC-ITS1F/ITS2 (Gardes and Bruns, 1993) for the first and second round of PCR amplifications, respectively. DGGE was performed using an 8 % (w/v) acrylamide gel with 30–70 % and 20–60 % denaturant gradient for bacteria and fungi, respectively, and run in a 1×TAE (Tris-acetate-EDTA) buffer for 14 h at 60 °C and 80 V with a DCode universal mutation detection system (Bio-Rad Lab, LA, USA). After electrophoresis, the gel was stained in 1:3300 (v/v) GelRed nucleic acid staining solution (Biotium, CA, USA) for 20 min. DGGE profiles were photographed with an AlphaImager HP imaging system (Alpha Innotech Crop., CA, USA) under UV light.

Real-Time PCR Assay Rhizosphere bacterial and fungal community sizes were estimated by SYBR Green real-time PCR assays with primer sets of 338f/518r (Muyzer et al., 1993) and ITS1F/ITS4 (White et al., 1990; Gardes and Bruns, 1993), respectively. Real-time PCR assays were conducted with an IQ5 real-time PCR system (Bio-Rad Lab, LA, USA) in a 20 μl volume mix containing 10 μl of 2× Real SYBR Mixture (Cowin Biotech, Beijing, China), 0.2 mM of each primer, 8 μg of bovine serum albumin (BSA), and 2.5 ng of purified soil DNA extracts. The PCR conditions were: 94 °C for 5 min; 94 °C for 45 sec; 56 °C for 45 sec for bacterial 16S rRNA gene (or 57.5 °C for 45 sec for fungal ITS region); 72 °C for 90 sec; 30 cycles in total; a final elongation at 72 °C for 10 min. Standard curves were created with a 10-fold dilution series of plasmids containing the 16S rRNA gene or ITS region from soil samples. Soil DNA extracts were tested for the inhibitory effect by diluting DNA extracts and by mixing a known amount of plasmid DNA with the soil DNA extracts. In all cases, no inhibition was detected. Sterile water was used as a negative control to replace templates. All amplifications were performed in triplicate. The specificity of the products was confirmed by melting curve analysis and agarose gel electrophoresis. The threshold cycle (*Ct*) values obtained for each sample were compared with the standard curve to determine the initial copy number of the target gene.

Statistical Analysis Data were analyzed following analysis of variance (ANOVA) and mean comparison between treatments was performed based on the Tukey's honestly significant difference (HSD) test at the 0.05 probability level with SAS 8.0 software. Banding patterns of the DGGE profiles and principal component analysis (PCA) were analyzed by

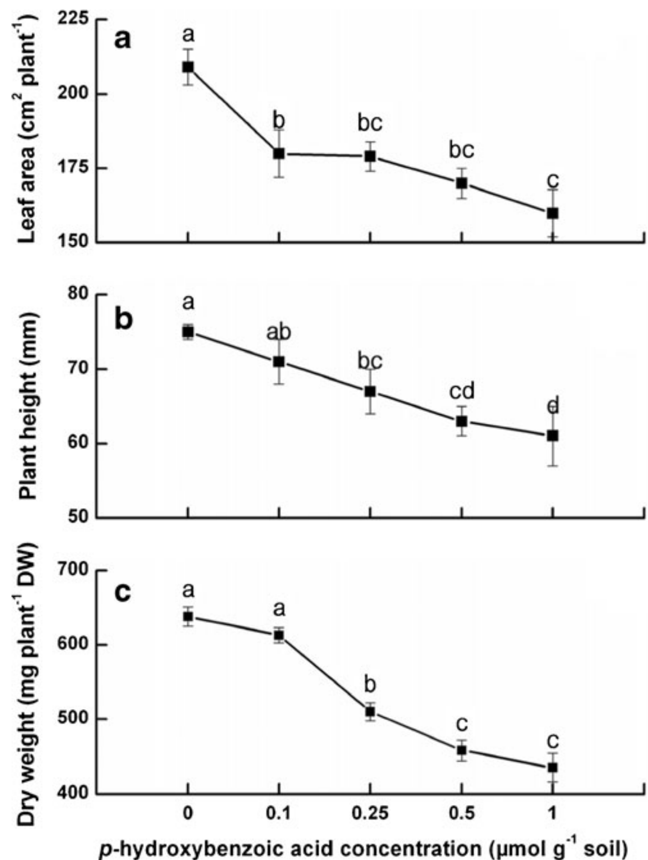

Fig. 1 Effects of exogenously applied *p*-hydroxybenzoic acid on cucumber seedling leaf area **a**, plant height **b**, and dry weight **c**. Data are represented as the means of three independent replicates with standard error bars. Different letters indicate significant differences among treatments ($P<0.05$, Tukey's HSD test)

the Quantity One software (version 4.5) and Canoco for Windows 4.5 software as described previously (Zhou et al., 2011), respectively. The diversity of bacterial and fungal communities was estimated by using the Shannon-Wiener index of diversity (H) following the equation: $H=-\sum P_i \log P_i$, where $P_i=n_i/N$, n_i is the height of peak, and N is the sum of all peak heights in the curve (Liu et al., 2007).

Results

Cucumber Seedling Growth Generally, PHBA inhibited cucumber seedling growth in a concentration dependent manner (Fig. 1). All concentrations of PHBA inhibited leaf area ($P<0.05$). Plant height and dry weight also were inhibited at concentrations≥0.25 μmol g^{-1} soil ($P<0.05$). Compared with the control, leaf area, plant height and dry weight had a reduction of 23.4, 18.7, and 31.8 % at the concentration of 1.0 μmol g^{-1} soil, respectively.

Rhizosphere Soil Dehydrogenase Activity and MBC Content *p*-Hydroxybenzoic acid increased the dehydrogenase activity and MBC content in the rhizosphere soil (Fig. 2). Dehydrogenase activity was stimulated even at the lowest concentration assayed (0.1 μmol g^{-1} soil, $P<0.05$). The highest MBC content was detected in soil treated with 0.5 μmol PHBA g^{-1} soil, which was about 1.65 times of the control.

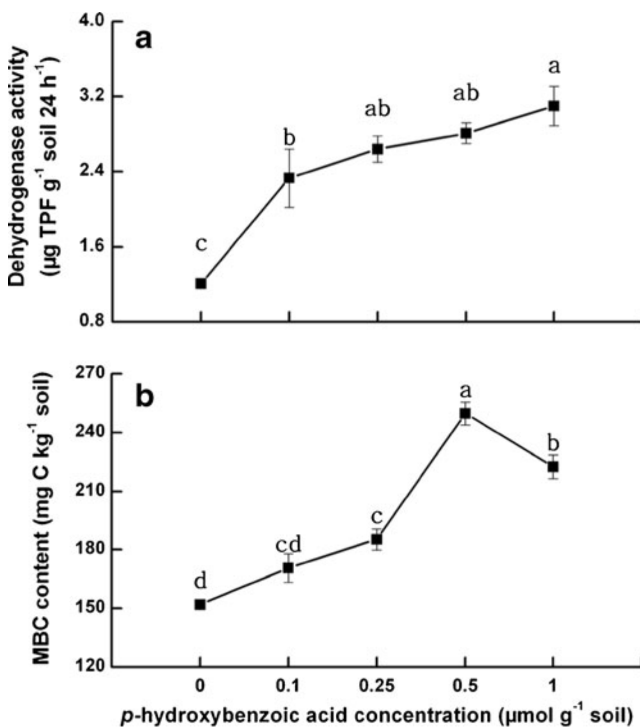

Fig. 2 Effects of exogenously applied *p*-hydroxybenzoic acid on cucumber rhizosphere soil dehydrogenase activity **a** and MBC content **b**. Data are represented as the means of three independent replicates with standard error bars. Different letters indicate significant differences among treatments ($P<0.05$, Tukey's HSD test)

Rhizosphere Bacterial and Fungal Community Sizes In this study, relative sizes of bacterial and fungal communities were estimated by measuring the densities of 16S rRNA and ITS rRNA genes, respectively. The PHBA significantly increased the sizes of rhizosphere bacterial and fungal communities ($P<0.05$) (Fig. 3a and b). The largest size of both bacterial and fungal communities was observed for soil treated with PHBA at 0.5 μmol g^{-1} soil. However, fungal community had a larger increase in size than the bacterial community. All concentrations of PHBA significantly inhibited the bacteria-to-fungi ratio ($P<0.05$) (Fig. 3c).

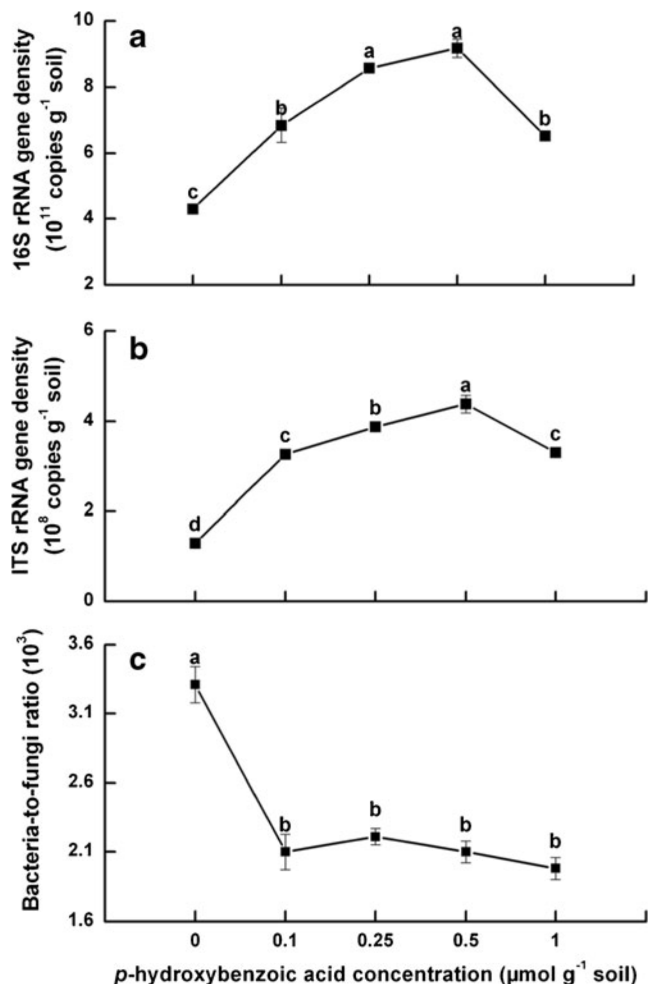

Fig. 3 Effects of exogenously applied *p*-hydroxybenzoic acid on 16S rRNA gene **a** and ITS rRNA gene **b** densities, and bacteria-to-fungi ratio **c** in the rhizosphere of cucumber. Data are represented as the means of three independent replicates with standard error bars. Different letters indicate significant differences among treatments ($P<0.05$, Tukey's HSD test)

Rhizosphere Bacterial and Fungal Community Structures PCR-DGGE analyses showed that the PHBA obviously changed rhizosphere bacterial (Fig. 4a) and fungal (Fig. 5a) community structures. Visual inspection of the DGGE profiles revealed different banding patterns for the bacterial and fungal communities in the soil before cucumber planting, the control soil and the soils treated with PHBA.

The Shannon-Wiener index (H) for bacterial community was smaller in the soil treated with PHBA than in the control soil and the soil before cucumber planting ($P<0.05$) (Fig. 6). The opposite trend was found in the Shannon-Wiener index for fungal community, which tended to increase with the concentration of PHBA. However, no definitive trend was observed in the Shannon-Wiener index for bacterial community among the four PHBA concentrations.

PCA analyses of bacterial (Fig. 4b) and fungal (Fig. 5b) DGGE banding patterns showed that soils treated with PHBA were clearly separated from the soil before cucumber planting and the control soil. For the rhizosphere bacterial community, soils treated with all concentrations of PHBA grouped together, indicating that there was a similar response of the rhizosphere bacterial community structure to different concentrations of PHBA. For the rhizosphere fungal community, soils treated with 0.5 and 1.0 μmol PHBA g^{-1} soil grouped together, whereas soils treated with 0.1 and 0.25 μmol PHBA g^{-1} soil formed their own cluster according to the PHBA concentration, indicating that high concentrations (0.5 and 1.0 μmol g^{-1} soil) and low concentrations (0.1 and 0.25 μmol g^{-1} soil) of PHBA had different influences on the rhizosphere fungal community structure.

Discussion

Phenolic compounds have shown detrimental effects on growth of crop plants, by influencing nutrient ion uptake, enzyme activities, water relations, and photosynthesis and respiration of plants (Inderjit and Duke, 2003). The physiological changes caused by allelochemicals are concentration dependent, and many phenolic compounds are bioactive at concentrations between 0.1 and 1 mM (Muscolo and Sidari, 2006). Consistent with previous findings, all concentrations of PHBA tested in this study inhibited cucumber seedling growth.

Phenolic compounds can increase soil dehydrogenase activity (Wang et al., 2008), soil microbial basal respiration (Wu et al., 2009a), MBC content (Wang et al., 2008), and the number of culturable soil microorganisms (Blum et al., 2000; Wang et al., 2008), and change microbial community structures (Qu and Wang, 2008; Wu et al., 2009a). In agreement with these findings, PHBA increased dehydrogenase activity, MBC content, and the sizes of bacterial and fungal communities as well as caused changes in the structures of bacterial and fungal communities. These results were not surprising, considering that, as low molecular weight compounds, phenolics can serve as carbon resources for soil microorganisms (Souto et al., 2000). However, phenolic and other carbon compounds (e.g., glucose, starch, xylose, and pine litter) show different influences on soil microbial phospholipid fatty acids profiles (Waldrop and Firestone, 2004; Brant et al., 2006). *p*-Hydroxybenzoic acid inhibited, while glucose and sucrose stimulated, the mycelial growth of *Fusarium oxysporum* (Wu and Wang, 2006; Wu et

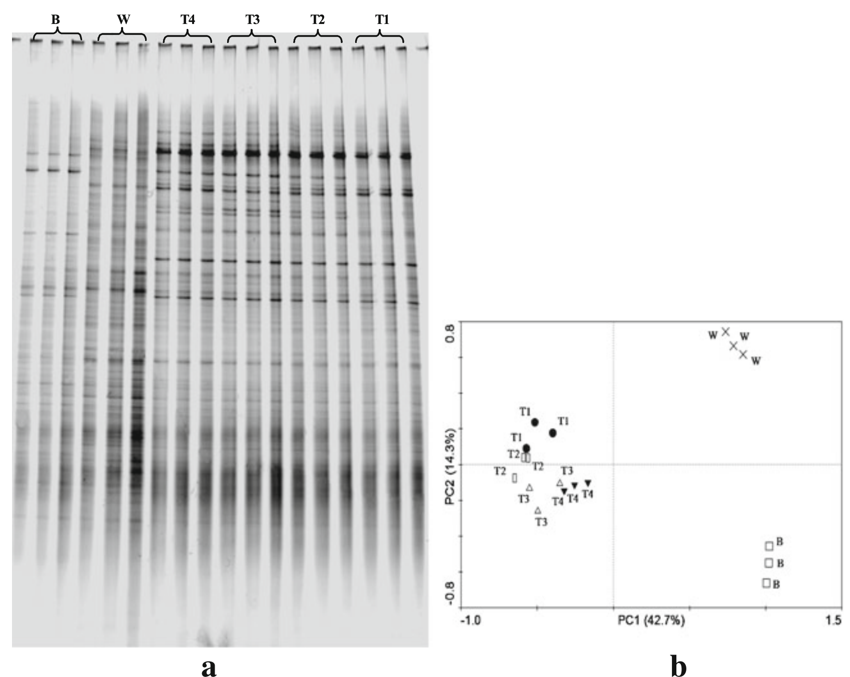

Fig. 4 DGGE profile **a** and PCA analysis **b** of bacterial 16 S rDNA fragment. B and W represent soil sample before cucumber planting and control, respectively. T1, T2, T3, and T4 represent treatments applied with *p*-hydroxybenzoic acid at the concentration of 0.1, 0.25, 0.5, 1.0 μmol·g^{-1} soil, respectively

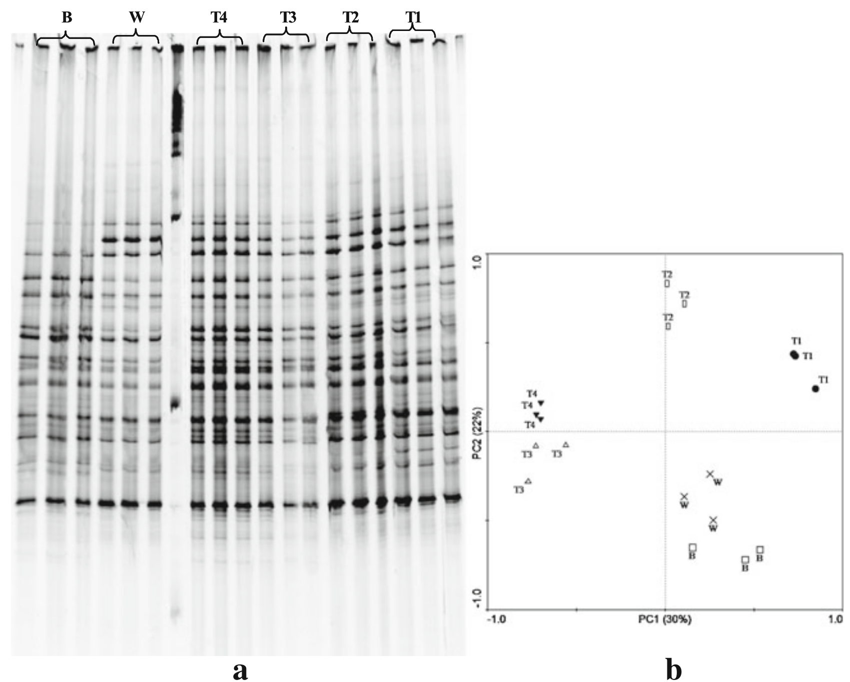

Fig. 5 DGGE profile (a) and PCA analysis (b) of partial fungal ITS sequences. B and W represent soil sample before cucumber planting and control, respectively. T1, T2, T3, and T4 represent treatments applied with *p*-hydroxybenzoic acid at the concentration of 0.1, 0.25, 0.5, 1.0 μmol·g^{-1} soil, respectively

al., 2009b; Liu et al., 2011). These findings suggest that phenolics might not act alone as carbon resources of soil microorganisms. However, no information is currently available about the detailed differences in the influences of phenolic compounds and other simple carbon compounds on soil microbial communities.

Increased soil microbial activity and biomass might compete with plants for limiting nutrients in soil, especially for nitrogen (Inderjit, 2005; Meier and Bowman, 2008). However, recent studies suggest that phytotoxicity, not microbial nitrogen immobilization, explains the plant litter inhibitory effects, because the addition of nitrogen solution could not eliminate the phytotoxicity (Meier et al., 2009; Bonanomi et al., 2011). Whether microbial nitrogen immobilization accounts for the phytotoxicity of PHBA to cucumber should be further addressed.

Changes in structure and size of bacterial communities exposed to low weight carbon substrates (glucose, glycine, and citric acid) (Eilers et al., 2010) or selected root exudate solutions (quinic, lactic, maleic acids, and glucose, sucrose, fructose) seem to be taxa dependent (Shi et al., 2011). In this study, increases in the rhizosphere bacterial community sizes and decreases in the Shannon-Wiener index of soil bacterial community as affected by exogenously applied PHBA suggest that PHBA stimulated certain species of soil bacteria and inhibited others in the cucumber rhizosphere.

p-Hydroxybenzoic acid seemed to have differential effects on rhizosphere bacterial and fungal communities. Changes in the structure of fungal community were concentration dependent, a situation not observed for the bacterial community. Moreover, PHBA decreased the Shannon-Wiener index for the bacterial community while it increased it for the fungal community. These findings might be due to a difference in the preferential use of labile carbon compounds between the soil fungi and bacteria (de Graaff et al., 2010). Bacteria have showed a greater ability to use readily available organic compounds than fungi (de Boer et al., 2005; Rinnan and Bååth, 2009). Our results also showed that PHBA decreased the bacteria-to-fungi ratio, a situation usually observed in 'sick' soils from continuous monocropping systems (Li et al., 2010). *p*-Hydroxybenzoic acid, therefore, might be linked to the increase of soil fungal community size in continuous monocropping systems.

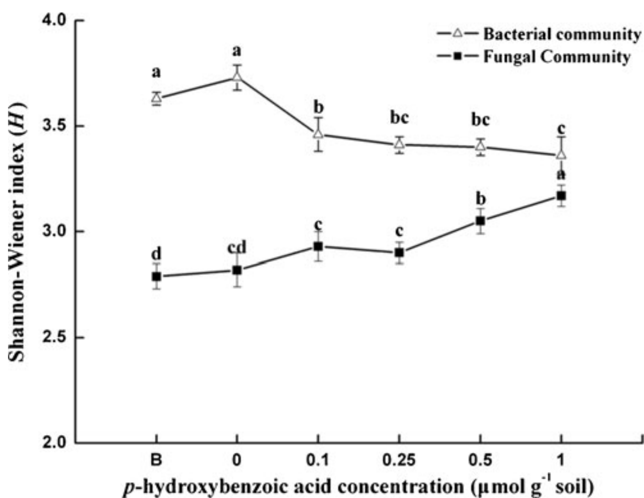

Fig. 6 Shannon-Wiener index (*H*) based on DGGE analyses of soil bacterial and fungal communities. B represents soil sample before cucumber planting. Data are represented as the means of three independent replicates with standard error bars. Different letters indicate significant differences among treatments ($P<0.05$, Tukey's HSD test)

The performance of plants can be influenced greatly by interactions with the abiotic and biotic soil environment (Wardle et al., 2004; Kulmatiski et al., 2008). Both beneficial and pathogenic microorganisms are important players in the rhizosphere of plants because they influence plant growth, physiological response to stress, and the ability to take nutrients and water (Reinhart and Callaway, 2006; Hoshino and Matsumoto, 2007). Changes in soil microbial communities may modify functional roles, which could consequently affect plant growth (Kourtev et al., 2002; Sharma et al., 2004; Wolfe and Klironomos, 2005; Batten et al., 2008; Bever et al., 2010). Thus, the PHBA might indirectly inhibit cucumber growth by changing cucumber rhizosphere microbial communities.

Soil-borne pathogens play a role in many negative plant-soil feedbacks (Mangla et al., 2008; Inderjit and van der Putten, 2010; Zhang et al., 2010). We previously found that PHBA promoted *F. oxysporum* f.sp. *cucumerinum*, a host-specific soil-borne pathogen of cucumber, in the soil (Wu and Wang, 2006), and that rhizosphere fungal and *Fusarium* community structures changed during the continuous monocropping of cucumber, and that the active fungal and *Fusarium* community sizes were larger in the season that cucumber performed poorly (Zhou and Wu, 2012). In this study, PHBA changed rhizosphere fungal community structure and increased rhizosphere fungal community size, indicating that, as an autotoxin of cucumber, PHBA might play some role in the soil sickness of cucumber indirectly through creating soil microbial conditions that are harmful in the continuous monocropping system.

Soil microorganisms can influence the persistence, availability and biological activities of allelochemicals in the soil (Inderjit, 2005; Jilani et al., 2008). On the other hand, root exudates or allelochemicals are known as one of the most important factors that affect soil microbial parameters (Bais et al., 2004; Welbaum et al., 2004; Broeckling et al., 2008). Thus, allelopathy can be better understood in terms of soil microbial ecology (Inderjit, 2005). Root residues and aboveground litters, which contain autotoxic substances, should accumulate in the soil under continuously cropped conditions. Phenolics thus should be maintained at a relatively stable level in soils continuously cropped with cucumber (Zhou et al., 2012). These autotoxins will likely affect soil microbial communities over time, and these changes in microbial communities could in turn negatively affect cucumber growth.

Overall, our results have validated the hypothesis that *p*-hydroxybenzoic acid can negatively affect cucumber growth and change soil microbial communities, which helps us to better understand why cucumber performs poorly in a continuous monocropping system. In addition, autotoxins might also be important in mediating negative plant-soil feedbacks through altering soil microbial communities. However, we analyzed only the effects of PHBA on the general bacterial and fungal communities. Influences on specific microbial functions implicated in ecosystem functioning and productivity need to be evaluated in the future.

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Costs of Jasmonic Acid Induced Defense in Aboveground and Belowground Parts of Corn (*Zea mays* L.)

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Abstract Costs of jasmonic acid (JA) induced plant defense have gained increasing attention. In this study, JA was applied continuously to the aboveground (AG) or belowground (BG) parts, or AG plus BG parts of corn (*Zea mays* L.) to investigate whether JA exposure in one part of the plant would affect defense responses in another part, and whether or not JA induced defense would incur allocation costs. The results indicated that continuous JA application to AG parts systemically affected the quantities of defense chemicals in the roots, and *vice versa*. Quantities of DIMBOA and total amounts of phenolic compounds in leaves or roots generally increased 2 or 4 wk after the JA treatment to different plant parts. In the first 2 wk after application, the increase of defense chemicals in leaves and roots was accompanied by a significant decrease of root length, root surface area, and root biomass. Four weeks after the JA application, however, no such costs for the increase of defense chemicals in leaves and roots were detected. Instead, shoot biomass and root biomass increased. The results suggest that JA as a defense signal can be transferred from AG parts to BG parts of corn, and *vice versa*. Costs for induced defense elicited by continuous JA application were found in the early 2 wk, while distinct benefits were observed later, i.e., 4 wk after JA treatment.

Keywords Corn · Jasmonic acid · Defense chemicals · Induced defense responses · Aboveground and belowground parts · Allocation costs

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Introduction

Plants have evolved several direct and indirect defense strategies that protect against insect herbivores or plant pathogens (Karban and Baldwin, 1997; Agrawal et al., 1999; Heil, 2008). Both direct and indirect defenses are commonly inducible (Karban and Baldwin, 1997). Such induced responses can occur in leaves (van Dam et al., 2003, 2004), and also in roots (Rasmann et al., 2005; Rasmann and Agrawal, 2008; Rasmann and Turlings, 2008). Most of these responses are systemic and cross the border between the roots and the aerial compartments (Baldwin et al., 1994; van Dam et al., 2001, 2003; Kaplan et al., 2008; Feng et al., 2010). Interactions between above- and below-ground communities recently have focused on the close connection between aboveground (AG) parts and belowground (BG) parts of a plant (Heil, 2011; van Dam and Heil, 2011, Soler et al., 2012). Attack of leaves by herbivores is well-known to systemically induce chemical defense responses in roots, and *vice versa* (Bezemer et al., 2003; van Dam et al., 2003; Wäckers and Bezemer, 2003; Bezemer and van Dam, 2005; van Dam and Heil, 2011). Jasmonic acid (JA) and its volatile equivalent, methyl jasmonate (MeJA), are key signal compounds involved in plant induced responses (McConn et al., 1997; Rojo et al., 2003; Wasternack, 2005; Bodenhausen and Reymond, 2007). Previous studies indicated that exogenous JA exposure to AG (or BG) parts of a plant can systemically induce the defense responses in roots (or leaves) (Dammann et al., 1997;

Ludwig-Müller et al., 1997; van Dam et al., 2001, 2004; Feng et al., 2010).

Plants possess a limited pool of resources that can be invested either in growth or in defense, each at the expense of the other (Coley et al., 1985). Hence, induced defense against herbivores and pathogens is costly (Baldwin, 1998; Agrawal, 2000; Heil et al., 2000; Purrington, 2000; Cipollini, 2002; Zavala et al., 2004). Such costs may manifest in various ways, such as genetic, allocation, autotoxicity, opportunity, and ecological costs (Heil, 2002). So-called allocation costs generally are linked with limited resources, which then cannot be used for other fitness-relevant functions such as further growth or reproduction (Heil, 2002; Steppuhn and Baldwin, 2008). Studies that have focused on allocation costs have revealed negative fitness effects of induced defense under enemy-free conditions (Baldwin, 1998; Agrawal et al., 1999; Heil et al., 2000; Redman et al., 2001; Cipollini, 2002). There is increasing agreement that induced defense causes relevant costs (Baldwin, 1998; Agrawal et al., 1999; Thaler, 1999; Redman et al., 2001; Boughton et al., 2006). Studies have focused mostly on the application of JA or MeJA to AG parts of plants (Thaler, 1999; Cipollini and Sipe, 2001; Redman et al., 2001; Cipollini, 2002; van Dam et al., 2004; Bower et al., 2005; Heijari et al., 2005; Barbosa et al., 2008; Schmidt et al., 2010). Allocation costs of induced defense also may occur when BG parts of plants are treated with JA or MeJA (van Dam et al., 2004; Bower et al., 2005; Schmidt et al., 2010), or when AG and BG parts are simultaneously exposed to JA or MeJA (van Dam et al., 2004), but such costs rarely have been demonstrated or quantified.

Costs of induced defense have been studied especially in *Brassica* spp. (Cipollini and Sipe, 2001; van Dam et al., 2004), *Arabidopsis thaliana* (Cipollini, 2002; Schmidt et al., 2010), tomato (Thaler, 1999; Redman et al., 2001), cotton (Barbosa et al., 2008), sugarcane (Bower et al., 2005), and Scots pine (Heijari et al., 2005), but not in corn, a major cereal crop in the world. In fact, corn (*Zea mays* L.) shows direct or indirect defense responses to diseases and insect pests in both leaves and roots (Rasmann et al., 2005; Xu et al., 2005; Wang et al., 2007; Rasmann and Turlings, 2008; Feng et al., 2010). DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) and phenolic acids are the major defense chemicals in corn (Classen et al., 1990; Sicker et al., 2000; Nuessly et al., 2007; Rostas, 2007; Erb et al., 2009a,b; Butrón et al., 2010). Our previous study showed that exogenous JA application to AG parts of the corn variety 'Gaoyou 115' for 1 wk could significantly increase the quantities of defense chemicals and induce expression of defense-related genes in both leaves and roots (Feng et al., 2010). Similar findings were obtained when JA was applied to BG parts, or simultaneously applied to AG plus BG parts of the corn (Feng et al., 2010). It is, however, still unclear whether such induced defenses can cause allocation costs after different plant parts are treated continuously with JA.

The specific objective of this study was to investigate whether JA application to different parts of corn would cause allocation costs. Costs of induced defense responses typically are measured as reduction in plant fitness (e.g., growth or reproduction associated parameters). In the present study, JA was applied continuously to different parts of the corn variety 'Gaoyou 115' every 4 d for 2 wk or 4 wk in order to investigate the costs of induced defense responses. Quantities of DIMBOA and total amounts of phenolic compounds (here referred to as "total phenolics") were chosen as indicators for induced defense responses. Growth-associated parameters such as plant height, leaf area, root length, root area, shoot biomass, and root biomass were selected as indicators for allocation costs.

Methods and Materials

Materials Seeds of corn cultivar 'Gaoyou 115' were obtained from the College of Agriculture and Biotechnology, China Agricultural University, China. Soil used was collected from the surface layer soil (0–20 cm) in the corn land of South China Agricultural University, Guangzhou, China. Soil samples were air dried at room temperature, sieved through a mesh (1 mm diam), and stored for further use. The soil contained 18.2 $g{\cdot}kg^{-1}$ organic matter, 0.9 $g{\cdot}kg^{-1}$ total N, 0.70 $g{\cdot}kg^{-1}$ total P, 16.75 $g{\cdot}kg^{-1}$ total K, and had a pH 6.16.

Plant growth and management Corn seeds were germinated in the laboratory in plastic pots (18×18 cm diam) containing 2500 g soil with 3 seeds per pot. One seedling in each plastic pot was selected for further growing. All plants were cultivated in a glasshouse, and supplied with 50 ml water every 2 d. In addition, pots were irrigated with 50 ml nutrient solution every 7 d [2 mM KNO_3, 0.5 mM $Ca(NO_3)_2\ 4H_2O$, 0.75 mM $MgSO_4\ 7H_2O$, 0.5 mM KH_2PO_4, 0.25 mM NaCl, 0.25 mM K_2SO_4, 60 μM Fe-Na EDTA, 50 μM H_3BO_3, 15 μM $MnCl_2\ 4H_2O$, 2 μM $ZnSO_4\ 7H_2O$, 0.25 μM $CuSO_4\ 5H_2O$, and 0.2 μM $Na_2MoO_4\ 2H_2O$]. The first JA treatment began when plants had two fully expanded leaves.

Treatment and sampling of plants Corns plants were subjected to four treatments: (1) CK1/CK2, control without JA application on either AG or BG parts; (2) JA1/CK2, JA application to AG parts; (3) CK1/JA2, JA application to BG parts; and (4) JA1/JA2, simultaneous JA application to AG plus BG parts. All fully expanded leaves of corn plants were treated by brushing with water solution (CK1/) or 100 μM JA solution (JA1/), both containing 0.02 % ethanol and 0.05 % Tween-20. Roots of corn plants were irrigated

with 50 ml water solution (/CK2) or 10 μM JA solution (/JA2), both containing 0.002 % ethanol. Jasmonic acid was applied continuously to AG parts, or BG parts, or AG plus BG parts every 4 d after the first JA treatment.

Plants were harvested 2 or 4 wk after the first JA treatment. Leaves (the 3rd leaf from top) and roots of the 4 plants from each treatment were harvested at random to determine the contents of DIMBOA and total phenolics. Shoots and roots of the 6 plants from each treatment were harvested at random to analyze growth associated parameters including plant height, leaf area, root length, root area, shoot biomass (dry weight), and root biomass (dry weight).

Determination of DIMBOA content The procedure of sample preparation for DIMBOA measurement was slightly modified from Ni and Quisenberry (2000). Samples were weighed and ground into powder with a mortar in 10 ml distilled water. Aqueous extracts were incubated for 20 min, and samples were diluted with methyl alcohol in a ratio of 1:1. The methanol-diluted extract was centrifuged at 12 000 $r \cdot min^{-1}$ for 15 min and then filtered. The filtrate was evaporated to dryness under vacuum. The residue was dissolved in 2 ml mixed solution (acetonitrile: 0.5 % aqueous acetic acid, 1: 1, v/v). Extracts were filtered through 0.45 μm membrane filters, and then samples were stored at −20 °C for further measurement.

DIMBOA concentrations in samples were quantified by High Performance Liquid Chromatography (HPLC) (Agilent 1100, USA) [column, Hypersil ODS C18 column (250×4 mm)] with a DAD detector by using external standard curves. Gradient elution was performed with a gradient of A (acetonitrile) and B (0.5 % aqueous acetic acid), i.e., 25–45 % of A from 0–10 min and 45–25 % of A from 10–15 min. Solvent flow rate was set at 1 $ml \cdot min^{-1}$. The injection volume was 20 μl, and the detection wavelength was 262 nm. DIMBOA concentrations in leaves and roots were determined according to the standard calibration curve obtained by measuring peak areas of a series of concentrations of DIMBOA standard samples.

Determination of total phenolics Total phenolics were quantified according to Randhir and Shetty (2005) and determined as gallic acid equivalents. Samples were weighed and ground into powder in liquid nitrogen, soaked in 10 ml of 95 % ethanol, and then kept in a freezer for 48 h. The sample was centrifuged at 12 000 $r \cdot min^{-1}$ for 10 min and filtered. The filtrate (1 ml) was transferred into a test tube, with an addition of 1 ml of 95 % ethanol, 5 ml of distilled water, and 0.5 ml of Folin–Ciocalteu phenol reagent. After an incubation period of 5 min, 1 ml of 5 % Na_2CO_3 was added, mixed well, and kept in dark for 1 h. Samples were vortexed, and absorbance was measured at 725 nm using a UV spectrophotometer.

Determination of growth parameters Two or 4 wk after JA treatment, samples were separated into leaves, stems, and roots after the plant height was recorded. Leaf area was measured using a LI-3000 Area Meter (LI-COR, Walz Co., OR, USA). Then, the samples (leaves and stems) were dried in a forced-air oven at 75 °C for 48 h, and shoot biomass was determined. Root length and root area were measured using special photo-analysis software (WinRHizo, Canada, Regent Instruments Company). After root length and root area were analyzed, samples were dried in a forced-air oven at 75 °C for 48 h, and root biomass was determined.

Statistical analysis Data of all measurements at the same sampling time were analyzed by one-way analyses of variance (ANOVA) using SAS 9.0. Means values were considered significantly different at $P<0.05$ as determined by Duncan's Multiple Range Test (DMRT).

Results

Effects of continuous JA application to different parts of corn on DIMBOA content After leaves or roots were continuously treated with JA, DIMBOA content in leaves increased, but that in roots significantly decreased at the 4th week after the first treatment (Fig. 1). Jasmonic acid application to AG parts for 2 wk led to a remarkable increase of DIMBOA content by 152 % in leaves as compared to the control; meanwhile JA application to AG parts for 4 wk led to a decrease of DIMBOA content by 30 % in roots. JA application to BG parts for 2 or 4 wk triggered a significant increase of DIMBOA by 163 % or 112 % in leaves, while DIMBOA content in roots was reduced by 21 % after JA application for 4 wk. Simultaneous JA treatment to AG plus BG parts for 2 wk elicited an increase of DIMBOA content by 170 % in leaves, but content in roots was reduced by 32 % after simultaneous JA treatment for 4 wk.

Effects of continuous JA application to different parts of corn on total phenolics Continuous JA application resulted in an increase of total phenolics in leaves compared to the non-treated control, while total phenolics in roots was affected depending on time period and plant parts exposed to JA (Fig. 2). Jasmonic acid exposure to AG parts for 2 or 4 wk led to a significant increase of total phenolics by 21 % or 24 % in leaves; meanwhile, total phenolics in roots increased by 34 % after JA exposure to AG parts for 4 wk. Total phenolics in roots was notably reduced by 23 % after JA exposure to BG parts for 2 wk; whereas that in leaves increased by 17 % after JA exposure to BG parts for 4 wk. Simultaneous JA exposure to AG plus BG parts for 2 wk led to an increase of 39 % of total phenolics in leaves, and an increase of 39 % in roots in 4 wk.

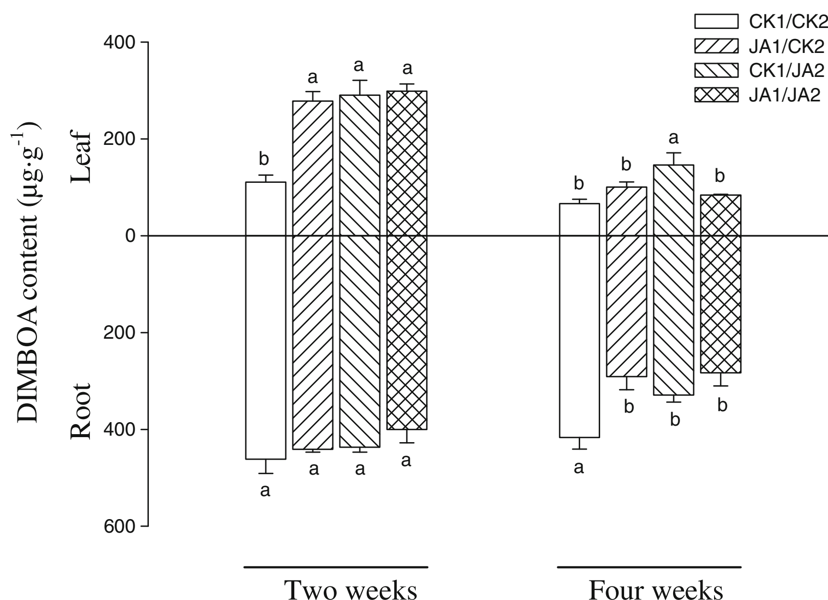

Fig. 1 Effects of continuous jasmonic acid (JA) application to different parts of corn on DIMBOA content (means+standard error). CK1 or JA1 = treatment of aboveground (AG) plant parts. CK2 or JA2 = treatment of belowground (BG) plant parts. CK1: treatment with water; JA1: treatment with 100 μM JA solution (water and JA solution both containing 0.02 % ethanol and 0.05 % Tween-20). CK2: treatment with water; JA2: treatment with 10 μM JA solution (water and JA solution both containing 0.02 % ethanol). For the same part of plant sampled at the same time, means followed by the same letter are not significantly different at $P<0.05$ (Duncan's Multiple Range Test, DMRT)

Effects of continuous JA application to different parts of corn on plant growth parameters In general, continuous JA application to corn plants changed plant growth parameters by reducing them in the 2nd wk but increasing them in the 4th wk (Table 1). In contrast to the control, JA application to AG parts for 2 wk led to an increase of leaf area by 5 %, to a reduction of root length by 58 %, and of root area by 22 %. However, JA1/CK2 treatment induced an increase of plant height by 7 % for 4 wk. JA treatment to BG parts for 2 wk led to a reduction of leaf area by 5 %, of root length by 34 %, and of root area by

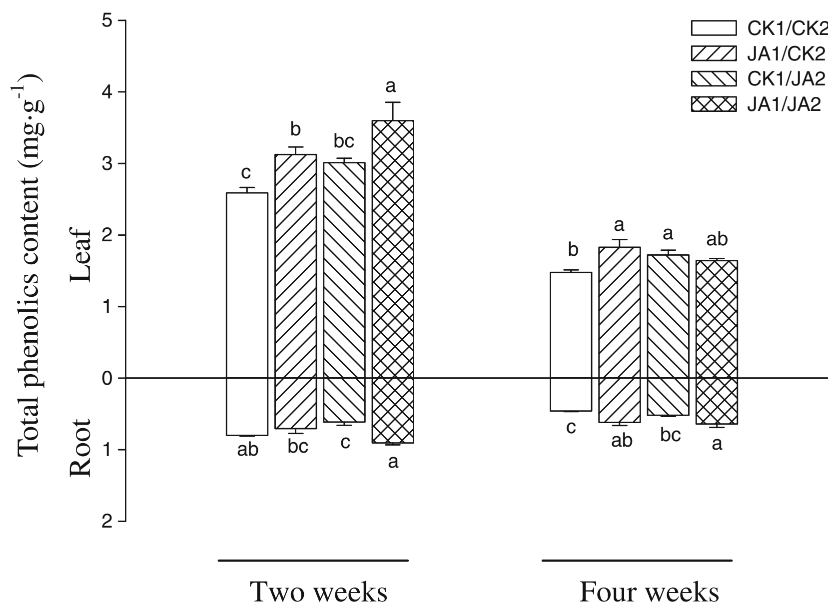

Fig. 2 Effects of continuous jasmonic acid (JA) application to different parts of corn on total phenolics content (means+standard error). CK1 or JA1 = treatment of aboveground (AG) plant parts. CK2 or JA2 = treatment of belowground (BG) plant parts. CK1: treatment with water; JA1: treatment with 100 μM JA solution (water and JA solution both containing 0.02 % ethanol and 0.05 % Tween-20). CK2: treatment with water; JA2: treatment with 10 μM JA solution (water and JA solution both containing 0.02 % ethanol). For the same part of plant sampled at the same time, means followed by the same letter are not significantly different at $P<0.05$ (Duncan's Multiple Range Test, DMRT)

Table 1 Effects of continuous jasmonic acid (JA) application to different parts of corn on plant growth parameters

JA-treated duration	Treatments	Plant height (cm)	Leaf area (cm^2)	Root length (cm)	Root area (cm^2)
Two weeks	CK1/CK2	93.3±1.4 a	725.0±13.7 b	1651.2±42.0 a	737.0±31.5 a
	JA1/CK2	93.9±1.3 a	763.8±7.8 a	698.5±18.9 c	573.6±11.7 b
	CK1/JA2	93.7±1.8 a	688.7±7.1 c	1083.9±21.5 b	626.2±6.9 b
	JA1/JA2	92.5±1.2 a	690.7±11.6 c	1044.4±28.2 b	467.7±21.4 c
Four weeks	CK1/CK2	123.0±1.5 b	1669.5±10.1 a	2261.7±51.5 c	921.8±41.6 b
	JA1/CK2	131.5±1.9 a	1646.6±37.3 a	2603.9±234.1 bc	901.4±50.2 b
	CK1/JA2	130.3±1.9 a	1598.2±21.7 a	2797.9±97.7 b	990.8±45.7 ab
	JA1/JA2	127.2±1.4 ab	1639.4±8.4 a	3373.4±41.5 a	1077.2±17.4 a

The data in the table are means±standard error. CK1 or JA1 = treatment of aboveground (AG) plant parts. CK2 or JA2 = treatment of belowground (BG) plant parts. CK1: treatment with water; JA1: treatment with 100 μM JA solution (water and JA solution both containing 0.02 % ethanol and 0.05 % Tween-20). CK2: treatment with water; JA2: treatment with 10 μM JA solution (water and JA solution both containing 0.02 % ethanol). Means in a column with the same letters are not significantly different at $P<0.05$ (Duncan's Multiple Range Test, DMRT).

15 %. In contrast, CK1/JA2 treatment increased plant height by 6 % and root length by 24 % for 4 wk. Simultaneous JA application to AG plus BG parts for 2 wk led to a reduction of leaf area by 5 %, of root length by 37 %, and of root area by 37 %; but JA1/JA2 treatment elicited an increase of root length by 49 % and of root area by 17 % for 4 wk.

Effects of continuous JA application to different parts of corn on biomass After corn plants were continuously treated with JA solution, shoot biomass slightly increased; root biomass significantly decreased after treatment for 2 wk but both increased significantly 4 wk after the onset of the treatment (Fig. 3). Jasmonic acid treatment to AG parts led to an increase of shoot biomass by 18 % in 2 wk and 21 % in 4 wk. Additionally, root biomass was reduced by 29 % in 2 wk, but increased by 32 % in 4 wk after JA treatment to AG parts. Jasmonic acid treatment to BG parts led to an increase of shoot biomass by 10 %, and a reduced root biomass by 25 % in 2 wk; root biomass was increased by 22 % after 4 wk of JA treatment. Similarly, shoot biomass increased by 23 % or 17 % after simultaneous JA application to AG plus BG parts for 2 or 4 wk; root biomass decreased by 31 % in 2 wk but increased by 33 % in 4 wk.

Discussion

Effects of JA treatment of AG and BG plant parts on corn chemical defense Our study showed that DIMBOA and total phenolics in leaves or roots generally increased 2 or 4 wk after JA treatment to different plant parts, suggesting that the resistance of AG and BG parts to insect herbivores or pathogens was increased.

Continuous 4 wk JA application to AG parts of corn led to an increase of total phenolics in the roots. Similar studies have shown that exogenous JA or MeJA application to AG parts of plants result in a systemic increase in the level of chemical defense in roots (e.g., glucosinolate content in *Brassica campestris*, Ludwig-Müller et al., 1997; activity of proteinase inhibitor in *Nicotiana attenuata*, van Dam et al., 2001; gene expression of proteinase inhibitor (*MPI*) in corn, Feng et al., 2010).

Jasmonic acid application to BG parts of corn systemically increased DIMBOA content in 2 or 4 wk, and total phenolics in leaves in 4 wk. A small number of studies have shown that exogenous JA or MeJA application to BG parts of plants can cause a systemic increase in the level of chemical defense in leaves (e.g., glucosinolate content of *Brassica oleracea*, van Dam et al., 2004; activity of proteinase inhibitor of *Nicotiana attenuata*, van Dam et al., 2001; gene expression of cathepsin D inhibitor (*Cdi*) and proteinase inhibitor II (*Pin2*) of *Solanum tuberosum*, Dammann et al., 1997; gene expression of *MPI* and farnesyl pyrophosphate synthase (*FPS*), and DIMBOA content of corn, Feng et al., 2010). Thus, it can be hypothesized that signals of JA are transferred from AG parts to BG parts, and also from BG parts to AG parts, hence affecting the plant's chemical defense.

Effects of JA treatment of AG and BG corn plant parts as a factor of sampling time Continuous JA application to AG parts or BG parts, or simultaneous application to AG plus BG parts for a longer time (4 wk) enhanced chemical defense levels in both leaves and roots of corn. The JA-induced defense responses in corn were coupled with growth-associated costs in the early stage, but not in the later stage when induced defense responses caused no cost and even exerted obvious benefit. For example, continuous JA application to AG parts for 2 wk increased DIMBOA and total phenolics in leaves, increased leaf area and shoot biomass, but reduced root length, root area, and root biomass. These findings suggest that the increase of defense levels in AG parts was at the cost of biomass accumulation

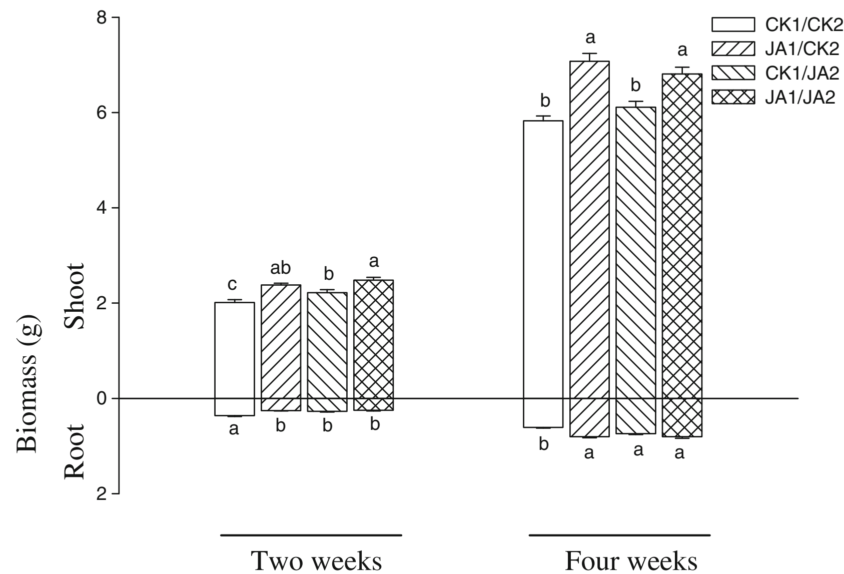

Fig. 3 Effects of continuous jasmonic acid (JA) application to different parts of corn on biomass (means+standard error). CK1 or JA1 = treatment of aboveground (AG) plant parts. CK2 or JA2 = treatment of belowground (BG) plant parts. CK1: treatment with water; JA1: treatment with 100 μM JA solution (water and JA solution both containing 0.02 % ethanol and 0.05 % Tween-20). CK2: treatment with water; JA2: treatment with 10 μM JA solution (water and JA solution both containing 0.02 % ethanol). For the same part of plant sampled at the same time, means followed by the same letter are not significantly different at $P<0.05$ (Duncan's Multiple Range Test, DMRT)

in BG parts of corn. On the other hand, after AG parts were continuously treated with JA for 4 wk, total phenolics in both leaves and roots increased, while DIMBOA in roots decreased, and plant height, shoot biomass, and root biomass increased as compared with the control. Our results showed that JA application to different parts of corn plants inhibit the accumulation of root biomass in the early stage. However, this effect vanished after 4 wk of JA application. This suggests that the negative effects of JA on root growth are only temporary. Van Dam et al. (2004) discovered that after JA was applied to AG or BG parts, or simultaneously applied to AG plus BG parts of *Brassica oleracea* for 1 wk, glucosinolate content in leaves significantly increased, but shoot biomass and root biomass were not affected (van Dam et al., 2004). This is different from our finding, which may be due to differences in species, the chemicals examined, and/or the time period for treatment.

Effects of JA treatment of AG and BG plant parts on growth of other plants Several studies have investigated the effects of JA application to AG parts on the growth of plants (Cipollini and Sipe, 2001; Redman et al., 2001; Bower et al., 2005; Heijari et al., 2005; Barbosa et al., 2008; Schmidt et al., 2010). Exogenous MeJA application to AG parts of Scots pine seedlings significantly decreased seedling height, shoot fresh weight, root fresh weight, and net photosynthesis when compared to control, but no difference was observed in root length (Heijari et al., 2005), indicating apparent allocation costs. Nevertheless, recent studies have demonstrated that induced defense responses caused by JA or MeJA application to AG parts require no growth-associated allocation costs, since JA or MeJA application did not affect the growth of the root tip (V_{Tip}) of *Arabidopsis thaliana* (Schmidt et al., 2010), root dry weight or shoot dry weight of sugarcane (Bower et al., 2005), plant height of *Brassica kaber* (Cipollini and Sipe, 2001), or shoot biomass or root biomass of tomato (Redman et al., 2001). Barbosa et al. (2008) observed that JA application to AG parts of cotton increased shoot dry weight, but had no obvious effects on plant height.

Induced defense responses that have been triggered by JA application to BG parts of plants are known to be costly (Bower et al., 2005; Schmidt et al., 2010). For instance, continuous MeJA application to BG parts of sugarcane plants posed a significantly adverse effect on plant growth by decreasing root dry weight and shoot dry weight (Bower et al., 2005). The application of JA or MeJA to BG parts reduced root growth (V_{Tip}) in *Arabidopsis thaliana* (Schmidt et al., 2010). Yet, there are some contrasting results that suggest such defense responses require no apparent costs. JA application to BG parts of *Brassica oleracea* systemically increased the content of glucosinolates, particularly aliphatic glucosinolates in leaves, but had no significant effects on shoot biomass or root biomass (van Dam et al., 2004).

In the present study, costs were detected in the early stage of JA-induced defense responses in corn. Continuous JA application to BG plant parts resulted in reduced accumulation of root biomass, a finding that is consistent with previous results obtained with other plant species (Bower et al., 2005; Schmidt et al., 2010). However, in the late stage (after 4 wk) JA-induced defense responses of corn caused no

apparent cost and even exerted obvious benefit. Hence, allocation costs of induced defense responses of JA application to different parts seem to be dependent on the plant species, treatment time, and parameters measured.

Other Costs of JA-treatment Reproduction-associated allocation costs of JA induced defense have been described in earlier studies (Agrawal et al., 1999; Thaler, 1999; Cipollini and Sipe, 2001; Redman et al., 2001). Those studies suggested that exogenous JA application to AG parts of tomato increased levels of two defensive enzymes, polyphenol oxidase (PPO) and peroxidase (POD) of leaves, but led to fewer fruits, longer ripening time, delayed fruit-set, fewer seeds per plant, and fewer seeds per unit of fruit weight (Redman et al., 2001). Jasmonic acid application to AG parts of *Arabidopsis thaliana* increased trypsin inhibitor activity in leaves by 41 %, and reduced total seed mass by 18 % overall (Cipollini, 2002). Exogenous JA application to AG parts of *Brassica kaber* increased trypsin inhibitor activity, peroxidase activity, and glucosinolate concentration in leaves, but slightly reduced time to first flower (Cipollini and Sipe, 2001). However, Agrawal et al. (1999) showed that costs of JA induced defenses in terms of seed number and seed weight in wild radish were not significant, although JA treatment delayed flowering and reduced pollen production. Thaler (1999) also found no reduction in yield of tomato plants when AG parts were treated with JA.

Future studies should be directed to whether JA induced defense responses require reproduction-related allocation costs in different parts of corn. In-depth studies are needed to elucidate other costs such as genetic, autotoxicity, opportunity, and ecological costs (van Dam and Baldwin, 2001; Heil, 2002; Strauss et al., 2002; Steppuhn and Baldwin, 2008; Heil, 2011; van Dam and Heil, 2011). This will provide a more solid base for the understanding of the overall effects of JA application and will be helpful for the development of new strategies in plant pest control.

In summary our results indicate that signals of JA can transfer from AG parts to BG parts of corn, and *vice versa*. Jasmonic acid-induced defense responses require growth related allocation costs of roots in the early stage (2 wk), while they display obvious benefit in the later stage (4 wk), which implies that induced plants can compensate, at least in part, for the initial growth depression eventually, and that resources can be shifted back to growth again. The induced tolerance response in roots stimulated by continuous JA exposure may benefit the growth of plants in the later stage. Studies are needed to investigate the mechanisms underlying such phenomena.

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RAPID COMMUNICATION

Evolutionary Potential of Root Chemical Defense: Genetic Correlations with Shoot Chemistry and Plant Growth

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Abstract Root herbivores can affect plant fitness, and roots often contain the same secondary metabolites that act as defenses in shoots, but the ecology and evolution of root chemical defense have been little investigated. Here, we investigated genetic variance, heritability, and correlations among defensive phenolic compounds in shoot vs. root tissues of common evening primrose, *Oenothera biennis*. Across 20 genotypes, there were roughly similar concentrations of total phenolics in shoots vs. roots, but the allocation of particular phenolics to shoots vs. roots varied along a continuum of genotype growth rate. Slow-growing genotypes allocated 2-fold more of the potential pro-oxidant oenothein B to shoots than roots, whereas fast-growing genotypes had roughly equivalent above and belowground concentrations. Phenolic concentrations in both roots and shoots were strongly heritable, with mostly positive patterns of genetic covariation. Nonetheless, there was genotype-specific variation in the presence/absence of two major ellagitannins (oenothein A and its precursor oenothein B), indicating two different chemotypes based on alterations in this chemical pathway. Overall, the presence of strong genetic variation in root defenses suggests ample scope for the evolution of these compounds as defenses against root herbivores.

Keywords Allocation · Below-ground · Chemical defense · Growth · Phenolics · Roots · Trade-off

Introduction

Roots and shoots often both contain defense phytochemicals (Rasmann and Agrawal, 2008), suggesting that roots are chemically defended against herbivores. However, allocation patterns among shoots vs. roots can vary widely depending on the class and function of chemical compounds investigated, plant family and species, plant chemotype within a species, and damage history (Kaplan et al., 2008). Optimal defense theory predicts that allocation patterns are adaptive and can illuminate the forces driving natural selection for chemical defenses, but there are still relatively few investigations comparing chemical defense levels in above vs. belowground tissues (van Dam, 2009). Furthermore, although root defense chemicals may evolve if there is heritable variation that affects plant fitness, genotypic variation for root chemicals, and potential trade-offs with levels of shoot defenses, have been little explored.

Here, we quantified the concentrations of defensive phenolic compounds in the shoots and roots of 20 genotypes of common evening primrose *Oenothera biennis* L. (Onagraceae). We focused on ellagitannins, a group of phenolics that have a high potential for toxicity via their oxidative capacity (Salminen and Karonen, 2011). We examined the genetic relationships between defense investment and growth by asking three questions: 1) Are phenolic concentrations and plant growth rate in shoots and roots heritable? 2) What is the genetic covariation

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within and among phenolic concentrations in shoots vs. roots? 3) Are there tissue-specific trade-offs between defense levels and plant growth rate?

Methods and Materials

Oenothera biennis has high genetic variability for functional traits, including life-history strategy, biomass allocation among roots vs. shoots, and secondary chemistry (reviewed in Johnson, 2011). Secondary chemicals are dominated by phenolics, in particular hydrolyzable tannins, ('ellagitannins') ranging in size from dimers to undecamers (Karonen et al., 2010). Variation in life-history strategy and the abundance of phenolics has been linked to herbivory by both aboveground insects (Johnson et al., 2009) and belowground mammals (Parker et al., 2010), but to date the chemical profiles of phenolics in above vs. belowground tissues of *O. biennis*, and their relationship to plant growth strategy, have not been reported.

We conducted a controlled growth experiment with 20 genotypes from separate *O. biennis* populations around Ithaca, New York (distinguished by microsatellites, Johnson et al., 2009), to investigate the interactions between plant growth rate and defensive chemistry. We germinated seeds from each genotype (N=3–6 per genotype) and grew them for 51–57 d on an open-air rooftop patio on the Cornell University campus, after which we collected, froze, lyophilized, and finely ground the leaf and root tissues from each plant. We analyzed phenolics in above and belowground plant tissues with high-performance liquid chromatography with a diode array detector following the methods of Johnson et al. (2009), where hydrolyzable tannins were quantified in pentagalloyl glucose equivalents (280 nm acquisition wavelength) and flavonoid glycosides in quercetin equivalents (349 nm). Plant growth rate was calculated as the dry biomass of roots plus shoot tissues divided by the number of days the plant had been alive.

We used restricted maximum likelihood (REML) to estimate the variance explained by plant genotype for each phenolic compound, total phenolics, and plant growth rate, with genotype included as a random effect, and significance tested using log-likelihood ratio tests. We calculated broad-sense heritability as $H^2=V_g/V_T$, where V_g is the total genetic variance (additive and nonadditive) and V_T is the total phenotypic variance (genetic and environmental). We assessed genetic covariation among traits using Pearson correlation coefficients on least squares mean values for all 300 pairwise combinations of phenolic correlations across genotypes (N=20 genotypes per correlation). The genetic covariance among traits was calculated according to the equation: $cov_g=r_g(G_{11}G_{22})^{0.5}$, where r_g is the genetic Pearson correlation coefficient between two traits, and G_{ii} is the genetic variance of each trait from REML. The statistical significance of genetic covariances was assessed as the P-value from the t-statistic of r_g. Binomial expansion tested whether the frequency of significant genotypic correlations differed from random expectations, and ANOVAs determined whether the strength of significant correlations differed among shoots and roots.

Results

Concentrations of total phenolics (i.e., sum of individual phenolics) and two ellagitannins comprising the bulk of the phenolics (≥68 %, oenothein B and A) were similar across shoots and roots (Fig. 1, Table S1). The composition of minor phenolics, however, differed. Shoot tissues contained chlorogenic acid and at least seven flavonoids that were not in root tissues, whereas root tissues contained at least eight ellagic acids that were not in shoot tissues (Table S1).

There was significant genetic variation for growth rate and phenolic concentrations. All but one of the phenolic groupings (a small group of unidentified flavonoids) were significantly heritable, with nearly 70 % of the variation in total above- and belowground phenolics attributable to plant genotype (Table S1). Qualitatively, four genotypes contained virtually no oenothein B in shoots, instead containing predominantly oenothein A (Fig. 1A, B). Plant growth rate varied 3-fold among genotypes (Fig. 1D–F), with half attributable to heritable variation (H^2=0.50, P<0.001).

There were mostly positive patterns of genetic covariation among phenolics. Out of 300 pairwise genetic associations among phenolics, we found 54 significant correlations (P≤0.05), 43 of which were positive (Table S2, overall P< 0.001, binomial expansion test). Genetic correlations among phenolic compounds generally were stronger within root tissues (mean r=0.29±0.04) than correlations within shoot tissues (mean r=0.04±0.04), or between shoot and root tissues (mean r=0.09±0.02; $F_{2,297}$, P<0.001, ANOVA). Phenolics also were positively correlated with plant growth rate, both within shoot tissues (mean r=0.26±0.05) and within root tissues (mean r=0.39±0.07), with no difference in the strength of these correlations across shoots vs. roots ($F_{1,23}$, P=0.154, ANOVA).

Slow-growing genotypes contained roughly twice as much oenothein B in their shoots vs. root tissues, whereas fast-growing genotypes had equivalent levels in shoots and roots (Fig. 1D).

Discussion

Our study demonstrates that although total levels of defense compounds may be similar across roots and shoots, this

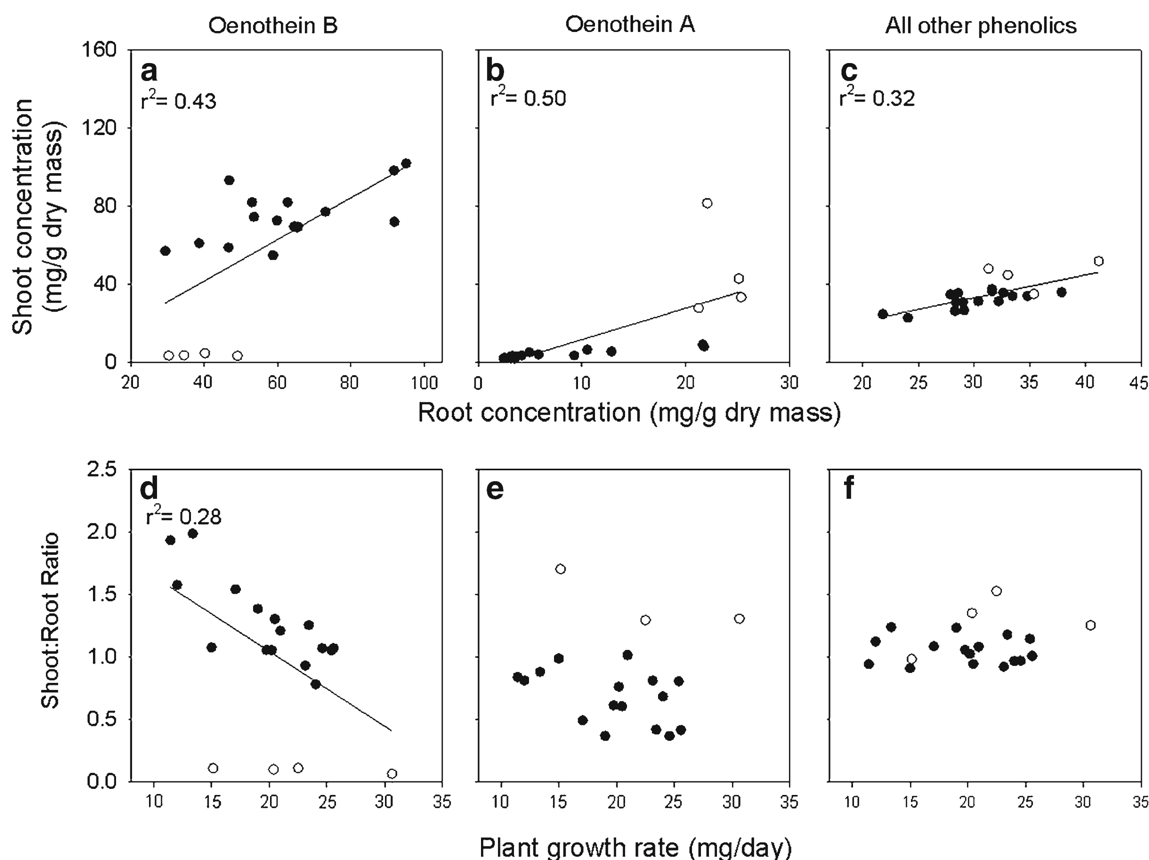

Fig. 1 Correlations between the concentrations of the major ellagitannins oenothein B and A and all other phenolics in shoots vs. root tissues of evening primrose, *Oenothera biennis* (Panels A–C). Each symbol is a mean value for an individual genotype (N=20). Four genotypes (open symbols) produced distinct shoot chemical profiles that were abundant in oenothein A but generally lacked its precursor oenothein B. Panels D–F show correlations between the ratio of phenolics in shoot over root tissues vs. the mean growth rate (mg/day) for each genotype. All regression lines are significant at $P<0.05$

pattern may underestimate differential patterns across genotypes. For example, one of the findings in our study was the observation of two *Oenothera biennis* chemotypes based on the two major compounds oenothein B and A. Twenty percent of genotypes produced essentially no oenothein B in their shoots, instead producing only oenothein A. Oenothein B is the dimeric precursor to the trimer oenothein A (Karonen et al., 2010). Thus, we posit the presence of a genetic polymorphism affecting either the structure or regulation of the enzyme that causes trimerization of oenothein B to oenothein A. Genotypes lacking oenothein B in their shoots retained it in their roots, and we found ellagic acids only in root tissues while flavonoids were only in leaf tissues. These differences in above vs. belowground phytochemical profiles could result from physiological constraints on allocation patterns or from differential selection pressures.

Our estimates of heritability and genetic variation for growth and phenolic compounds are similar to other published values in *O. biennis* (Johnson et al., 2009), indicating strong genetic differentiation for life history strategy and chemical defense. However, we saw few of the growth-defense trade-offs that define allocation costs to defense, and we did not find significant evidence of negative trade-offs among phenolic compounds. Instead, we found generally positive covariation among compounds, and a generally positive relationship between chemical defense and genotype growth rate. Similarly, other studies have failed to detect a negative relationship between growth and defense, or shown a positive relationship (Koricheva, 2002), consistent with analyses showing that the linkages between herbivory, chemical defense, and growth rate are complex and constrained by numerous genetic and physiological constraints (Carmona et al., 2011). Nevertheless, slow-growing genotypes invested more heavily in aboveground defenses, suggesting a tissue-specific trade-off that could be indicative of an adaptive response to aboveground vs. belowground herbivory that needs further exploration. Overall, the presence of strong genetic variation in root phytochemistry of *O. biennis* suggests ample raw material for natural selection to drive the evolution of root chemical defenses against root herbivores.

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Too Low to Kill: Concentration of the Secondary Metabolite Ranunculin in Buttercup Pollen does not Affect Bee Larval Survival

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Abstract Growing evidence suggests that the freely accessible pollen of some plants is chemically protected against pollen-feeding flower visitors. For example, a diet of pollen from buttercup plants (*Ranunculus*) recently was shown to have a deleterious effect on developing larvae of several bee species not specialized on *Ranunculus*. Numerous *Ranunculus* species contain ranunculin, the glucosyl hydrate form of the highly reactive and toxic lactone protoanemonin, that causes the toxicity of these plants. We tested whether the presence of ranunculin is responsible for the lethal effects of *R. acris* pollen on the larvae of two bee species that are not *Ranunculus* specialists. To investigate the effect on bee larval development, we added ranunculin to the pollen provisions of the *Campanula* specialist bee *Chelostoma rapunculi* and the Asteraceae specialist bee *Heriades truncorum,* and allowed the larvae to feed on these provisions. We quantified ranunculin in pollen of *R. acris* and in brood cell provisions collected by the *Ranunculus* specialist bee *Chelostoma florisomne*. We demonstrated that although ranunculin was lethal to both tested bee species in high concentrations, the concentration in the pollen of *R. acris* was at least fourfold lower than that tolerated by the larvae of *C. rapunculi* and *H. truncorum* in the feeding experiments. Ranunculin concentration in the brood cells of *C. florisomne* was on average even twentyfold lower than that in *Ranunculus* pollen, suggesting that a mechanism different from ranunculin intoxication accounts for the larval mortality reported for bees not specialized on *Ranunculus* pollen.

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Keywords *Ranunculus* · Ranunculin · Protoanemonin · Pollen · Plant defense · Osmiini · *Chelostoma* · *Heriades* · Megachilidae

Introduction

Bees, including solitary native species, provide important ecosystem services as pollinators of flowering plants (Kremen et al., 2007). However, they exact considerable costs on plants, because they require enormous quantities of pollen to feed their broods (Müller et al., 2006). Flowers are expected to balance the need to attract bees for pollination with the need to restrict extensive pollen losses (Praz et al., 2008a; Sedivy et al., 2011). Various mechanisms have evolved that limit pollen loss by narrowing the spectrum of pollen-collecting flower visitors (Westerkamp, 1997; Westerkamp and Classen-Bockhoff, 2007) or by reducing the pollen quantity withdrawn by pollinators per flower visit. Examples of these mechanisms include specialized anthers, pollen-concealing flower structures, and portioned pollen release over extended time periods (Vogel, 1993; Harder and Barclay, 1994; Müller, 1996; Castellanos et al., 2006).

Growing evidence suggests that some plants that possess freely accessible pollen might also chemically protect the pollen. For example, the pollen of *Stryphnodendron polyphyllum* (Mimosoideae) is poisonous to the larvae of the honeybee (De Carvalho and Message, 2004). Similarly, the pollen of *Ranunculus* (Ranunculaceae) did not support larval development of three strict pollen-specialist bees specialized on

Campanula, Echium, and Asteraceae, respectively, as well as one highly pollen-generalist bee (Praz et al., 2008a; Sedivy et al., 2011). The larval mortality pattern in these four species was characterized by rapid death upon onset of feeding, suggesting that *Ranunculus* pollen may contain secondary metabolites that are toxic to bee larvae. *Ranunculus* pollen also is known to be toxic to adult honeybees, which suffer high rates of mortality when feeding primarily on *Ranunculus* pollen, a phenomenon known as "Bettlacher May sickness" (Morgenthaler and Maurizio, 1941).

Fresh plants of the genus *Ranunculus* are known for their toxic effect on livestock (Kingsbury, 1964). This effect arises from high concentrations of the glucoside ranunculin, the precursor of the toxic protoanemonin, present in the plant tissue (Fig. 1) (Benn and Yelland, 1968). The content of ranunculin in *Ranunculus* species normally oscillates around 10 mg per g dry weight (d.w.) (Ruijgrok, 1966), but can reach nearly 200 mg/g d.w. in *R. cymbalaria* (Bai et al., 1996). Upon infliction of mechanical damage to plant tissue, the non-toxic ranunculin is hydrolyzed by endogenous β-glucosidase, an enzyme stored in the vacuole (Mauch and Staehelin, 1989), to yield the highly reactive anhydroaglycone protoanemonin (2,3-dihydro-5-methylidenefuran-2-one), a volatile lactone (Hill and Van Heyningen, 1951). When ingested, protoanemonin can cause gastric distress in livestock (Kingsbury, 1964). Applied to human skin, protoanemonin may produce erythema and blistering (Benn and Yelland, 1968). In addition, protoanemonin has antimicrobial properties (Campbell et al., 1979; Mares, 1987; Martin et al., 1990) and exhibits insecticidal effects on fly larvae of *Drosophila melanogaster* (Drosophildae), adult beetles of *Tribolium castaneum* (Tenebrionidae), and ant workers of *Pheidole pallidula* (Formicidae) (Bhattacharya et al., 1993; Varitimidis et al., 2006).

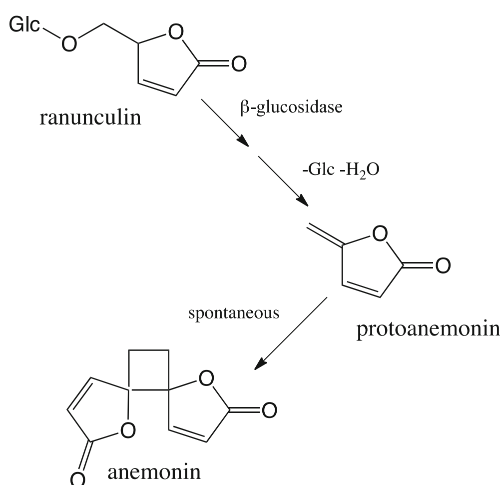

Fig. 1 The transformation of the glucoside ranunculin to the unstable and toxic lactone protoanemonin in plant tissue and spontaneous transformation to anemonin. Modified after Benn and Yelland (1968)

Some herbivorous insects are able to cope with high concentrations of ranunculin in their diet. Larvae of several leaf and stem mining species of agromyzid flies of the genus *Phytomyza* are specialized on *Ranunculus* and other ranunculin-containing genera of the Ranunculaceae, e.g., *Anemone*, *Clematis*, and *Helleborus* (Spencer, 1990). Larvae of *P. ranunculi* and *P. ranunculivora* often are found in leaves of *R. acris* (Pitkin et al., 2010), where they can be exposed to ranunculin concentrations of about 28 mg/g d.w. (Bai et al., 1996). The physiological basis of the ability of these herbivorous insects to tolerate ranunculin and/or protoanemonin remains unknown.

While stems, leaves, and the androecium of *Ranunculus* contain ranunculin in considerable amounts (Ruijgrok, 1966; Bonora et al., 1988; Bai et al., 1996), no attempt has been made to quantify ranunculin in *Ranunculus* pollen. The high relative amounts of protoanemonin released from pollen and anthers of some *Ranunculus* species (Bergström et al., 1995) as well as the high concentration of protoanemonin in the androeceum of *R. ficaria*, which was found to be twice as high as the concentration measured in the whole plant (Bonora et al., 1988), may indicate the presence of substantial quantities of ranunculin in the pollen of *Ranunculus*. Furthermore, by thermal desorption of anthers of several Ranunculaceae, Jürgens and Dötterl (2004) detected high relative amounts of protoanemonin in three *Ranunculus* species, and protoanemonin from *Ranunculus* pollen seems to be used in host-plant recognition by the *Ranunculus* specialist bee *Chelostoma florisomne* (Dobson and Peng, 1997).

We hypothesized that protoanemonin released from the secondary metabolite ranunculin is responsible for the toxicity of *R. acris* pollen to the larvae of bee species that are not *Ranunculus* specialists. To investigate the effect of ranunculin on larval development, we selected two solitary bee species specialized on pollen of plants other than *Ranunculus*. We tested larval performance of *Chelostoma rapunculi*, a *Campanula* pollen specialist, and *Heriades truncorum*, an Asteraceae pollen specialist, on diets consisting of pollen from their natural host plants mixed with ranunculin in various concentrations. In addition, we quantified ranunculin in pollen and flower buds of *R. acris* as well as in brood cell provisions of *Chelostoma florisomne*, which is a pollen-specialist species that collects pollen exclusively from *Ranunculus* flowers (Sedivy et al., 2008).

Material and Methods

Bee Species To assess the effect of ranunculin on larval development of solitary bees, we selected two species belonging to the same taxonomic group (Osmiini; Megachilidae) as the *Ranunculus* specialist *Chelostoma florisomne*. These species, *Chelostoma rapunculi* and *Heriades*

truncorum, are specialized on *Campanula* (Campanulaceae) and on Asteraceae, respectively (Westrich, 1989; Praz et al., 2008b; Sedivy et al., 2008). Neither can develop on a *Ranunculus* pollen diet (Praz et al., 2008a). All these species nest in pre-existing cavities such as insect-bored holes in dead wood and hollow stems, and they can easily be reared in hollow bamboo stalks. Once provisioning of the brood cells with pollen and nectar is complete, an egg is deposited onto the pollen diet, and the female bee closes the cell with a thin wall of clay or resin. Successful larval development ends with spinning a cocoon, in which the bee enters metamorphosis to the adult stage. For the experiments, we used eggs and brood cell provisions from bees in nesting stands on the campus of ETH Zurich.

Bee Larval Performance The larvae of *C. rapunculi* and *H. truncorum* were experimentally reared on a *Campanula* and Asteraceae pollen diet, respectively, obtained from conspecific nests and mixed with ranunculin in five increasing concentrations: 0 (control), 10, 20, 50, and 100 mg per g pollen dry weight (d.w.), henceforth referred to as the control treatment, 10, 20, 50, and 100 mg/g treatment. These concentrations are in line with natural ranunculin concentrations reported for *Ranunculus* plants (Ruijgrok, 1966; Bonora et al., 1988; Bai et al., 1996). Pollen dry weight of the brood cell provisions averages approximately 27% in *C. rapunculi* and 33% in *H. truncorum* (A. Bühler and A. Müller, unpublished). Ranunculin (>99% pure) originating from extractions of *Ranunculus* plants was obtained from Michael H. Benn (University of Calgary, Canada). To prepare the experimental pollen diet, ranunculin was ground to fine powder and thoroughly mixed with the brood cell provisions in a mortar. The mixing process was conducted in a careful and gentle way to prevent destruction of the pollen grains and, thus, to prevent the release of β-glucosidase followed by hydrolysis of ranunculin.

Rearing of bee larvae was conducted in individual artificial brood cells (for details see Sedivy et al., 2011). Freshly completed bee nests were collected daily from the nesting stands. Each egg was detached with a thin spatula from the brood cell provision and transferred onto 60 mg of the experimental pollen diet previously placed into the artificial cell. Larvae hatched and started feeding between 1 and 3 d after transfer onto the experimental pollen diet. Each larva was allowed to feed individually in a single artificial cell to mimic natural conditions. For each species and treatment, 24–31 eggs were transferred. Development took place in a climate chamber (E7 / 2; Conviron, Winnipeg, Canada) in darkness at 25±0.5 °C for 16 h followed by a 4 h gradual decrease to 10±0.5 °C, followed by a 4 h gradual increase back to 25±0.5 °C, at a constant 70±0.5 % relative humidity. Egg hatching, initiation of larval feeding, cocoon completion, and incidences of death were recorded every second day. Survival time was considered the time between onset of feeding and either death or completion of the cocoon. Unhatched eggs were removed from statistical analyses.

Statistical Analysis Kaplan–Meier survival statistics was used to compare larval survival among the different treatments following Lee and Wang (2003). The number of days between hatching and completion of the cocoon was considered as 'censored data'; individuals that died before the completion of the cocoon represented the exact observations for which the event (death) occurred, while those that completed the cocoon were the censored observations. The latter were considered survivors and were withdrawn from survival calculations. To test for differences among survival distributions, the *log-rank test* was applied with Bonferroni correction using the option 'pairwise for each stratum' implemented in the software when comparing two groups. For each species, we tested for differences in survival according to pollen-diet treatment, and larval survival of the two species was compared for each ranunculin concentration. For statistical analyses, SPSS 19.0.0 for Macintosh OS X (SPSS Inc., Chicago, IL, USA) was used.

Ranunculin Recovery To test whether the ranunculin concentration in the experimental pollen provisions remained stable during feeding experiments, we added 10 mg/g of ranunculin to brood cell provisions of *Heriades truncorum* and quantified the ranunculin content by LC-MS analysis (see below) immediately after mixing, after 8 d and after 22 d (N=5 for each time interval). For this experiment, we used the same methodological procedure including climate chamber conditions as for the bee larval performance experiments.

Plant Material Ranunculin was quantified in pollen and flower buds of *Ranunculus acris* as well as in the brood cell provisions of the *Ranunculus* specialist *Chelostoma florisomne*. At each of 8 different locations in Switzerland, which spanned a geographic range of approximately 130 km (comprising locations around Neuchâtel, Solothurn, Aarau, and Zurich), 250 freshly opened flowers of *R. acris* were collected in April 2011. Flowers were bundled and fixed in an upside-down position over a parchment paper cone large enough to collect released pollen. After 24 h, the pollen that accumulated at the bottom of the cone was sieved through a 90 μm-pore sieve and stored at −80°C until extraction and analysis. Our pollen collection procedure closely matched the pollen collecting behavior of bees, which harvest pollen from dehisced anthers before they deposit it in the brood cells. At each of the 8 locations, a single flower bud (close to blooming) was collected from each of 5 different plants, was immediately frozen in liquid nitrogen, and stored at −80°C until extraction and analysis.

At a large nesting site of *C. florisomne* in the surroundings of Neuchâtel (Gletterens), where *R. acris* was the near exclusive pollen source for this species, we collected 11 freshly completed nests. From each nest, the provision of the outermost (i.e., the most recently completed) brood cell was removed and immediately stored at −80°C until extraction and analysis.

Extractions of Plant Material For extraction of ranunculin we basically followed the method described by Bai et al. (1996). All samples, i.e., pollen, flower buds, and brood cell provisions, were freeze-dried and individually extracted with methanol (3×15 ml) by repeatedly and thoroughly grinding in a mortar. To ensure that the mechanical damage inflicted on the plant material did not lead to a significant loss of ranunculin due to the action of β-glucosidase, the grinding was conducted in methanol. To test whether the hard pollen exine was successfully disrupted in order to extract the complete contents of the pollen grains, the ground pollen was examined microscopically. Extracts were filtered through a cotton plug, evaporated to dryness, and stored at −60 °C until LC-MS analysis.

LC-MS Analysis For the LC-MS analysis, the total dried extract was quantitatively dissolved in the mobile phase, and if necessary, an aliquot of the solution was further diluted. High-performance liquid chromatography (HPLC) was performed on an Agilent 1200 HPLC system (Agilent Ltd., Santa Clara, CA, USA) equipped with a binary solvent pump. Separation was performed on a reversed-phase 4.6-mm×250-mm, 5 μm, Phenomenex ODS Aqua column (Phenomenex, Torrance, CA, USA). An isocratic mode with MeOH/H_2O (0.05% ammonium acetate) (6:4) at a flow rate of 1 ml/min (total run time, 5 min) was employed. The sample injection volume was 5 μl.

Mass spectrometry (MS) was performed on an electrospray ionization-quadrupole-time of flight (ESI-Q-TOF) MS system (maXis, Bruker Daltonics, Bexhill-on-Sea, UK). The instrument was operated in a wide-pass quadrupole mode, and the TOF data was collected for *m/z* 50–1300 with low-collision energy of 8 eV. The optimized ion source and mass analyzer conditions were as follows: drying gas, N_2 (99.99%) at 8.0 l/h and temperature of 200 °C; nebulizer pressure 1.6 bar; capillary and endplate voltages 500 V and 4,500 V, respectively; TOF tube voltage 9,880 V; reflection voltage 2,004 V; pusher voltage 1,640 V; MCP detector voltage 2,927 V. The system was mass calibrated in the positive-ion mode using a methanol solution of sodium formate on the enhanced quadratic algorithmic mode.

The signal of the extracted ion chromatogram at *m/z* 299.1 ($[M+Na]^+$) was employed for the quantification of ranunculin as sodium adduct. Quantification was performed using a five-point calibration curve obtained with pure ranunculin using the Data Analysis 4.0 and Quant Analysis 2.0 software (Bruker Daltonics, Bexhill-on-Sea, UK).

Results

Bee Larval Performance All larvae of *Chelostoma rapunculi* died within 4−10 d (median, 6 d) when feeding on the 50 mg/g and the 100 mg/g ranunculin treatment (Fig. 2a), while 29 % of the larvae survived on the 20 mg/g treatment (Table 1). Larval survival did not differ significantly between the control and the 10 mg/g and 20 mg/g treatments, respectively (*log-rank test*, χ^2=5.688, *P*=0.17 and χ^2=3.682, *P*=0.55 after Bonferroni correction), but differed significantly between the 10 mg/g and the 20 mg/g treatment (*log-rank test*, χ^2=17.998, *P*<0.001). The larvae feeding on the control treatment required 20–34 d (median, 28 d) until completion of the cocoons compared to 34–56 d (median, 42 d) on the 10 mg/g treatment and 32–50 d (median, 44 d) on the 20 mg/g treatment.

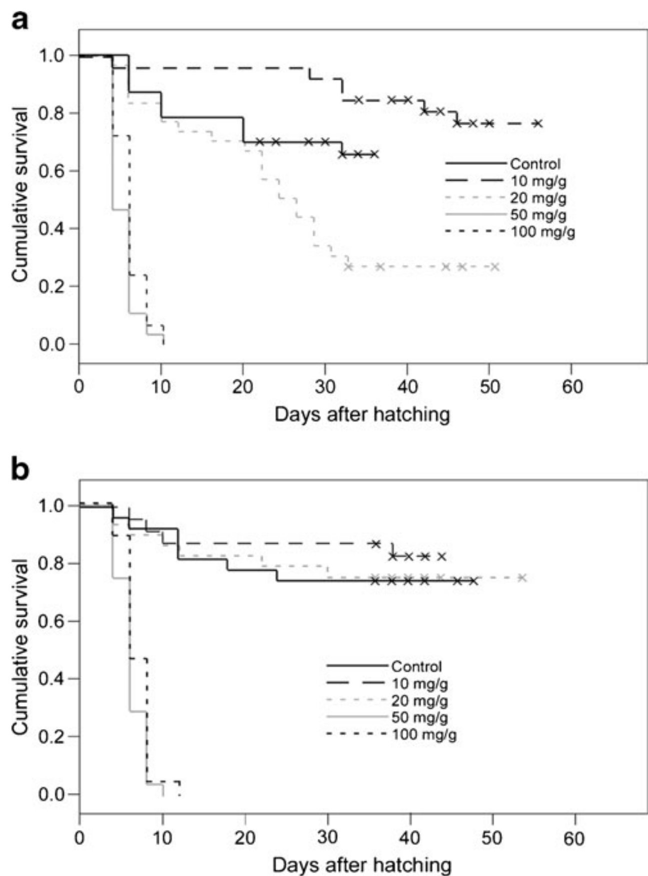

Fig. 2 Cumulative survival of larvae of bees specialized on plants other than *Ranunculus* when reared on their host pollen diet admixed with different quantities of ranunculin. Crosses indicate the days after hatching at which at least one individual reached the cocoon stage (censored data). **a** the *Campanula* specialist *Chelostoma rapunculi*, and **b** the Asteraceae specialist *Heriades truncorum*

Table 1 Larval survival of the two bee species *Chelostoma rapunculi* and *Heriades truncorum* when reared on their host pollen diet mixed with ranunculin

Bee species	Ranunculin concentration (mg/g d.w.)	Hatched eggs (unhatched)	Surviving larvae			Group heterogeneity	
		N	*N*	%	Survival time (days)[a]	*P*	Groups
C. rapunculi	0 (control)	23 (1)	15	65.2	26.30±2.35	<0.001	a, b
	10	28 (2)	22	78.6	49.17±2.59		a
	20	31 (0)	9	29.0	26.71±2.99		b
	50	28 (2)	0	0	5.21±0.30		c
	100	30 (1)	0	0	6.07±0.32		c
H. truncorum	0 (control)	29 (1)	23	79.3	35.52±2.39	<0.001	a
	10	25 (5)	21	84.0	39.37±2.34		a
	20	29 (1)	22	75.9	43.93±3.43		a
	50	24 (6)	0	0	6.17±0.34		b
	100	28 (2)	0	0	6.86±0.32		b

[a] Survival time gives the Kaplan-Meier survival time in days (mean±SEM) of the larvae on each pollen diet. Group heterogeneity was tested with the pairwise *log-rank test* between all treatments. Diets sharing the same letter did not differ significantly at $P<0.05$ (*post hoc test*: pairwise *log-rank test* using Bonferroni corrections)

All larvae of *Heriades truncorum* died within 4–12 d (median, 6 d) when feeding on the 50 mg/g and 100 mg/g ranunculin treatment (Fig. 2b). Larval survival neither differed significantly between the control and the 10 mg/g and 20 mg/g treatments, respectively (*log-rank test*, $\chi^2=0.294$, $P=0.59$ and $\chi^2=0.118$, $P=0.73$ after Bonferroni correction), nor between the 10 mg/g and 20 mg/g treatment (*log-rank test*, $\chi^2=0.549$, $P=0.459$; Table 1). Larvae feeding on the control treatment required 30–42 d (median, 34 d) until completion of the cocoons compared to 36–44 d (median, 38 d) on the 10 mg/g treatment and 36–54 d (median, 42 d) on the 20 mg/g treatment.

A comparison between the two bee species tested revealed largely parallel performances across treatments; no significant differences were observed between the survival of the two species in the control, the 10 mg/g, the 50 mg/g, or the 100 mg/g treatments (*log-rank test*, $\chi^2=1.48$, $P=0.224$; $\chi^2=0.995$, $P=0895$; $\chi^2=3.833$, $P=0.050$; $\chi^2=3.032$, $P=0.082$). In the 20 mg/g treatment, survival of *H. truncorum* larvae was greater than that of *C. rapunculi* larvae (*log-rank test*, $\chi^2=11.347$, $P<0.001$).

Ranunculin Recovery Recovery rate of ranunculin amounted to 62.5–71.0% (mean, 66.0%, $N=5$) immediately after its addition to the experimental pollen provision, to 55.4–70.5% (mean, 60.8%, $N=5$) after 8 d, and to 56.5–72.5% (mean, 63.2%, $N=5$) after 22 d. These results indicate that the concentration of ranunculin mixed to the pollen provisions remained constant over a substantial period of time, and that about 60% of the added 10 mg/g ranunculin was biologically available.

Ranunculin Content To quantify the range of ranunculin in the field-collected samples, we assessed its concentration in pollen, flower buds, and brood cells (Table 2). Ranunculin concentration in the pollen of *Ranunculus acris* (mean, 0.55 mg/g) was almost forty times lower than that in the flower buds (mean, 19.45 mg/g), but almost twenty times greater than that in the brood cell provisions of *Chelostoma florisomne* (mean, 0.03 mg/g). The maximum concentration of ranunculin found in the pollen of *R. acris* was 1.34 mg/g (Table 2). Neither protoanemonin, the ranunculin anhydroaglycone, nor anemonin, the product of spontaneous dimerization of protoanemonin, were detected in any of the samples.

Discussion

Results of the feeding experiments provide evidence that the two tested solitary bee species not specialized on *Ranunculus* tolerated the 10 mg/g ranunculin treatment without any measurable effects on larval survival. Based on the ranunculin recovery experiment, the 10 mg/g ranunculin treatment corresponds to an approximate concentration of biologically available ranunculin of at least 5.54 mg/g d.w.

Table 2 Content of ranunculin quantified by LC-MS in pollen and flower buds of *Ranunculus acris* and in brood cell provisions of the *Ranunculus* specialist bee *Chelostoma florisomne*

Source	*N*	Mean±SEM [mg/g]	Range [mg/g]
Pollen	8	0.55±0.18	0.03–1.34
Flower buds	8	19.45±5.20	3.72–53.93
Brood cell provisions[a]	11	0.03±0.01	0.003–0.12

[a] The *Ranunculus* pollen in the brood cell provisions was collected by foraging *C. florisomne* females from *R. acris*, which was the nearly exclusive pollen source for this species at the location where the brood cells were collected. Ranunculin amounts were calculated for pollen dry weight by subtracting average water and nectar contents in the brood cells

However, ranunculin in the pollen of *R. acris* quantified by LC-MS analysis amounted to maximally 1.34 mg/g d.w., a concentration that is at least fourfold lower than that tolerated by *Chelostoma rapunculi* and *Heriades truncorum*. The maximum ranunculin concentration in the brood cell provisions of the *Ranunculus* specialist *Chelostoma florisomne* was one order of magnitude lower (0.12 mg/g d.w.) than in the freshly collected pollen of *R. acris*, although the pollen in the analyzed brood cell provisions was derived from this *Ranunculus* species. Ranunculin is lethal when added to the natural pollen provisions of *C. rapunculi* and *H. truncorum* at very high concentrations (50 mg/g and 100 mg/g treatments). However, these concentrations greatly exceed natural concentrations of ranunculin found in *R. acris* pollen. Hence, the presence of ranunculin cannot explain the mortality of these bees when reared on a *Ranunculus* pollen diet, contrary to hypotheses proposed previously (Praz et al., 2008a; Sedivy et al., 2011).

Survival of *C. rapunculi* larvae feeding on the still high 10 mg/g and 20 mg/g ranunculin treatments was not significantly affected compared to the control treatment. However, mean survival values at the 20 mg/g treatment were low, and difference to the survival at the 10 mg/g treatment was significant, pointing to some adverse effects of the 20 mg/g treatment on larval survival. A sublethal effect (Piskorski et al., 2011a) of both the 20 mg/g and 10 mg/g treatments on *C. rapunculi* was noted as development times of the larvae were prolonged compared to the larvae in the control treatment.

Ranunculin admixed to the pollen provision at 10 mg/g could be recovered at a range of approximately 60%, irrespective of whether the incubation period lasted 0, 8, or 22 d. This finding indicates that a minor proportion of the ranunculin was deactivated during mixing, either through an adsorption or a chemical degradation. This result further indicates that the biologically available ranunculin concentration remained constant over long periods after mixing, thus underlining the validity of the conclusions drawn here.

Two previous studies described protoanemonin, the compound derived from hydrolysis of ranunculin, as the most prominent volatile in the headspace of pollen samples of *R. acris* (Bergström et al., 1995) and after thermal desorption of anthers of *R. acris* and other Ranunculaceae species (Jürgens and Dötterl, 2004). In both cases, only relative amounts were provided, and no information was given regarding absolute quantities of this lactone present in a volatile profile poor in other compounds. In another study, in which protoanemonin in different organs of *R. ficaria* was quantified after steam distillation, the androeceum was found to emit almost twice as much protoanemonin as the whole plant (Bonora et al., 1988). In that study, however, ranunculin was not quantified in the pollen itself. Thus, the high amounts of protoanemonin measured in the headspace of the androeceum of *R. ficaria* might have been derived from a high concentration of ranunculin in the anther filaments, or in the anther tissue surrounding the pollen sacs prior to pollen release, rather than directly from the pollen. The low concentrations of ranunculin found in the pollen of *R. acris* in the current study is in line with the trace amounts of protoanemonin recently reported from an analysis of pollen volatiles of *R. bulbosus* (Piskorski et al. 2011b). The ranunculin concentrations we measured in the flower buds of *R. acris*, amounting to up to 54 mg/g d.w., corresponds to published levels of ranunculin in other ranunculin-containing Ranunculaceae species (Ruijgrok, 1966; Bai et al., 1996), thus validating our extraction and quantification methods.

Surprisingly, we found that the level of ranunculin in the pollen provision collected by *C. florisomne* was on average twentyfold lower than in pure pollen. Pollen in the cell provisions is diluted with nectar admixed by the foraging females at a ratio of approximately 1:1 (based on dry weight; A. Bühler and A. Müller, unpublished), explaining some but not all of the discrepancy noted between ranunculin concentrations in the flower pollen and in the bees' brood cell provisions. One process that substantially reduces ranunculin content in cut *Ranunculus* plants is drying, which triggers β-glucosidase-mediated autolysis of ranunculin with release of protoanemonin, yielding hay that is non-toxic to livestock (Majak, 2001). A similar process can likely be ruled out in the pollen harvested by *C. florisomne*, since the pollen is neither dried nor mechanically damaged during pollen collection, deposition, and storage in the brood cell. We hypothesize that the high sugar concentration originating from the nectar surrounding the pollen grains in the brood cell provisions may lead to an osmotic stress that provokes the release of protoanemonin from its precursor ranunculin, similar to the situation in drying *Ranunculus* plants.

In summary, the pollen of *R. acris* contains the secondary metabolite ranunculin in concentrations considerably below the lethal threshold for the tested bee larvae. Thus, we found no evidence that the incapability of several bee species to develop on a *Ranunculus* pollen diet is caused by ranunculin (Praz et al., 2008a; Sedivy et al., 2011). Hence, a different mechanism likely causes larval mortality of bees not specialized on *Ranunculus* pollen, such as the presence of another still unknown toxic pollen compound or the lack of essential nutrients in the pollen, e.g. certain sterols or amino acids.

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Identification and Field Evaluation of Pear Fruit Volatiles Attractive to the Oriental Fruit Moth, *Cydia molesta*

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Abstract Plant volatiles play a key role in host plant location of phytophagous insects. *Cydia molesta* is an important pest of pear fruit late in the growing season. We identified and quantified volatiles from immature and mature fruits of six pear varieties by using gas chromatography–mass spectrometry (GC-MS). Attractiveness of synthetic blends to adults based on gas chromatography-electroantennogram detection (GC-EAD) activity was investigated in both field and laboratory. Consistent electroantennographic activity was obtained for 12 compounds from headspace collections of the mature fruits of the six pear varieties. Qualitative and quantitative differences were found among six odor profiles. Among the six mixtures, the mixture of 1-hexanol, nonanal, ethyl butanoate, butyl acetate, ethyl hexanoate, hexyl acetate, hexyl butanoate, and farnesene (different isomers) with a 1:1:100:70:7:5:1:4 ratio from the variety Jimi and the mixture of nonanal, ethyl butanoate, 3-methylbutyl acetate, ethyl hexanoate, hexyl acetate, and farnesene with a 1:100:1:32:1:2 ratio from the variety Huangjin were highly attractive to both sexes in the field. However, male captures were much higher than those of females. Further wind tunnel tests proved that both sexes exhibited upwind flight to the lures, but only males landed on the source. Our finding indicates that mixtures mimicking Jimi and Huangjin volatiles attract both females and males of *C. molesta*, and these host volatiles may be involved in mate finding behavior.

Keywords Oriental fruit moth · *Cydia molesta* · Pear · Field experiments · Wind tunnel · Host plant volatiles · Lepidoptera · Tortricidae

Introduction

Plant-derived volatiles play a significant role in guiding female herbivores insects to host plants for oviposition (Angioy et al., 2003; Mitchell et al., 2008). Evidence has been provided that plant volatiles elicit specific responses in females of several species of insects both in the laboratory (Tasin et al., 2005, 2007, 2009; Casado et al., 2008) and in the field (Cha et al., 2008; Anfora et al., 2009; Bruce et al., 2011; Zhang et al., 2011).

The oriental fruit moth (OFM), *Cydia* (=*Grapholitha*) *molesta* (Busck) (Lepidoptera: Tortricidae), presumably originating from north-west China, has become widely distributed throughout the stone fruit-growing area in the world (Roehrich, 1961). All of its host plants belong to the family Rosaceae, including mainly peach, pear, apple, but also nectarine, plum, cherry, and others. In North China, the moth first infests the growing peach and apple shoots in the early growing season and then shifts to pear and apple fruits. In the late season, pear trees are the primary OFM host plants. Females mainly lay eggs on mature pear fruits (Rothschild and Vickers, 1991; Dorn et al., 2001; Myers et al., 2006; Yang and Liu, 2010). Because of the economic importance of the OFM, its sex pheromone has been studied since the 1960s (Roelofs et al., 1969), and pheromone-based techniques have been established to monitor and control its populations (Trimble et al., 2004; Stelinski et al., 2005; Kovanci et al., 2009). However, an important drawback in practice is that use of sex pheromone affects only males, while females are directly responsible for infestations by

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laying eggs on fruits and making inter-orchard flights (Yetter and Steiner, 1932; Steiner and Yetter, 1933). The flight performance of females significantly exceeds that of males (Hughes and Dorn, 2002), and thereby females represent a serious threat to adjacent orchards.

For the OFM, olfactory cues from either shoots or fruits of peach and apple have been studied extensively. Excised peach shoots emitting (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenol, and benzaldehyde at 4:1:1 mixture proportions were attractive to mated OFM females (Natale et al., 2003). Behavioral experiments were performed to identify the key components among all the volatiles emitted from peach shoots (Piñero and Dorn, 2007). Volatiles emitted from peach and apple fruits were also attractive to mated females in a dual choice arena (Natale et al., 2004), and the level of attraction of OFM females to peach shoots and fruits varied at three phenological stages (Piñero and Dorn, 2009). However, all of these attraction studies were conducted in the laboratory. The only field work was reported by Il'ichev et al. (2009), who carried out headspace analysis of volatiles from intact young shoot tips of peach. They found that a mixture of (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene and (*E*)-β-farnesene at mixture proportions 1:2:2 was the best attractant, but only OFM males and no females were captured in the field.

Up to now, there are no reports on olfactory orientation of OFM to pear fruit. Pears are substantial post-peach resources during the late fruit-growing season. In China and some other Asian countries, pears are as important as peaches, and inter-planting and mixed cultivation of pears and peaches is common (Makaji, 1987; Zhao et al., 1989; Yang et al., 2002). Such a situation poses a serious threat to pear cultivation in the vicinity of peach trees (Zhao et al., 1989; Yang and Liu, 2010). In the peach-pear system, the role of volatile organic compounds (VOCs) of pear fruits still is little understood, but such knowledge should be helpful to elucidate mechanisms underlying the host shift of OFMs from peach to pear.

The objectives of the present study were: (1) to identify potentially attractive VOCs from six pear varieties; and (2) to test behavioral responses of OFM adults to synthetic blends of these VOCs in a laboratory wind tunnel and in the field.

Methods and Materials

Insects The OFMs used were obtained from an experimental orchard at the Institute of Forestry and Pomology (IFP), Beijing Academy of Agriculture and Forestry (BAAF), Beijing, China (39°58′N, 116°13′E). Larvae were collected from field-infested shoots of peach, *Prunus persica* L. Batsch cv. Dajiubao, in late June, and were maintained in a climatic chamber at 24±1 °C and 65–70 % RH under a photo:scoto regime of 16 L: 8 D, with the photophase starting at 05:00 hr am. Larvae were mass-reared on apple, *Malus domestica* L. Borkh. cv. Hongfushi, in a glass container (27 cm diameter, 13 cm high) for 3 instars and then transferred individually to smaller glass containers (2.5 cm diam., 8 cm high) until eclosion. Adults were maintained in a bell-shaped glass container (diam. of the two openings 6 and 15 cm, 41 cm high). Both sides of the container were covered with fine nylon mesh. From a hole pierced through the mesh in the small side, 15 % honey solution was provided on water-soaked cotton. The moths were reared in the laboratory for 3 generations before testing. For electrophysiological experiments (GC-EAD), 2- to 3-d-old females were used. For the wind-tunnel bioassay, 2- to 4-d-old mated female and male moths were chosen without a conscious bias. Mated females were obtained by placing groups of ca. 20 newly emerged females together with 30 males in the same cage for two scotophases to ensure mating. Adult moths were used only once and were not exposed to synthetic odor sources before the bioassay.

Chemicals Decane (99 %), tetradecane (99 %), pentadecane (99 %), hexadecane (99 %), nonadecane (99 %), 2-methylbutyl acetate (99 %), 3-methylbutyl acetate (97 %), ethyl pentanoate (98 %), butyl butanoate (98 %), 6-methyl-5-hepten-2-one (98 %), butyl hexanoate (98 %), (*E*)-β-ocimene (60 %), and (*E,E*)-α-farnesene (49 %) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The farnesene obtained from Sigma-Aldrich also contained in addition to (*E,E*)-alpha-farnesene (49 %) (*E*)-beta-farnesene (26 %), (*Z*)- beta-farnesene (18 %), and (Z,*E*)-alpha-farnesene (7 %). We used this mixture of farnesene isomers for our EAG and field studies and refer to it as "farnesene". Heptadecane (99 %), hexanol (99 %), 2-ethyl-1-hexanol (99 %), octanal (98 %), nonanal (97 %), decanal (97 %), pentyl acetate (99 %), hexyl acetate (99 %), and racemic linalool (97 %) were obtained from Fluka Production GmbH (Buchs, Switzerland). Ethyl butanoate (99 %) and butyl acetate (99 %) were bought from Acros Organics (New Jersey, USA). Ethyl hexanoate (98 %) and hexyl butanoate (98 %) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Compounds that did not elicit antennal responses, and for which no standards were available, were tentatively identified using the NIST-database.

Plant Materials Pears were cultivated in an experimental orchard at the IFP. Six varieties of 3 pear species were selected: Xinyali (XY), Xuehua (XH), and Jimi (JM) of *Pyrus bretschneideri*, Huangjin (HJ) and Fengshui (FS) from *Pyrus pyrifolia*, and Conference (KF) from *Pyrus communis*. All these varieties are commonly grown in the Beijing area. No insecticides or any specific treatment against OFM were used in the orchard during tests. Unripe

and ripe fruits were healthy and were picked from trees according to the phenological development of corresponding varieties in the Beijing area in 2009 and 2010 (Cao et al., 2000, 2006; Jiang et al., 2002). Fruits were picked within 20 min before sampling of VOCs or wind-tunnel assays.

Collection of VOCs We used a push-pull system to collect headspace VOCs. Fruits (ca. 1,500 g) were placed immediately after harvesting into a 2,000 ml glass jar for extraction. Air aspirated with a vacuum pump (Qianxi Air Company, Beijing, China) was filtered through an activated-charcoal filter, then passed through the jar at 300 ml/min, and finally through a sorbent cartridge (Porapak Q, 50 mg, 80/100 mesh, Supelco, Bellefonte, PA, USA). The sorbents were held between plugs of glass wool in a glass tube (10 cm long, 0.5 cm inner diam). Samples were collected for 8 hr at 24±1 °C and 65–70 % RH. Volatiles were desorbed by eluting the sorbent cartridge with 500 μl hexane (HPLC grade, Sigma-Aldrich) at room temperature. Five samples were collected for each unripe and ripe pear variety, respectively. Samples were analyzed by GC-EAD and GC-MS. For quantitative analyses, 0.5 μg benzaldehyde (99 %, Fluka, Switzerland) was added as an internal standard to each sample. Preliminary analyses showed that this compound was not detectable in the headspace of the pear varieties studied here. Sample volumes were reduced to 50 μl by using a slow stream of nitrogen and then analyzed. If not used immediately, extracts were sealed in glass vials and stored at −18 °C until used.

GC-MS Headspace VOCs of the unripe and ripe fruits of the 6 varieties and mixtures of synthetic compounds were analyzed with an Agilent Technologies 5973 MS (Agilent) coupled to an Agilent Technologies 6890 N GC (Agilent) equipped with polar DB-WAX fused-silica column (30 m× 0.25 mm ID, 0.25 μm film, J&W Scientific Inc., Folsom, CA, USA) or nonpolar DB-5 fused-silica column (30 m× 0.25 mm ID, 0.25 μm film, J&W Scientific Inc., Folsom, CA, USA). The temperature program was as follows: 50 °C for 1 min, then 3 °C/min to 120 °C, then 10 °C/min to 240 °C and finally held at 240 °C for 10 min. Windows NT/MASS Spectral Search Program (Version 1.7) software was used for data analysis. Injections were made in the splitless mode. Helium was used as carrier gas (1.0 ml/min). For electron impact (EI) mass spectra, the ionization voltage was 70 eV, and the temperatures of the ion source and of the interface were 230 °C and 280 °C, respectively. The emission current was 34.6 μA. Identification of VOCs was verified by comparison with authentic samples.

GC-EAD VOCs were analyzed with an Agilent Technologies 6890 N GC with a flame ionization detector, using a polar DB-WAX (30 m×0.25 mm ID, 0.25 μm film, J&W Scientific, Folsom, CA, USA) or nonpolar DB-5 (30 m× 0.25 mm ID, 0.25 μm film, J&W Scientific Inc., Folsom, CA, USA) capillary column coupled with an electroantennographic detector (EAD; Syntech, Hilversum, The Netherlands). The oven temperature program was the same as in the GC-MS analysis. Nitrogen was used as carrier gas (1.0 ml/min). A micromanipulator assembly (MP-15, Syntech) was connected to a stimulus controller (CS-55, Syntech). All signal sources were connected to a serial data acquisition interface (IDAC-4, Syntech). Antennae from the heads of OFM females were excised using microscissors. A few segments from the tips of antennae were clipped off and mounted on the antenna holder with two metal electrodes using conductive gel (Spectra 360, Parker Lab, NJ, USA), and then the electrode holder was inserted into the EAD probe. Testing began after a relative stable baseline had been achieved. The outlet of the GC column was split in a 2:1 ratio between the electroantennographic detector, a cut antenna of an OFM female, and the flame ionization detector (FID). The mounted antenna was positioned in the charcoal-filtered and humidified air stream that carried the VOCs eluting from the GC column. The antennal and FID signals were amplified and recorded simultaneously using Syntech software (GC-EAD 32, version 4.4, Syntech, Hilversum, The Netherlands). Each sample was tested 6 times. Identities of EAD-active compounds were verified by comparison of mass spectra and retention times with those of synthetic standards. Each tested antenna was derived from a different female. In addition, blends of 12 EAD-active synthetic compounds (1-hexanol, nonanal, ethyl butanoate, butyl acetate, 2-methylbutyl acetate, 3-methylbutyl acetate, pentyl acetate, ethyl hexanoate, hexyl acetate, butyl hexanoate, hexyl butanoate, and farnesene as a mixture of different isomers, see above) were analyzed by GC-EAD (10 ng of each).

Field Experiment 1 On the basis of the results of the GC-EAD analyses, VOCs from the mature fruits of 6 varieties eliciting antennal responses in female OFM were formulated in blends for the field tests. Six blends of synthetic compounds were prepared in the ratios of GC-EAD-active VOCs as emitted by the corresponding 6 varieties (Table 1). The formulations contained 100 mg of the most abundant compound and the others in the same proportion as in the natural volatile mixture. Preliminary field experiments showed that such concentrations can provide an adequate emission under field conditions. The rubber septa are goblet shaped (10 mm depth, 6 mm inter diam.) and have a maximum volume of 400 μl. They were made to order by Shunyi Rubber Company, Beijing, China. This volume is enough to load all the treatment solutions in the study. The mixtures of compounds were prepared 1–2 hr prior to bioassays in the field trial. They were added into the rubber

Table 1 Components and blend ratios for each blend used in the field experiment 1 and 3

Compounds[a]	Amount loaded on rubber septum in six varieties[b] (mg)					
	JM	KF	XY	HJ	XH	FS
Alcohols						
1-hexanol	1	2	1		3	2
Aldehyde						
Nonanal	1		1	1	2	1
Esters						
Ethyl butanoate	100		100	100	96	100
Butyl acetate	70	100	39		100	8
2-methylbutyl acetate		2				
3-methylbutyl acetate			6	1		1
Pentyl acetate		3	1			
Ethyl hexanoate	7	1	11	32	11	15
Hexyl acetate	5	41	4	1	8	3
Butyl hexanoate			1			
Hexyl butanoate	1				2	
Terpenoid						
Farnesene[c]	4	1	1	2	6	1

[a]In order of elution during gas chromatography

[b]The six varieties of 3 pear species were Xinyali (XY), Xuehua (XH), and Jimi (JM) of *Pyrus bretschneideri*, Huangjin (HJ) and Fengshui (FS) from *Pyrus pyrifolia*, and Conference (KF) from *Pyrus communis*

[c]Farnesene = mixture of (*E,E*)-alpha-farnesene (49 %), (*E*)-beta-farnesene (26 %), (*Z*)- beta-farnesene (18 %), and (*Z,E*)-alpha-farnesene (7 %)

septa and then fixed upward on the bottom of sticky delta trap in the dusk, during which OFM-flight intensity peaks, for all the treatments. We deployed the rubber septa with solutions directly in the trap, before the mixture solution was fully impregnated into the rubber septa. All odor blends were deployed in this way. Unbaited traps (HPLC-grade hexane, Sigma-Aldrich) and OFM sex-pheromone lures (Geruibiyuan Technology Company, Beijing, China) were used as controls.

The field test was conducted in a 7-hectare peach orchard and a 6.5-hectare pear orchard at the IFP with a history of OFM infestation. The peach orchard was chosen with the aim of avoiding full overlap between the background odor in the pear orchard and synthetic blends derived from pear fruits. A sticky delta trap (35 cm long×20 cm high×20 cm wide) was used to trap the insects. Trials were carried out in a randomized complete-block design. In each block, baited traps were installed approximately 1.5 m above the ground, and were set up at a distance of at least 30 m to minimize interference between traps. Each treatment was replicated four times in four blocks.

Traps were monitored twice weekly. Captured OFMs were transferred to the laboratory, and their number and sex were recorded. Trials lasted 2 week. The data of captures over a 14-d-period were pooled. The field test was conducted from late June to early August 2010 in peach and pear orchards, during the 2nd and 3rd OFM flights in the Beijing area.

Field Experiment 2 In field experiment 1, an eight-component mixture of 1-hexanol, nonanal, ethyl butanoate, butyl acetate, ethyl hexanoate, hexyl acetate, hexyl butanoate, and farnesene (different isomers, see above) at 1:1:100:70:7:5:1:4 proportions in JM and a six-component mixture of nonanal, ethyl butanoate, 3-methylbutyl acetate, ethyl hexanoate, hexyl acetate, and farnesene at 1:100:2:32:1:2 proportions in HJ were more attractive to the moths than the other blends. For field experiment 2, the common VOCs and the specific VOCs in two mimics were grouped separately based on the ratio in HJ and JM, and then six blends—JM, JMS, JMC, HJ, HJS, and HJC—were compared for their attractiveness to the moths in the peach orchard (Table 2).

Field Experiment 3 Six mixtures of synthetic VOCs in the proportions of GC-EAD-active VOCs emitted by the corresponding six varieties were further evaluated in a peach orchard in 2011 (Table 1). The field test was conducted from late June to early July, during the second OFM flight period, in a 15-hectare peach orchard located in the fruit-production area of Tianbaoyuan, Huairou, Beijing. All other details of the experiment were the same as for field experiment 1.

Wind Tunnel Assay The laboratory wind tunnel measured 1.6 m long, 0.5 m wide, 0.5 m high. A fan at the upwind end generated a steady airflow into the tunnel, set at 0.3 m/s at the point of release of moths. The light intensity in the tunnel was about 250 lux. The room was kept at 23±2 °C and 50–70 % RH.

Moths were transferred into the test room 2 hr before experiments. Batches of 10 moths were placed in a small screen metal release cage (7 cm diam., 9 cm height) with a

Table 2 Components and blend ratios for each blend used in the field experiment 2

Compounds[a]	Amount loaded on rubber septum in six blends[b] (mg)					
	JM	JMS	JMC	HJC	HJS	HJ
Alcohols						
1-hexanol	1	1				
Aldehyde						
Nonanal	1		1	1		1
Esters						
Ethyl butanoate	100		100	100		100
Butyl acetate	70	70				
3-methylbutyl acetate					1	1
Ethyl hexanoate	7		7	32		32
Hexyl acetate	5		5	1		1
Hexyl butanoate	1	1				
Terpenoid						
Farnesene[c]	4		4	2		2

[a]In order of elution during gas chromatography

[b]The six blends were the head-space VOC mimics of two pear varieties, Jimi (JM) and Huangjin (HJ), and their subsets, specific VOCs (JMS, HJS) and common VOCs (JMC, HJC)

[c]Farnesene = mixture of (*E,E*)-alpha-farnesene (49 %), (*E*)-beta-farnesene (26 %), (*Z*)- beta-farnesene (18 %), and (*Z,E*)-alpha-farnesene (7 %)

side door. The air could flow through the release cages. Tests began 2 hr before the beginning of the scotophase and lasted 3 hr. The release cage containing a moth was placed on a holder at the downwind end of the tunnel, 30 cm above its floor and ca. 140 cm from the VOC source. The side door faced the upwind end of the tunnel, and the door was opened to allow the moths to leave the cage. Each batch of 10 mated moths was tested for 20 min, and 6 batches of moths were used per day. Each VOC mixture was tested with 9 batches of moths on different days. Moths were tested only once. Synthetic compounds were loaded in rubber septa. The septum loaded with one of the mixtures was placed on a holder at the upwind end in the center of the tunnel, 10 cm from the upwind end. Individual moths were scored for the following behaviors: (1) departure from the release cage and flight upwind; (2) arrival within 10 cm of the VOC source; and (3) landing on the source. For data analysis, we categorized each moth based on the most complete behavior that it displayed within 20 min.

For these experiments, mature pear fruits (ca. 220 g, 8–10 cm diam.) were freshly cut and immediately transferred into the laboratory for bioassays. VOCs collected from the fruit were diluted with hexane (HPLC grade, Sigma-Aldrich). Mixtures were prepared in proportions of GC-EAD-active VOCs found in the natural blends emitted by the corresponding varieties (Table 3). The formulation of the lure was similar to that in the field test. The predominant VOC in the mixture was dosed at 0.5 mg. Preliminary tests in the wind tunnel showed that these concentrations were adequate to elicit moth responses.

Data Analysis Mean numbers of OFM males and females captured in traps baited with different lures in the field and attractiveness of mated OFM females and males to the VOC source in the wind tunnel were analyzed by one-way ANOVA. The means were separated by Tukey's multiple range tests ($P < 0.05$). Significant differences in three flight behaviors between both sexes were analyzed by Mann–Whitney *U*-tests. All data were analyzed with the statistical program SPSS (version 13.0).

Results

Chemical Identification of VOCs from Different Pear Varieties In order to understand which volatiles attract OFMs to pears, we analyzed the VOCs of unripe and ripe fruits of six different varieties. Several compounds were identified, belonging to different chemical classes: hydrocarbons, alcohols, aldehydes, esters, benzene derivatives, ketones, and terpenoids (Table 3). Profiles of the six varieties consisted of the same chemical classes, but differed greatly between ripe and unripe fruits. In particular, decanal, octanal, methyl salicylate, (*E*)-2-hexenyl benzoate, ocimene, and linalool were characteristic of unripe pears and absent from ripe fruits, whereas the concentrations of other VOCs, such as several hydrocarbons, 2-ethyl-1-hexanol, nonanal, and (*E,E*)-α-farnesene, decreased with maturation. In mature fruits, esters were the dominant VOCs, mainly ethyl butanoate, butyl acetate, ethyl hexanoate, and hexyl acetate. In particular, butyl acetate was the most abundant VOC identified in KF and XH varieties, and ethyl butanoate in the others (Table 3).

Antennal Responses to VOCs from Ripe Pears By using GC-EAD, we identified the compounds detected by the antenna of the moth (Fig. 1). Their chemical identities were

Table 3 Relative quantities of volatile compounds collected in the headspace of immature and mature fruits of six pear varieties

Compounds	S [a]	Varieties											
		JM		KF		XY		HJ		XH		FS	
		I [b]	M [b]	I	M	I	M	I	M	I	M	I	M
Hydrocarbons													
Decane*	SA	42						<1					
Tetradecane*	SA	69	<1	1	<1	<1	<1	28	<1	21	<1	23	<1
Pentadecane*	SA		1		<1		<1		<1	66	<1	71	<1
Hexadecane*	SA		1	5	<1	38	1	100	<1	100	<1	100	<1
Heptadecane*	F		1		<1		<1		<1		<1		<1
6-methyl-octadecane			<1	<1		<1		25		18		33	
Nonadecane*	SA	<1	<1	1		16		37		45		44	
Alcohols													
1-hexanol*	F		1	2	2		<1				3		2
2-ethyl-1-hexanol*	F	92	<1	1	<1	11	<1	28	<1	31	<1	39	
2-decenol		<1											<1
2-methyl-1-hexadecanol			<1		<1						<1		
Aldehydes													
Octanal*	F	31				<1		<1		<1		<1	
Nonanal*	F	90	<1	<1		6	<1	12	<1	16	2	20	1
Decanal*	F	53				<1		12		15		<1	
Esters													
Ethyl butanoate*	AO		100			<1	100		100	4	96		100
Butyl acetate*	AO		70	100	100	22	39			6	100		8
2-methylbutyl acetate*	SA			<1	2								
3-methylbutyl acetate*	SA			<1			6		1				<1
Ethyl pentanoate*	SA								<1				<1
Pentyl acetate*	F			6	3		<1						
Butyl butanoate*	SA		<1	6	<1								
Ethyl hexanoate*	TC		7		<1	<1	11		32		11		15
Hexyl acetate*	F		5	19	41	<1	4		<1		8		3
Hexyl propanoate				1									
Heptyl acetate					<1								
Butyl hexanoate*	SA			3			<1						
Hexyl butanoate*	TC		1	2							2		
Ethyl octanoate							1		<1				
Hexyl hexanoate			<1		<1						<1		
Benzenoids													
Methyl salicylate		53						<1					
(*E*)-2-hexenyl benzoate		59											
Ketones													
6-methyl-5-hepten-2-one*	SA	42				<1		<1		<1		20	<1
Terpenoids													
(*E*)-β-ocimene*	SA	56											
Linalool*	F	25											
(*E,E*)-α-farnesene*	SA	100	4	4	1	100	1	26	2	33	6		<1

The asterisked compounds had been conclusively identified by comparison of spectra and retention times with those of an authentic standard. Compounds in bold face type elicited antennal responses in GC-EAD experiments (also see Table 4). Compounds within each class were listed according to retention times on a polar DB-Wax fused silica column

The six varieties of 3 pear species were Xinyali (XY), Xuehua (XH), and Jimi (JM) of *Pyrus bretschneideri*, Huangjin (HJ) and Fengshui (FS) from *Pyrus pyrifolia*, and Conference (KF) from *Pyrus communis*. Quantities expressed relative to the most abundant compound (set to a value of 100) in the two stages. The average amount of the most abundant compound collected from 100 g of fruits in the two phenological stages was: JM) 2.98±1.31 ng/hr of (*E, E*)-α-farnesene in immature fruits (*N*=5); 253.57±64.56 ng/hr of ethyl butanoate in mature fruits (*N*=5); KF) 251.65±94.50 ng/hr of butyl acetate in immature fruits (*N*=5); 1140.38±157.06 ng/hr of butyl acetate in mature fruits (*N*=5); XY) 18.58±6.05 ng/hr of (*E, E*)-α-farnesene in immature fruits (*N*=5); 329.57±99.69 ng/hr of ethyl butanoate in mature fruits (*N*=5); HJ) 7.79±7.21 ng/hr hexadecane in immature fruits (*N*=5); 222.06±88.68 ng/hr of ethyl butanoate in mature fruits (*N*=5); XH) 7.97±5.38 ng/hr hexadecane in immature fruits (*N*=5); 45.78±22.05 ng/hr of butyl acetate in mature fruits (*N*=5); FS) 8.72±5.71 ng/hr hexadecane in immature fruits (*N*=5); 257.93±97.27 ng/hr of ethyl butanoate in mature fruits (*N*=5)

[a] S - source of authentic standards. The standards were obtained from Sigma-Aldrich Co., St. Louis, MO, USA(SA), Fluka Production GmbH, Buchs, Switzerland (F), Acros organics, New Jersey, USA (AO), Tokyo Chemical Industry CO., Tokyo, Japan (TC)

[b] I and M stand for immature and mature fruits, respectively

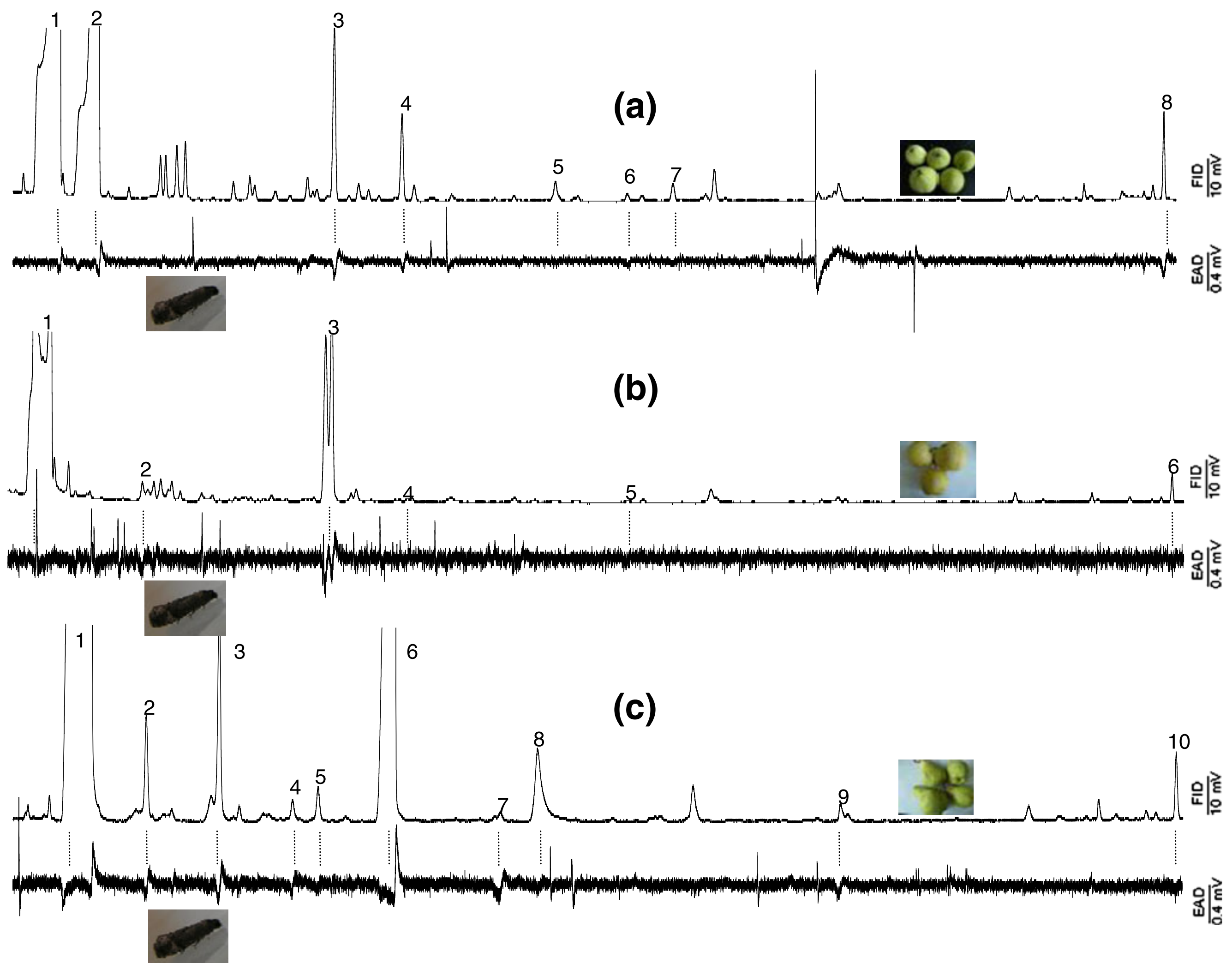

Fig. 1 Simultaneously recorded GC-EAD responses to VOCs collected from mature fruits of the varieties Jimi (JM) of *Pyrus bretschneideri* (**a**), Huangjin (HJ) of *Pyrus pyrifolia* (**b**) and Conference (KF) of *Pyrus communis* (**c**) using a polar DB-WAX capillary column. The upper trace is the flame ionization detector response (FID), and the lower displays the *Cydia molesta* female antennal response (EAD). Consistently EAD-active compounds in six different tests were showed as follows: For JM (a): (1) ethyl butanoate; (2) butyl acetate; (3) ethyl hexanoate; (4) hexyl acetate; (5) 1-hexanol; (6) nonanal; (7) hexyl butanoate; (8) (*E, E*)-α-farnesene. For HJ (b): (1) ethyl butanoate; (2) 3-methylbutyl acetate; (3) ethyl hexanoate; (4) hexyl acetate; (5) nonanal; (6) (*E, E*)-α-farnesene. For KF (c): (1) butyl acetate; (2) 2-methylbutyl acetate; (3) pentyl acetate; (4) unidentified; (5) ethyl hexanoate; (6) hexyl acetate; (7) unidentified; (8) 1-hexanol; (9) unidentified; (10) (*E, E*)-α-farnesene

then verified by MS, and comparison of mass spectra and retention times were made with those of authentic compounds.

In total, 12 compounds from the headspace of ripe fruits belonging to six varieties elicited antennal responses from OFM females: 1-hexanol, nonanal, ethyl butanoate, butyl acetate, 2-methylbutyl acetate, 3-methylbutyl acetate, pentyl acetate, ethyl hexanoate, hexyl acetate, butyl hexanoate, hexyl butanoate, and (*E, E*)-α-farnesene (Fig. 1). The EAD-active VOCs were different in quality and quantity among the six varieties. However, ethyl hexanoate, hexyl acetate, and (*E, E*)-α-farnesene were common components of all 6 varieties, while 1-hexanol, nonanal, ethyl butanoate, and butyl acetate were present in five of them (Table 3). Different esters frequently were present among GC-EAD-active volatiles in the mature fruits of all varieties.

In order to quantify antennal sensitivity, a second series of GC-EAD analyses was performed using 10 ng of each VOC. The strongest antennal response in OFM mated females was evoked by farnesene (Table 4).

Field Experiment 1 Based on the results of the GC-EAD analysis, six mixtures, each containing the most GC-EAD-active VOCs from the mature fruits of each variety, were evaluated both in a peach and a pear orchard in the early season (Fig. 2). A larger OFM population apparently was present in the peach orchard than in the pear orchard, based on the number of male moths trapped with the sex

Table 4 Antennal responses of *Cydia molesta* females to synthetic compounds identified from mature pear fruit as determined by GC-EAD

Compounds[a]	Antennal response (mV ± SD)[b]	Relative response (%) vs. Farnesene[c]	Source of volatiles[d]
Alcohols			
1-hexanol	0.094±0.017	59	JM, KF, XY, XH, FS
Aldehyde			
Nonanal	0.116±0.030	73	JM, XY, HJ, XH, FS
Esters			
Ethyl butanoate	0.016±0.009	10	JM, XY, HJ, XH, FS
Butyl acetate	0.018±0.008	11	JM, KF, XY, XH, FS
2-methylbutyl acetate	0.032±0.024	20	KF
3-methylbutyl acetate	0.038±0.024	24	XY, HJ, FS
Pentyl acetate	0.072±0.033	46	KF, XY
Ethyl hexanoate	0.108±0.035	68	JM, KF, XY, HJ, XH, FS
Hexyl acetate	0.112±0.047	71	JM, KF, XY, HJ, XH, FS
Butyl hexanoate	0.036±0.018	23	XY
Hexyl butanoate	0.052±0.015	33	JM, XH
Terpenoid			
Farnesene[e]	0.158±0.013	100	JM, KF, XY, HJ, XH, FS

[a] In order of elution during gas chromatography

[b] Mean antennal response (mV ± SD) using 10 ng of standard compounds (N=6)

[c] Mean antennal response in relation to stimulus amount

[d] The six varieties of 3 pear species were Xinyali (XY), Xuehua (XH), and Jimi (JM) of *Pyrus bretschneideri*, Huangjin (HJ) and Fengshui (FS) from *Pyrus pyrifolia*, and Conference (KF) from *Pyrus communis*

[e] Farnesene = mixture of (*E,E*)-alpha-farnesene (49 %), (*E*)-beta-farnesene (26 %), (*Z*)- beta-farnesene (18 %), and (Z,*E*)-alpha-farnesene (7 %)

pheromone. Traps baited with the blends mimicking the odor of variety JM (1-hexanol, nonanal, ethyl butanoate, butyl acetate, ethyl hexanoate, hexyl acetate, hexyl butanoate, and farnesene in a 1:1:100:70:7:5:1:4 ratio) and variety HJ (nonanal, ethyl butanoate, 3-methylbutyl acetate, ethyl hexanoate, hexyl acetate, farnesene with a 1:100:1:32:1:2 ratio) caught significantly more OFM males than the control or the traps baited with the other four blends. Only a few females were trapped with no significant difference between traps, including the control (Fig. 2a). In the pear orchard, where in the early season a much smaller OFM population was present based on the monitoring by sex pheromone-baited traps (9.25±2.22 males/trap), no moths were caught in the traps baited with pear volatiles.

Another field test also was performed in late July in both peach and pear orchards. Similar numbers of OFMs were found in both orchards, as monitored by sex pheromone (172.50±4.04 and 174.25±4.92 males/trap in peach and pear orchards, respectively). As in the early season test, traps baited with blends of the JM and HJ varieties caught significantly more OFM males than the other traps (Fig. 2b).

Field Experiment 2 In order to screen the VOCs essential for moth attraction in the synthetic odor blends that mimicked JM and HJ, the components of the JM and HJ blends were further evaluated in the field for their importance in attraction. We set up the following treatments: (1) JM mimic blend; (2) HJ mimic blend; (3) JMC: common components released by both HJ and JM, using the component ratio typical for JM; (4) HJC: common components released by both HJ and JM, using the component ratio typical for HJ; (5) JMS: VOCs selectively released from JM; and (6) HJS: VOCs selectively released from HJ (Fig. 3). All the other details of the experiment were same as for field experiment 1. The six blends were compared for attractiveness to moths in the peach orchard. The complete JM mixture was the most effective, followed by the complete HJ mixture, and the complete mixtures were significantly more effective than the reduced mixtures (Fig. 3).

Field Experiment 3 The field trials to evaluate further the response of OFMs to six blends that mimicked the six varieties were replicated in 2011. A larger OFM population was present in the peach orchard during the early season in 2011 than in 2010, based on the high number of male moths trapped with the sex pheromone (Figs. 2 and 4). As in the first field test in 2010, traps baited with mixtures of the JM and HJ varieties caught significantly more OFMs than the control, and the traps baited with the other four mixtures, but the total captures were significantly greater in 2011 than 2010 (Fig. 2 and 4). In particular, female captures significantly increased in 2011. The JM blend was the most effective attractant for females.

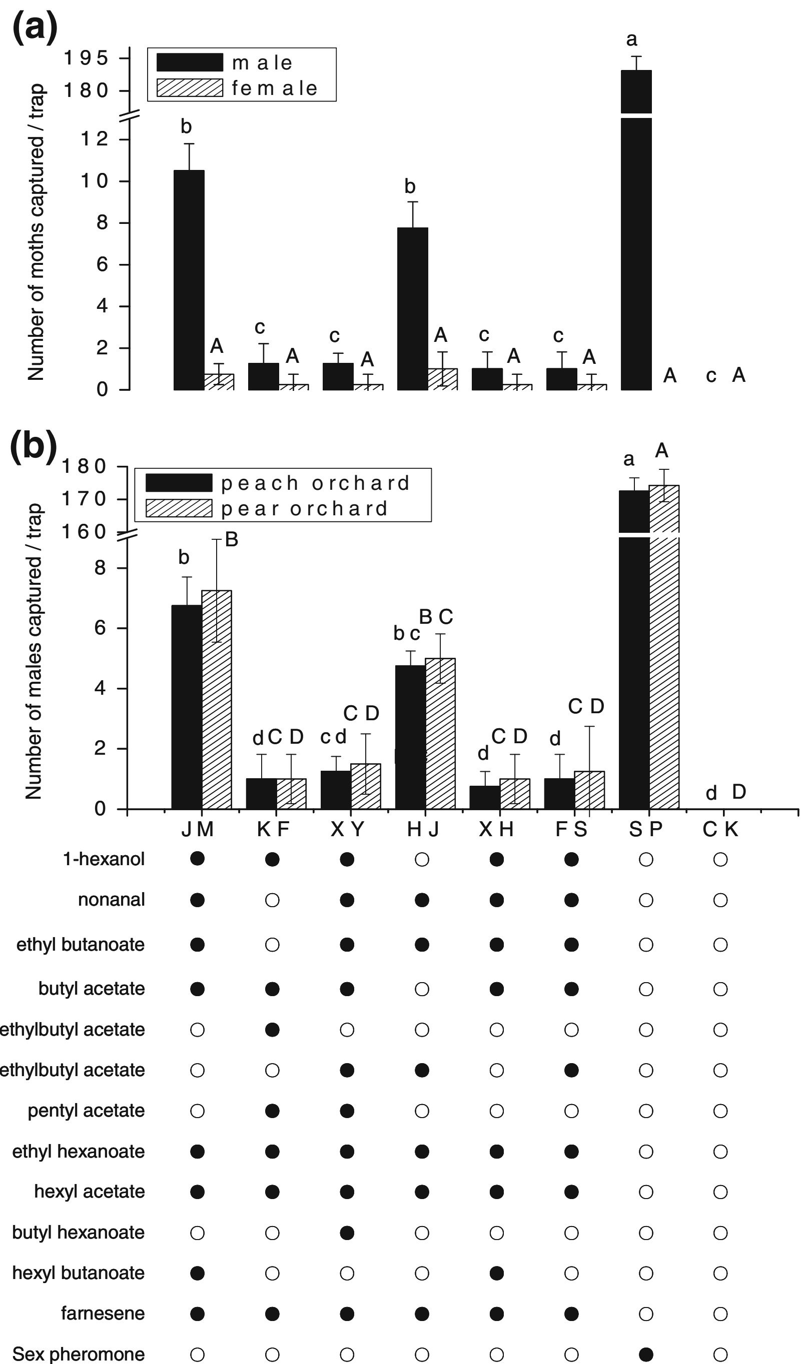

Fig. 2 Mean total number ± SD of *Cydia molesta* males and females captured in each trap (*N*=4) in a peach orchard on June 26-July 9, 2010 (**a**) and males captured in each trap (*N*=4) in peach and pear orchard on July 21-August 3, 2010 (**b**). Each lure baited with rubber septa with synthetic VOC mixtures from different varieties, a hexane control (CK) and sex pheromone (SP). The six varieties of 3 pear species were Xinyali (XY), Xuehua (XH), and Jimi (JM) of *Pyrus bretschneideri*, Huangjin (HJ) and Fengshui (FS) from *Pyrus pyrifolia*, and Conference (KF) from *Pyrus communis*. Formulation of each synthetic lure is indicated below the graph. Each lure contained 100 mg of the most abundant component. Farnesene = mixture of (*E,E*)-alpha-farnesene (49 %), (*E*)-beta-farnesene (26 %), (*Z*)-beta-farnesene (18 %), and (*Z,E*)-alpha-farnesene (7 %). The experiment was conducted at an experimental orchard at the Institute of Forestry and Pomology (IFP), Beijing Academy of Agricultural and Forestry (BAAF), Beijing, China. Different letters on bars indicate significant differences (one-way ANOVA followed by Tukey's multiple comparison test, $P<0.05$)

Wind Tunnel Bioassays In field experiments, we found that VOC mixtures attracted significantly more male than female OFMs. In order to evaluate the sexual differences in the flight performance toward synthetic mixtures in the laboratory, we used the eight-component JM mixture and the six-component HJ mixture as lures in wind-tunnel tests. Mature JM fruits also were applied as lures. We then investigated in more detail the differences between males and females in responding to JM and HJ mixtures, as well as to mature fruits of the JM variety. All three samples stimulated upwind oriented flight in both females and males (Fig. 5a, b), but only males reached and contacted the source (Fig. 5b). Males were more strongly attracted than females to synthetic mixtures.

Mature pear fruits of variety JM proved to be the most attractive to both sexes in the wind tunnel. In such experiments, 67 % of the females flew upwind and 50 % arrived within 10 cm of the source, but none landed on the fruit (Fig. 5a). By contrast, 96 % of males showed upwind orientation, 91 % arrived within 10 cm of the source, and 17 % landed on the source (Fig. 5b). In summary, the behaviors of female and male OFMs to JM pears were very similar to those observed with the corresponding artificial VOC blends (Fig. 5a, b).

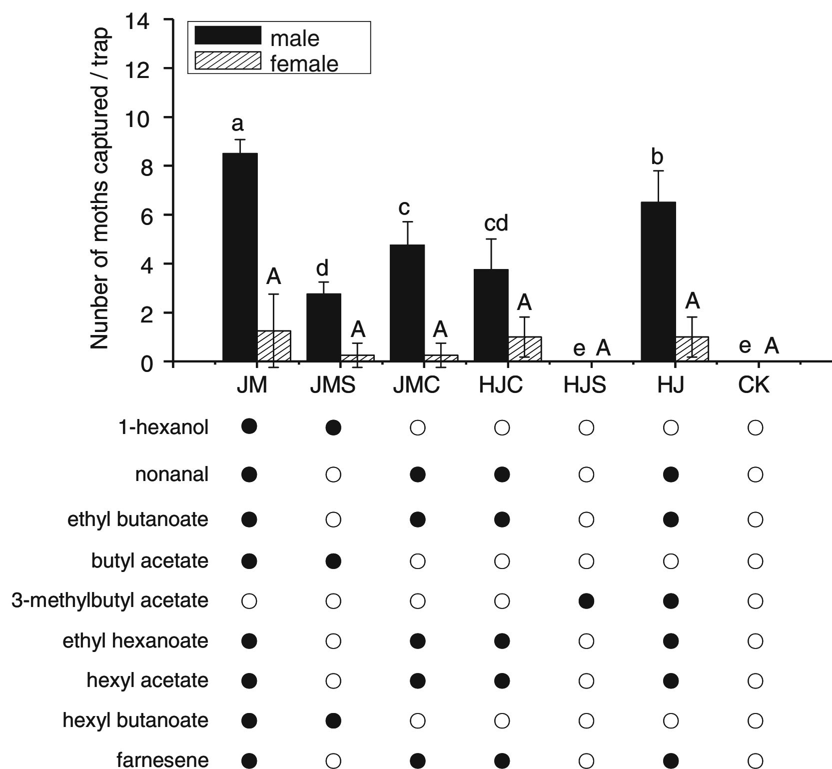

Fig. 3 Mean total number ± SD of oriental fruit moth (OFM) males and females captured in each trap *(N*=4), each baited with rubber septa with the synthetic VOC mixtures based on headspace VOC composition from mature fruits of the varieties Jimi (JM) of *Pyrus bretschneideri* and Huangjin (HJ) of *Pyrus pyrifolia,* their subsets and a hexane control (CK) in a peach orchard. Formulation of synthetic lures is indicated below the graph. Each lure contained 100 mg of the most abundant component. Farnesene = mixture of (*E,E*)-alpha-farnesene (49 %), (*E*)-beta-farnesene (26 %), (*Z*)- beta-farnesene (18 %), and (Z, *E*)-alpha-farnesene (7 %). The experiment was conducted in Beijing, on July 10-July 23, 2010. Different letters (capital letters for males captured and small letters for females) on bars indicate significant differences (one-way ANOVA followed by Tukey's multiple comparison test, $P<0.05$)

Discussion

We identified 12 EAD-active VOCs from 6 pear varieties. The VOC mixtures from mature JM fruit and mature HJ fruit were significantly more attractive in the field to OFM females and males than the blends from other varieties. The results indicate that olfactory cues from pears play a role in orientation to host plants by both sexes of OFM. Most studies on plant VOC mixtures that attract OFM have been performed in the laboratory. Our studies verified the attractiveness of synthetic host-plant VOCs to female and male moths under field conditions.

Different VOC profiles influence OFM behavior at different phenological stages. At the beginning of the season, the pest mainly infests the growing shoots of peach. Natale et al. (2003) reported that a mixture of (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenol, and benzaldehyde from peach shoots (4:1:1) elicited significant bioactivity in dual-choice tests with a bioassay-assisted fractionation that used different sorbent polymers. Piñero and Dorn (2007) found that a mixture of benzaldehyde, benzonitrile, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenol, and (*E*)-2-hexenal from peach shoots was as attractive as natural VOCs in a Y-tube olfactometer by using a subtraction approach. In field tests, Il'ichev et al. (2009) reported that a mixture of (*Z*)-3-hexenyl acetate, (*E*)-β-farnesene, and (*E*)-β-ocimene at 1:2:2 proportions was the most effective lure.

We found that headspace collections from pear fruits were greatly different from peach-shoot VOCs. Esters were the major components from mature pear fruits, and several of them elicited antennal responses. The increase in ester production in mature fruits corresponded with pest damage in mature fruit. The seasonal flight period for OFM starts before the onset of pear-fruit ripening, and females oviposit on the mature fruit. Our findings suggest that esters are essential for attraction of OFMs to pear fruits during the late fruit-growth stage. Some multi-voltine insects such as the OFM exploit several plant species as their hosts in different phenological stages. This is an important strategy for optimization of resources in order to complete the life cycle, and it is important for understanding the evolution of VOC-based recognition of host plants by herbivorous insects and for development of a possible semiochemical-based synthetic lure for use in the field.

By comparing the attractiveness of different VOC mixtures from pear fruits, we found both quality and quantity of

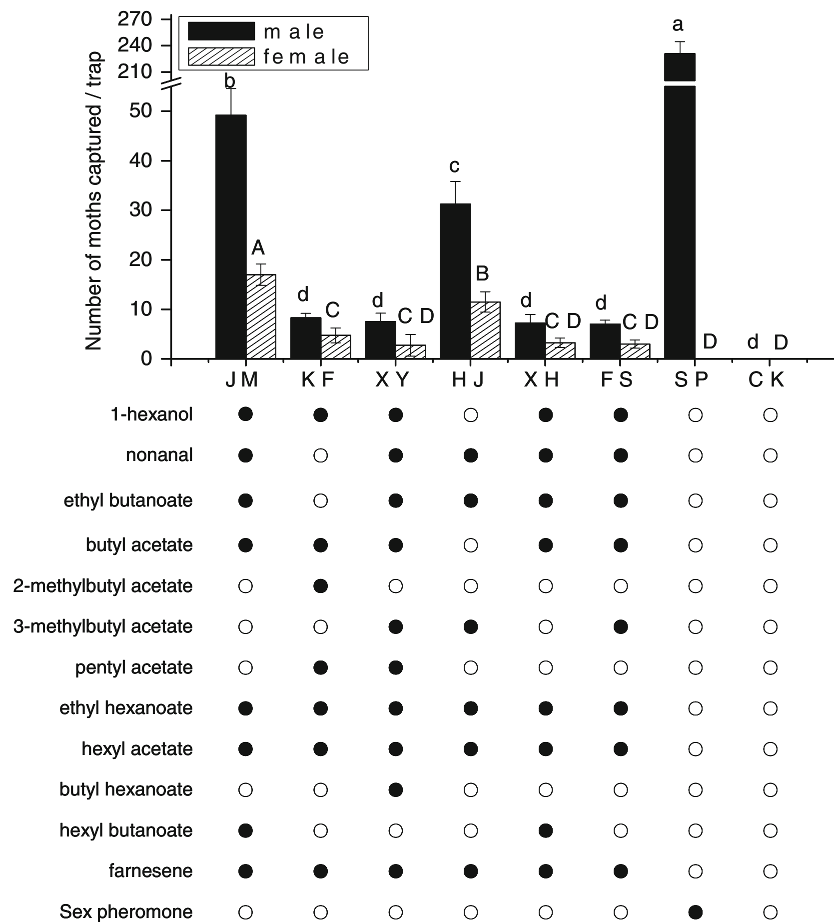

Fig. 4 Mean total number ± SD of *Cydia molesta* males and females captured in each trap *(N*=4) in a peach orchard on June 28–July 11, 2011. Each trap was baited with a rubber septum with synthetic VOC mixtures corresponding to those emitted by different varieties, a hexane control (CK), and sex pheromone. The six varieties of 3 pear species were Xinyali (XY), Xuehua (XH), and Jimi (JM) of *Pyrus bretschneideri*, Huangjin (HJ) and Fengshui (FS) from *Pyrus pyrifolia*, and Conference (KF) from *Pyrus communis*. Formulation of synthetic lure is indicated below the graph. Each lure contained 100 mg of the most abundant compounds. Farnesene = mixture of (*E,E*)-alpha-farnesene (49 %), (*E*)-beta-farnesene (26 %), (*Z*)- beta-farnesene (18 %), and (Z,*E*)-alpha-farnesene (7 %). The experiment was conducted in a peach orchard located in the fruit production area of Tianbaoyuan, Huirou, Beijing. Different letters on bars indicate significant differences (one-way ANOVA followed by Tukey's multiple comparison test, $P < 0.05$)

components in the mixtures to be important. First, minor components are essential for optimal attraction. Ethyl butanoate and butyl acetate were the most abundant active VOCs from six varieties, but the minor components were significantly different, suggesting that they are responsible for the different attractiveness of the different varieties. The minute amounts of benzonitrile from peach shoots have been shown to be important for olfactory responses in OFMs (Piñero et al., 2008). Second, the mixture proportions of ubiquitous plant VOCs are crucial for attraction of OFMs. Many insects use specific compounds to recognize host plants (Fraenkel, 1959; Szafranek et al., 2006; Hilker and McNeil, 2008). In our studies, however, EAD-active compounds were relatively ubiquitous plant VOCs. In such a case, species-specific mixture proportions of common VOCs would be crucial for insects to locate their hosts reliably. The mixture proportions of common plant VOCs are well-known to be important for host-plant recognition (Visser and Avé, 1978; Buttery et al., 1984; Reddy and Guerrero, 2000; Bruce et al., 2005). Similarly, in our study system, JM emitted the same EAD-active VOCs as XH, and the mixture proportions of these compounds were responsible for the significantly higher attractiveness of JM in the field.

Male captures, however, were higher although the lures were based on the EAD-active compounds for females. Similar results have been reported by Il'ichev et al. (2009). They identified potentially effective attractants from young peach shoot tips, but traps with all tested mixtures in all concentrations caught only males in the field. In our windtunnel study, we showed that females also were attracted by true pear fruits and synthetic lures although the performance of males exceeded that of females especially at close range

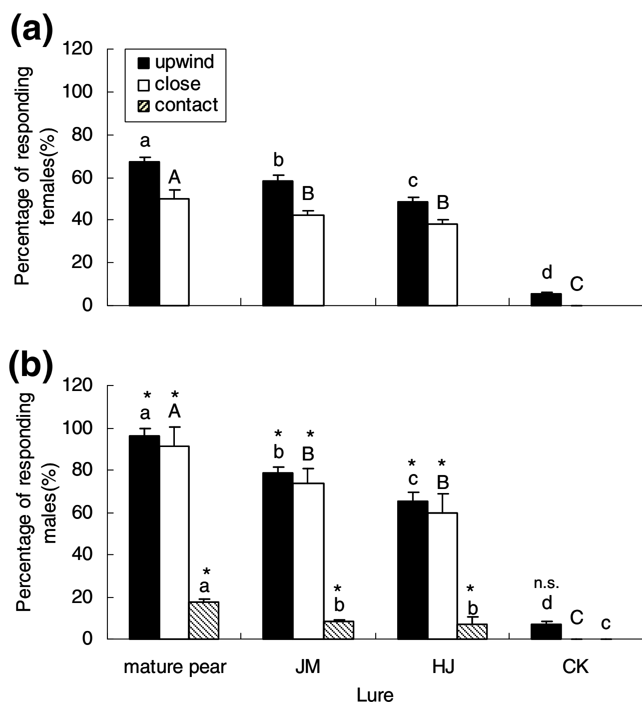

Fig. 5 Attraction of mated *Cydia molesta* females (**a**) and males (**b**) in a wind tunnel to mature pear synthetic VOC mixtures mimicking headspace composition of mature fruits of the varieties Jimi (JM) of *Pyrus bretschneideri* and Huangjin (HJ) of *Pyrus pyrifolia*. JM mimic = 1-hexanol, nonanal, ethyl butanoate, butyl acetate, ethyl hexanoate, hexyl acetate, hexyl butanoate, farnesene; HJ mimic = nonanal, ethyl butanoate, 3-methylbutyl acetate, ethyl hexanoate, hexyl acetate, farnesene with ratios shown in Table 3. Farnesene = mixture of (*E,E*)-alpha-farnesene (49 %), (*E*)-beta-farnesene (26 %), (*Z*)- beta-farnesene (18 %), and (*Z,E*)-alpha-farnesene (7 %). Within columns with same color, means followed by different letters are significantly different (one-way ANOVA followed by Tukey's multiple comparison test, $P<0.05$). Moths were scored for upwind orientation (*black bar*), arrival at the source within 10 cm (*white bar*), and contact with the lures (*grey bar*). Significant differences in three flight behaviors between both sexes were analyzed by the Mann–Whitney *U*-test ($P<0.05$; ns, no significant difference)

(Fig. 5). Similar results have been found for the codling moth, *Cydia pomonella,* an important pest of apple (Wildbolz, 1958; Coracini et al., 2004). Female codling moths have frequently been observed to fly upwind over several meters toward branches with green apples, but contact the apple less frequently, which suggests that the females employ a different search strategy to find a suitable oviposition site, especially at close range (Witzgall et al., 1999).

Why are more male OFMs than females captured by traps with lures that emit plant-derived VOCs? Some authors have hypothesized that males use plant VOCs to distinguish environments where they can find females more easily (Ansebo et al., 2004). A partial explanation might be related to the large number of olfactory receptor neurons that are found in male antennae (Bäckman et al., 2000). Moreover, the flight mode of males may be different from that of females. Males fly toward the plant in search of females, and their orientation behavior is adapted to locate a point source of sex pheromone. By contrast, females searching for suitable oviposition sites may not be attracted to point sources of plant VOCs, and therefore trap design could be quite important. Furthermore, females may use optical cues in addition to VOCs to orient to an oviposition site. The greatest flight activity of mated and unmated male and female OFMs occurred during the first hour of dusk, during which time light intensity decreased from 3,750 to 57 lux, suggesting that the females use optical cues during orientation to oviposition sites (Hughes and Dorn, 2002). Females may land at the oviposition site based on both chemical and visual cues. The grape berry moth, *Paralobesia viteana*, a crepuscular species, oviposits less in the absence of light (Clark and Dennehy, 2002). In addition, physical structure and low-volatility VOCs present on the fruit surfaces could also affect the landing of females. In our study, OFMs always laid eggs on the smooth surface of waxed paper in the laboratory. The sticky base of the traps might be a repellent for female landing. The high male capture rates in our study could be applied in further research to establish a novel approach for monitoring males, especially in orchards treated with sex pheromone for mating disruption (Il'ichev et al., 2009). A logical follow-up, therefore, would be to investigate putative synergism between host-plant VOCs and the sex pheromone.

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Perception of Host Plant Volatiles in *Hyalesthes obsoletus*: Behavior, Morphology, and Electrophysiology

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Abstract The Palearctic planthopper *Hyalesthes obsoletus* is the natural vector of the grapevine yellow disease Bois noir. Grapevine is an occasional host plant of this polyphagous planthopper. To deepen our knowledge of the role of plant volatile organic compounds for *H. obsoletus* host plant searching, we carried out behavioral, morphological, and electrophysiological studies. We tested the attraction of *H. obsoletus* to nettle, field bindweed, hedge bindweed, chaste tree, and grapevine by using a Y-shaped olfactometer. The results showed a significant attraction of male *H. obsoletus* to chaste tree, and of the females to nettle. Male *H. obsoletus* were repelled by odor from hedge bindweed. Ultrastructural studies of the antennae showed at least two types of olfactory sensilla at the antennal pedicel: plaque organs and trichoid sensilla. Volatile organic compounds from nettle and chaste tree were collected, and the extracts were analyzed by coupling gas-chromatography to both mass-spectrometry and electroantennography. The volatile organic compounds that elicited electrophysiological responses in male and female antennae were identified. These findings are discussed with respect to behavior of *H. obsoletus* males and females in the field.

Keywords Planthopper · Phytoplasma vector · Volatile organic compounds · Y-tube bioassays · Electrophysiology · Antenna functional anatomy

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Introduction

Plant volatile organic compounds (VOCs) have critical roles in the evolution of host plant use by phytophagous insects (Linn et al., 2003). These insects detect VOCs via olfactory sensilla that are located on the antennae, and these cues provide information about food, mates, and oviposition sites (Visser, 1983; Schoonhoven et al., 2005). The role of olfaction in planthopper host location is still under investigation. The electroantennogram (EAG) technique and antenna morphology studies have been applied to *Nilaparvata lugens* Stal (Hemiptera: Fulgoromorpha: Delphacidae), and these have revealed olfactory receptors on the antennae that are responsive to plant volatiles (Aljunid and Anderson, 1983; Youn, 2002).

The insect investigated in the present study, the Paleartic planthopper *Hyalesthes obsoletus* Signoret (Hemiptera: Cixiidae), is known to use very different plants in different areas of its distribution (Table 1). Olfactometer assays and the EAG technique have shown that the chaste tree is the most attractive plant to *H. obsoletus* adults in Israel (Sharon et al., 2005). In the Marche region (central-eastern Italy), *H. obsoletus* larvae develop only on the nettle root system, and adult dispersion in a vineyard agroecosystem depends mainly on the spatial distribution of field bindweed (Riolo et al., 2007). In Germany, the formation of *H. obsoletus* host plant races on nettle and field bindweed has

Table 1 Host plants associated with *Hyalesthes obsoletus* in European and Mediterranean countries

Host plants		Country	Life stage
Family	Species		
Amaranthaceae	*Amaranthus retroflexus* L.	Israel[1]	A
Apiaceae	*Falcaria vulgaris* Bernh.	Turkey[2]	L, A
Asteraceae	*Ambrosia artemisiifolia* L.	Italy[3]	A
	Artemisia vulgaris L.	Italy[4]	A
	Tanacetum vulgare L.	Italy[4]	A
	Taraxacum officinale Weber	Switzerland[5]	A
	Taraxacum sp.	Turkey[2]	L, A
Boraginaceae	*Onosma armenum* DC.	Turkey[2]	L, A
Brassicaceae	*Crambe orientalis* L.	Turkey[2]	L, A
	Isatis glauca Gilib.	Turkey[2]	L, A
	Lepidium draba L.	†France[6]	L, A
		Switzerland[5]	A
		Turkey[2]	L, A
Convolvulaceae	*Calystegia sepium* (L.) R. Br.	France[6]	A
		†Germany[7]	L, A
		Italy[3,8]	L, A
		Switzerland[5]	A
	Convolvulus arvensis L.	†France[6]	L, A
		†Germany[7]	L, A
		Israel[1]	A
		Italy[4,8,9]	L, A
		Slovenia[10]	A
		Switzerland[5]	A
		Turkey[2]	L, A
Fabaceae	*Medicago sativa* L.	†Turkey[2]	L, A
	Melilotus officinalis (L.) Pall.	Turkey[2]	L, A
	Onobrychis viciifolia Scop.	Turkey[2]	L, A
Geraniaceae	*Geranium tuberosum* L.	Turkey[2]	L, A
Lamiaceae	*Lamium orvala* L.	Italy[8]	L, A
	Lavandula angustifolia Mill.	†France[6]	L, A
	Lavandula hybrida Reverchon	France[6]	A
	Satureja montana L.	France[6]	A
	Vitex agnus-castus L.	†Israel[1]	L, A
Myrtaceae	*Myrtus communis* L.	Israel[1]	A
Oleaceae	*Olea europaea* L.	Israel[1]	A
Plantaginaceae	*Linaria striata* L.	France[6]	A
	Plantago cynops L.	France[6]	A
	Plantago lanceolata L.	Switzerland[5]	A
Polygonaceae	*Polygonum aviculare* L.	Switzerland[5]	A
	Rumex sp.	Turkey[2]	L, A
Ranunculaceae	*Ranunculus bulbosus* L.	†Germany[7]	L, A
	Clematis vitalba L.	Switzerland[5]	A
Rubiaceae	*Galium verum* L.	France[6]	A
Urticaceae	*Urtica dioica* L.	France[11]	L, A
		†Germany[7]	L, A
		Hungary[12]	A
		†Italy[4,8,9]	L, A
		Slovenia[10]	A
		†Switzerland[5]	L, A

L: larvae; A: adults

†main host plants; [1]Sharon et al., 2005; [2]Güclü and Ozbek, 1988; [3]Picciau et al., 2008; [4]Lessio et al., 2007; [5]Kessler et al., 2011; [6]Sforza et al., 1999; [7]Langer et al., 2003; [8]Forte et al., 2010; [9]Riolo et al., 2007; [10]Petrovic et al., 2003; [11]Bressan et al., 2007; [12]Palermo et al., 2004

been hypothesized (Johannesen et al., 2008; Imo et al., 2011), similarly to what has been reported for *Alebra* leafhoppers (Hemiptera: Cicadellidae) (Aguin-Pombo, 2002). However, the use of different host plants by *H. obsoletus* might also be influenced by the availability of the plants, the soil type (preference for soils with natural cavities, suitable for larval development), the cultivation practices (Weber and Maixner, 1998), and the presence of organic mulch (Howard and Oropeza, 1998). *Hyalesthes obsoletus* has an important role as a vector of the stolbur phytoplasma, which is associated with grapevine yellow, a disease that is known as Bois noir (Maixner et al., 1994). Grapevines represent only an occasional host for planthopper adults (Lessio et al., 2007) that can transmit the phytoplasma during their feeding probing. Vineyard inter-row grass cover and border vegetation represent potential phytoplasma reservoirs (Maixner et al., 1995; Riolo et al., 2007).

The aim of this study was to investigate the influence of plant odor on *H. obsoletus* host plant searching. We determined the attractiveness of odor from nettle, field bindweed, hedge bindweed, chaste tree, and grapevine to females and males of *H. obsoletus* associated to nettle by using a Y-tube olfactometer. Furthermore, we recorded EAG responses to volatile extracts from the plants that were most attractive to both sexes of *H. obsoletus*, and we identified the VOCs that elicited positive EAG responses. Finally, the functional anatomy of the *H. obsoletus* antenna pedicel was studied.

Methods and Materials

Insects Adult *H. obsoletus* were collected from nettle plants in the Ancona district (43°32′ N; 13°23′ E), in the region of Marche, in Italy. These were transferred to the laboratory, separated according to sex, and caged with fresh shoots of nettle. Twenty-four hours before the beginning of the bioassays, adult planthoppers were transferred to cages containing sucrose solution (5 % sucrose, 0.5 % sorbitol), to avoid any influence on the plants used in the subsequent experiments. Planthoppers were kept at 26±1 °C and 60 %±10 % relative humidity, under a natural photoperiod.

Plants Chaste tree and grapevine cv. Chardonnay were grown in plastic pots (diam., 20 cm; height, 20 cm) in a greenhouse (temperature, 26±1 °C; relative humidity, 60 % ±10 %; natural photoperiod). Nettle, field bindweed, and hedge bindweed were collected in the field (in the Ancona district).

Y-Tube Olfactometer Bioassays The responses of *H. obsoletus* adults to plant VOCs were investigated using a dual choice Y-tube olfactometer (stem, 25 cm; arm length, 20 cm, arm angle, 75°; internal diam, 4 cm). Each arm of the Y-tube was connected to a glass cylinder (9×18 cm); one chamber served as the control, and the other held the test material. An airflow was maintained from each cylinder through the olfactometer arms, using an air pump with the airflow adjusted with a flow meter to 1.0 $l min^{-1}$. The incoming air was passed through activated charcoal and humidified with double-distilled, deionized water. The glass Y-tube was positioned with a slope of 10° from the horizontal plane.

Single planthoppers were introduced individually into the olfactometer at the entrance of the stem, and they were observed until they had walked at least 13 cm up one of the arms, or until 5 min had elapsed. Planthoppers that did not choose a side arm within 5 min were recorded as ‘no choice’. For each individual planthopper, their activation time, as the exit time from the release vial, and their choice time were recorded.

Samples were randomly assigned at the beginning of the bioassays, and they were reversed after having tested 5 planthopper adults in order to minimize any spatial effects on the choices of the planthoppers. Bioassays were conducted from ~15:00 to 19:00 hr. After each trial, the Y-tube was washed with detergent, rinsed with distilled water and absolute ethanol, and baked overnight at 200 °C. The responses of 60 planthoppers per sex were tested for each of the various treatments. Experiments were conducted in a laboratory at a temperature of 26±1 °C and a relative humidity of 60 %±10 %.

Volatiles Sources and Experiments Experiments were designed to investigate the adult *H. obsoletus* male and female responses to the volatiles from the different plant species. Parts of plants of nettle, field bindweed, hedge bindweed, chaste tree, and grapevine were collected 1 hr prior to the start of the bioassays. Fresh shoots were used (length, 10–15 cm; weight, ca. 5 g). The cut stem was wrapped in cotton wool and inserted into a 6-ml vial filled with distilled water. Cotton wool inserted into a 6-ml vial filled with distilled water was used as the blank. Vials were sealed with Teflon tape.

Headspace Collection Headspace collections were made from fresh shoots of nettle and chaste tree, which were the plants that elicited significant behavioral responses in the Y-olfactometer experiments (see Results section). Fifteen shoots (ca. 200 g) were placed in a 25×38 cm polyacetate bag (Toppits, Melitta, Sweden) for collection of the volatiles (Tasin et al., 2005; Faccoli et al., 2008). The ends of the shoots were placed into glass vials (4 ml) filled with water and sealed with parafilm. The air from the headspace of each bag was drawn out at 150 ml min^{-1} through an adsorbent cartridge trap (75 mg Super Q; Sigma-Aldrich, Milan, Italy) connected to a vacuum pump. Charcoal-filtered air was simultaneously pulled into the bag by the same pump, to maintain constant pressure. Collections

were carried out over 24 hr in a climatic chamber at a temperature of 25±2 °C, a relative humidity of 60 %±10 %, and a photoperiod of 16:8 (L:D), with 1,000 lx during the light period. Volatiles were eluted from the adsorbent cartridges by solvent desorption at room temperature, using 500 µl hexane (>99 % purity, Sigma-Aldrich). Three collections from different groups of each of the shoots were carried out. Some of these extracts were prepared for chemical quantification, and 0.5 µg heptyl acetate (≥99 % purity) was added as an internal standard (Bengtsson et al., 2001). The collected extracts were reduced to 50 µl using a slow stream of nitrogen, and then stored in 2-ml vials at −18 °C until use.

Gas Chromatography and Electroantennography Detection Two microliters of the concentrated plant extracts in hexane were injected into a Hewlett-Packard 5890 gas chromatography (GC) system. This used a polar Innowax column (30 m×0.32 mm; J & W Scientific, Folsom, CA, USA) programmed to increase from 60 °C (held for 3 min) at 8 °C min^{-1}, to 220 °C (held for 7 min), and was interfaced with the EAG apparatus (Arn et al., 1975). The outlet of the GC column was split in a 1:1 ratio between a flame ionization detector and an antenna of *H. obsoletus*.

We used an EAG technique that is similar to that described by Den Otter et al. (1996), using a standard EAG apparatus (Syntech, Hilversum, The Netherlands). A glass capillary indifferent electrode was filled with Kaissling solution (Kaissling, 1987), which contained 5.0 gl^{-1} polyvinylpyrrolidone K90 (Fluka Chemie, Buchs, Switzerland), and this was inserted into the head of the planthopper. The difference electrode was a similar glass capillary from which the tip was previously cut, and this was brought into contact with the distal end of an antenna. The GC-EAG responses of *H. obsoletus* antennae to nettle extracts were recorded for antenna activity. Compounds eluting from the capillary column were delivered to the antenna through a glass tube (12 cm×8 mm) in a charcoal-filtered and humidified airstream. The antenna signal and the flame ionization detector signal were amplified and recorded simultaneously using Syntech software.

Samples from both nettle and chaste tree extracts were tested on 5 different *H. obsoletus* males and females. A compound was considered electrophysiologically active when it elicited at least three antennal responses that were different from background noise (Zhang et al., 2001).

Chemical Analysis Three samples of each extract were analyzed by coupled GC and mass spectrometry (GC-MS). The analyses were performed on a Hewlett-Packard 5890 GC system, with a polar Innowax column (30 m×0.32 mm; J & W Scientific, Folsom, CA, USA) programmed to increase from 60 °C (held for 3 min) at 8 °C min^{-1}, to 220 °C (held for 7 min). This was interfaced with a Hewlett-Packard 5970B mass spectrometer that was operated using electron impact ionization (70 eV). The identities of most (84 %) of the compounds in the volatiles collections were verified by comparison with synthetic compounds, as indicated in Table 3. The compounds that did not elicit antennal responses and for which no standards were available, were tentatively identified using the Wiley mass spectra database. The identified compounds were quantified by comparing their peak areas to those of the internal standard.

Scanning Electron Microscopy Ten individuals of each sex were used for the scanning electron microscopy (SEM) observations. Planthoppers were anesthetized using CO_2 and kept at −18 °C until they died. Individual planthoppers then were dissected, to remove antennae from the head capsule. In some cases, the whole head was detached from the rest of the body, with the antennae in their natural positions. The specimens were dehydrated through a series of graded ethanol concentrations, from 50 to 99 %. After dehydration, the 99 % alcohol was substituted with pure hexamethyldisilazane (Sigma), and the specimens were left to dry under a chemical hood under room conditions. Five antennae were mounted on each aluminum stub, with care taken to position them with different orientations, to have a clear view of the ventral, dorsal, and both of the lateral sides. These mounted antennae were gold-sputtered using a Balzers Union SCD 040 unit. Observations were carried out using Philips XL 30 and Zeiss Supra scanning electron microscopes.

Transmission Electron Microscopy Ten individual planthoppers of each sex were anesthetized with CO_2 and immediately immersed in a solution of 5 % glutaraldehyde and 2.5 % paraformaldehyde in 0.1 M cacodylate buffer, 5 % sucrose, pH 7.2–7.3. For each antenna, the pedicel was separated from the scape and the flagellum to aid fixative penetration, and they were left at 4 °C for 2 hr. Samples were kept at 4 °C overnight in 0.1 M cacodylate buffer, 5 % sucrose, pH 7.2–7.3. Then, the antennae were post-fixed in 1 % OsO_4 (osmium tetroxide) for 1 hr at 4 °C, and rinsed in the same buffer. Dehydration through a graded ethanol series was followed by embedding in Epon-Araldite, with propylene oxide as the bridging solvent. Thin sections (90 nm) were cut with a diamond knife on a Nova LKB ultramicrotome, and mounted on formvar-coated 50-mesh grids. Finally, after staining with uranyl acetate (20 min, room temperature) and lead citrate (5 min, room temperature), the sections were examined with a Philips EM 208 electron microscope. Digital pictures were obtained (1,376×1,032 pixels, 8b, uncompressed grayscale Tiff files), using a high-resolution MegaViewIII (SIS) digital camera connected to the transmission electron microscope.

Statistical Analysis The Y-tube olfactometer data were analyzed by one-tailed binomial tests (H_0: insects do not prefer

the odor source compared with the blank). Differences between treatments (plants) were evaluated by contingency table analysis based on *Chi-square* (Zar, 1999). Percentages (arcsin transformed) of males and females leaving the release vials were tested by one-way ANOVA. The variabilities in the activation times and first choice times between the host plants and the sexes were determined by one-way ANOVA followed by least significant difference tests. The significance level of all of the statistical tests was set at $P<0.05$. These analyses were performed using Systat 11 (Systat Software Inc.). The individuals that did not make a choice were not included in the analyses.

Results

Bioassays The olfactometer assays showed that *H. obsoletus* males were significantly ($P<0.05$) attracted to the volatiles of chaste tree, whereas females were attracted to nettle, when these plant shoots were compared with blank (Figs. 1 and 2). Furthermore, the males were significantly ($P<0.05$) repelled by hedge bindweed when this plant was compared with blank (Fig. 1). However, no differences in *H. obsoletus* preferences were recorded between treatments (males: $\chi^2=6.878$, $df=4$, $P=0.142$; females: $\chi^2=5.025$, $df=4$, $P=0.285$).

The exit time from the release vial (i.e., the activation time) was significantly influenced by the sex of the planthoppers: males exited the release vial faster than the females (in average 15.7 sec for males vs. 23.9 sec for females) ($P<0.01$; Table 2). Similarly, overall, the males showed a shorter first choice time (mean 95.3 sec), while for females the first choice time was in average 119.8 sec ($P<0.001$; Table 2). In particular, when the bioassays were carried out with field bindweed, males showed a shorter activation time ($P<0.01$) and first choice time ($P<0.001$) than the females (Table 2). Furthermore, by comparing the different treatments, a higher mean choice time ($P<0.05$) was observed for males when nettle was tested vs. blank. Other differences were seen for male vs. female activation times when hedge bindweed ($P<0.01$) and grapevine ($P<0.05$) were assayed (Table 2). Moreover, the percentage of female planthoppers that made a choice between the arms of the olfactometer was lower than that of the male planthoppers (76 % females vs. 88 % males; $P<0.001$).

Chemical Analysis Compounds identified from the shoots of nettle and field bindweed are listed in Table 3. Hydrocarbons, alcohols, aldehydes, ketones, esters, aromatic compounds, monoterpenes, sesquiterpenes, and other terpenoids were identified from nettle, with a total of 41 compounds. Similarly for chaste tree, hydrocarbons, alcohols, aldehydes, esters, aromatic compounds, monoterpenes and sesquiterpenes were identified, with a total of 24 compounds.

The most abundant compound in the headspace collections for nettle was (*Z*)-3-hexenyl-acetate, with an average amount of 200 g of nettle shoots of 4.66±0.98 μg h^{-1} (74.3 %±15.1 % of the total ion abundance). The most abundant compound in the headspace collections for chaste tree was α-pinene, here with an average amount of 200 g of chaste tree shoots of 0.57±0.09 μg h^{-1} (44.1 %±13.20 % of the total ion abundance).

Gas Chromatography and Electroantennography Detection The GC-EAG analyses of the headspace collections from nettle shoots detected nine active compounds for the male *H.*

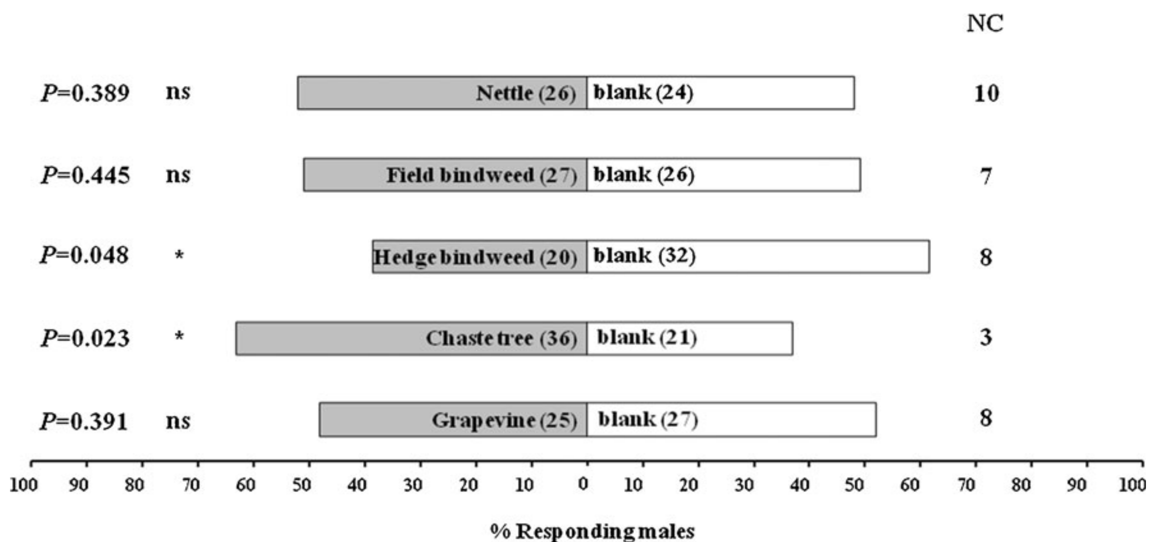

Fig. 1 Behavioral responses of *Hyalesthes obsoletus* males to the plants tested. The numbers in *brackets* indicate the number of insects that made a choice; the level of significance is indicated on the left (one-tailed binomial test: ns, non-significant; *$P<0.05$); NC, number of insects that did not complete a choice

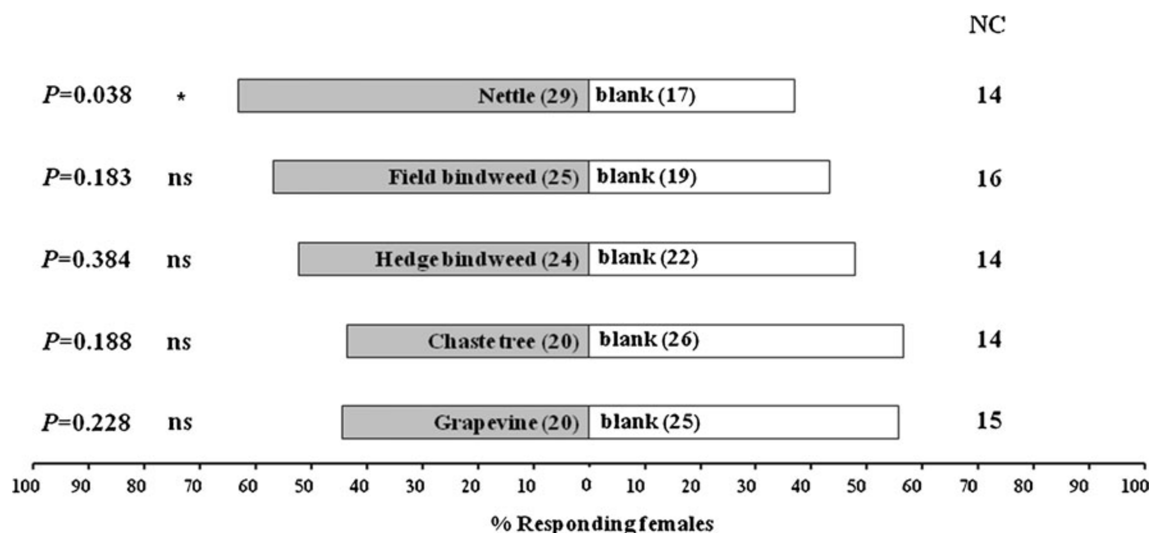

Fig. 2 Behavioral responses of *Hyalesthes obsoletus* females to the plants tested. As for legend to Fig. 1

obsoletus antennae, and 11 active compounds for the female *H. obsoletus* antennae (Table 3). The antennal responses of the males were elicited by (*Z*)-3-hexen-1-ol, (±)-linalool, (*E*)-β-caryophyllene, (*E*)-β-farnesene, (*Z*,*E*)-α-farnesene, (*E*,*E*)-α-farnesene, methyl salicylate, (*Z*)-jasmone, and benzothiazole, whereas the antennal responses of the females were elicited by 4,8-dimethyl-1,(*E*)-3,7-nonatriene, (*Z*)-3-hexenyl-acetate, (*Z*)-3-hexen-1-ol, (±)-linalool, (*E*)-β-caryophyllene, (*E*)-β-farnesene, (*Z*,*E*)-α-farnesene, (*E*,*E*)-α-farnesene, methyl salicylate, (*Z*)-jasmone, and benzothiazole (Table 3). *Hyalesthes obsoletus* males and females responded to the same eight volatile compounds collected from the chaste tree headspace (Table 3): 1,8-cineole, (±)-linalool, (*E*)-β-caryophyllene, methyl benzoate, (*E*)-β-farnesene, (*Z*,*E*)-α-farnesene, (*E*,*E*)-α-farnesene, and methyl salicylate.

Functional Anatomy In *H. obsoletus*, the antennae have three segments: the short, cylindrical scape that is devoid of sensilla, the pedicel, and the elongated flagellum. The sensory structures associated with the flagellum have previously been studied in detail (Romani et al., 2009). The pedicel is the largest antennomere, with 200 μm diam. and ca. 250 μm length (Fig. 3a, d). The surface of the pedicel has two types of multiporous sensilla: the sensilla placoidea, or 'plaque organs' (*sensu* Lewis and Marshall, 1970), and the sensilla trichoidea (Fig. 3d). No differences were found in the number and arrangement of these sensilla between these male and female planthoppers. The presence of abundant wax covering the antennal structures was difficult to prevent in all of the preparations.

Sensilla Placoidea These structures are present on most of the pedicel surface, with about 30 elements (Fig. 3a). Each sensillum appears as a rounded, flattened, 'rose-like' structure (diam ca. 35 μm) (Fig. 3b). The porous cuticle is folded several times, which gives rise to up to 15 elongated elements. The space between each of these structures is occupied by a cuticular spine (length, ca. 16 μm), which points

Table 2 Activation and first choice times of *Hyalesthes obsoletus* males and females

Treatment	Activation time [sec]		First choice time [sec]	
	Males	Females	Males	Females
Nettle vs. blank	23.6±5.8a	22.4±4.4a	124.7±10.8a	121.4±10.9a
Field bindweed vs. blank	13.3±3.1**a	28.5±3.3a	80.1±8.9***b	148.9±10.9a
Hedge bindweed vs. blank	8.9±1.9**a	27.2±5.8a	95.2±9.6b	109.8±11.9a
Chaste tree vs. blank	19.3±4.4a	15.1±2.1a	92.4±9.1b	108.4±10.9a
Grapevine vs. blank	13.6±4.2*a	26.3±4.5a	84.2±9.7b	110.9±10.2a
Overall means	15.7±3.9**	23.9±4.0	95.3±9.6***	119.8±11.0

Data are means ± SE

N=60 males or females for each comparison. Significance in male vs. female comparisons: *$P<0.05$; **$P<0.01$; ***$P<0.001$ (one-way ANOVA test). Identical letters within the same column indicate no difference between treatments (least significant difference test, $P<0.05$)

Table 3 Mean levels of volatile compounds [μg h^{-1}] collected in the headspace from shoots of nettle and chaste tree, and GC-EAG activity on *Hyalesthes obsoletus* male and female antennae

Compound[1,2]	Source of standards[4]	Plant shoots		GC-EAG activity			
		Nettle	Chaste tree	Nettle		Chaste tree	
				Male	Female	Male	Female
Hydrocarbons							
Dodecane	SA	–	0.65±0.15				
Tetradecane	F	–	0.45±0.07				
Pentadecane	F	0.16±0.07	0.31±0.09				
Alcohols							
3-hexanol	SA	0.46±0.11	0.03±0.01				
2-hexanol	SA	0.20±0.06		–			
(*Z*)-3-hexen-1-ol	SA	2.41±1.01	0.29±0.10	*	*		
(*E*)-3-hexen-1-ol	SA	0.18±0.04	–				
1-octen-3-ol	SA	0.69±0.17	–				
1-octanol	SA	0.03±0.01	–				
Aldehydes							
Hexanal	F	0.12±0.05	–				
Nonanal	SA	–	0.08±0.02				
Ketones							
3-hexanone	SA	0.38±0.13	–				
Acetophenone	SA	0.05±0.01	–				
4-ethyl-acetophenone[3]	–	0.05±0.02	–				
Esters							
2-hexyl-acetate[3]	–	0.08±0.03	–				
n-hexyl-acetate	SA	0.65±0.19	–				
(*Z*)-3-hexenyl-acetate	SA	74.29±15.06	0.03±0.02		*		
3-cyclohexenyl-acetate[3]	–	0.24±0.09	–				
Exobornyl-acetate[3]	–	0.06±0.04	–				
(*Z*)-3-hexenyl-benzoate	SA	0.11±0.03					
Aromatics							
Methyl benzoate	SA	0.02±0.01	0.04±0.02			*	*
Naphthalene	SA	0.04±0.03	–				
Methyl salicylate	SA	0.17±0.07	0.12±0.04	*	*	*	*
2-methyl-benzothiazole[3]	–	0.06±0.02	–				
Benzothiazole	SA	9.68±2.49	0.38±0.16	*	*		
Homoterpenes							
4,8-dimethyl-1,(*E*)-3,7-nonatriene	IS	0.55±0.21	–		*		
Irregular terpenoids							
(*Z*)-jasmone	SA	0.04±0.03	–	*	*		
Monoterpenes							
α-pinene	SA	0.48±0.18	44.07±13.20				
β-pinene	SA	0.05±0.01	–				
Myrcene	SA	0.02±0.01	5.37±1.92				
Ocimene	SA	0.12±0.03	0.29±0.07				
Limonene	SA	0.09±0.05	2.70±1.28				
Camphor[3]	–	0.10±0.06	–				
(±)-linalool	SA	0.02±0.01	0.02±0.01	*	*	*	*
1,8-cineole	F	–	20.00±4.07			*	*
α-terpinene	SA	–	0.61±0.19				
α-terpineol	SA	–	3.22±1.03				

Table 3 (continued)

Compound[1,2]	Source of standards[4]	Plant shoots		GC-EAG activity			
		Nettle	Chaste tree	Nettle		Chaste tree	
				Male	Female	Male	Female
Carene	SA	–	5.46±2.56				
p-cymene	SA	–	1.21±0.56				
Sesquiterpenes							
Longifolene[3]	–	0.03±0.02	–				
β-elemene[3]	–	0.08±0.01	–				
(*E*)-β-caryophyllene	SA	0.93±0.18	3.45±1.34	*	*	*	*
(*E*)-β-farnesene	B	0.24±0.09	1.27±0.34	*	*	*	*
α-humulene	SA	0.09±0.03	–				
Germacrene-D	AB	0.27±0.07	2.59±0.79				
(*Z*,*E*)-α-farnesene	F	0.34±0.18	0.46±0.12	*	*	*	*
(*E*,*E*)-α-farnesene	F	4.35±1.21	7.14±2.49	*	*	*	*
Farnesol	SA	0.08±0.03	–				
α-thujene	C	–	0.30±0.08				
Caryophyllene oxide	SA	0.11±0.05	–				

Data are means ± SE

N=3 for each plant species. Unit is μg h^{-1}

[1] Mean amount of (*Z*)-3-hexenyl-acetate collected from 200 g shoots of nettle was 4.66±0.98 μg h^{-1}

[2] Mean amount of α-pinene collected from 200 g shoots of chaste tree was 0.57±0.09 μg hr^{-1}

[3] Compounds tentatively identified with Wiley mass spectra database

[4] Standards were obtained from Sigma-Aldrich (Milan, Italy) (SA), Fluka Chemie (Buchs, Switzerland) (F), Bedoukian Research Inc. (Danbury, CT) (B), Firmenich (Geneva, Switzerland) (F), Ingve Stensrøm (Aas, Norway) (IS), Anna-Karin Borg-Karlson (Stockholm, Sweden) (AB)

[*] Compounds eliciting responses in *Hyalesthes obsoletus* male and female antennae for GC-EAG; a compound was considered electrophysiologically active when it elicited at least three antennal responses that were different from background noise (Zhang et al., 2001)

towards the center of the sensillum (Fig. 3b). A second series of shorter cuticular spines (length, ca. 10 μm) is arranged all around the sensillum. The TEM cross-sections revealed a substantial difference in the cuticular organization between the porous cuticle and the spines, with the cuticle being thin (150 nm) and pierced by numerous scattered pores, and the spines being thick (ca. 500 nm) and aporous (Fig. 4a–c). Each sensillum placoideum is innervated by a variable number of sensory neurons (from 45 to 80) that are arranged in units (Fig. 4d–f). Each unit is surrounded by a dendritic sheath, and is formed by two to 18 dendrites (Fig. 4e). At the level of the sensillum socket, each neuron gives rise to numerous dendritic branches that completely fill the space below the porous cuticle.

Sensilla Trichoidea These sensilla are grouped on a specific side of the pedicel, i.e., the side facing the compound eye, and there are ca. 140 of them (Fig. 3d). Each sensillum (base diam. ca. 1.6 μm; length, ca. 22 μm) is seen as a blunt-tipped hair, and they are slightly curved and inserted into the pedicel wall through an inflexible socket (Fig. 3e). The hair cuticle is densely covered with pores (Fig. 3f). The TEM shows that two sensory neurons innervate each sensillum (Fig. 5c, d). The dendrites have branches near the base of the sensillum which gives rise to some ramifications that extend up to the sensillum tip (Fig. 5a, b).

Discussion

Bioassay The bioassay results showed sex-specific responses by *H. obsoletus* to plant volatiles. Males responded positively to odor from chaste tree, whereas females were attracted by nettle volatiles, when these plant shoots were compared with blank. Males also showed a repellent response to the hedge bindweed volatiles. We observed shorter activation and first choice times for males, as compared to females, when field bindweed was assayed. Furthermore, comparing the different treatments, a higher mean choice time was observed for males when nettle was tested vs. blank.

The attraction of females to nettle volatiles was expected, as nettle is a primary host for *H. obsoletus* larval development. Previous studies have shown that *H. obsoletus* show plant affiliation with their primary hosts (Sharon et al.,

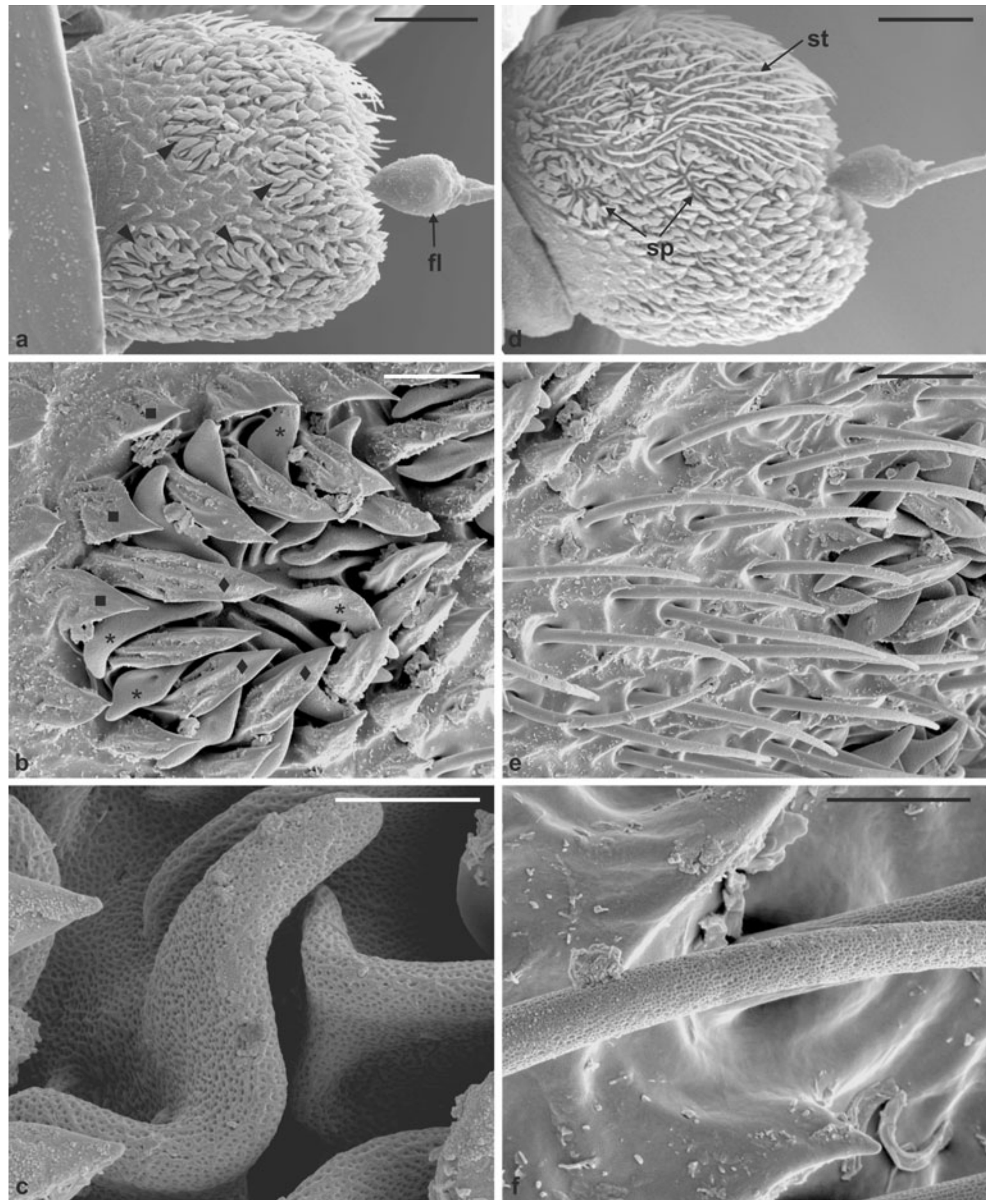

Fig. 3 SEM images from *Hyalesthes obsoletus*. **a** Lateral view of the pedicel, showing sensilla placoidea (*arrowheads*). FL, flagellum. **b** Close-up of a sensillum placoideum. The folded porous cuticle (*asterisk*), as well as the long cuticular spines (*black diamond suit*) and the short cuticular spines (*black square*), are clearly visible. **c** Detail of the porous cuticle. **d** Dorsal view of the pedicel showing the sensilla trichoidea (ST) and sensilla placoidea (SP). **e** Numerous sensilla trichoidea are visible. **f** High magnification detail of the sensilla trichoidea porous cuticle. Scale bars: a, d: 50 μm; b, e: 10 μm; c, f: 3 μm

2005; Maixner et al., 2009; Kessler et al., 2011) and that affiliation is established during its last larval instar (Kessler et al., 2011). The formation of sympatric host races in *H. obsoletus* also has been hypothesized (Imo et al., 2011). The attraction of *H. obsoletus* males to chaste tree (primary host in Israel) volatiles that was observed during this study is unexpected, as this plant is not found in the Italian agro-ecosystems. In olfactometer assays, Sharon et al. (2005) observed that chaste tree is the most attractive plant for both males and females when compared to bindweed and grapevine. Johannesen et al. (2008) reported that southern and western European populations of the planthopper have a common Levantine origin. This implies that the plant affiliation of *H. obsoletus* is not only based on associative learning of chemical and/or physical cues, but also determined by genetic factors (Papaj and Prokopy, 1989; Bernays, 2001).

Hyalesthes obsoletus males also show greater activity than the females in the field (Bressan et al., 2007). Field bindweed has been reported as the main primary host for *H. obsoletus* in France and Germany (Sforza et al., 1999; Langer et al., 2003). Moreover, the dispersion of this planthopper depends on the field bindweed spatial distribution when its primary host is unavailable (i.e., mowing) (Riolo et al., 2007). We suggest that in many Italian agroecosystems, field bindweed is not an *H. obsoletus* primary host because it grows in compacted loam soils that are not suitable for larva development. Indeed, despite the intrinsic genetic ability of *H. obsoletus* to change the rank order of its plant preference, the available host plants, the soil type (preference for soils with natural cavities), the cultivation practices (Weber and Maixner, 1998), and the presence of organic mulch (Howard and Oropeza, 1998) also have major roles in planthopper larval development.

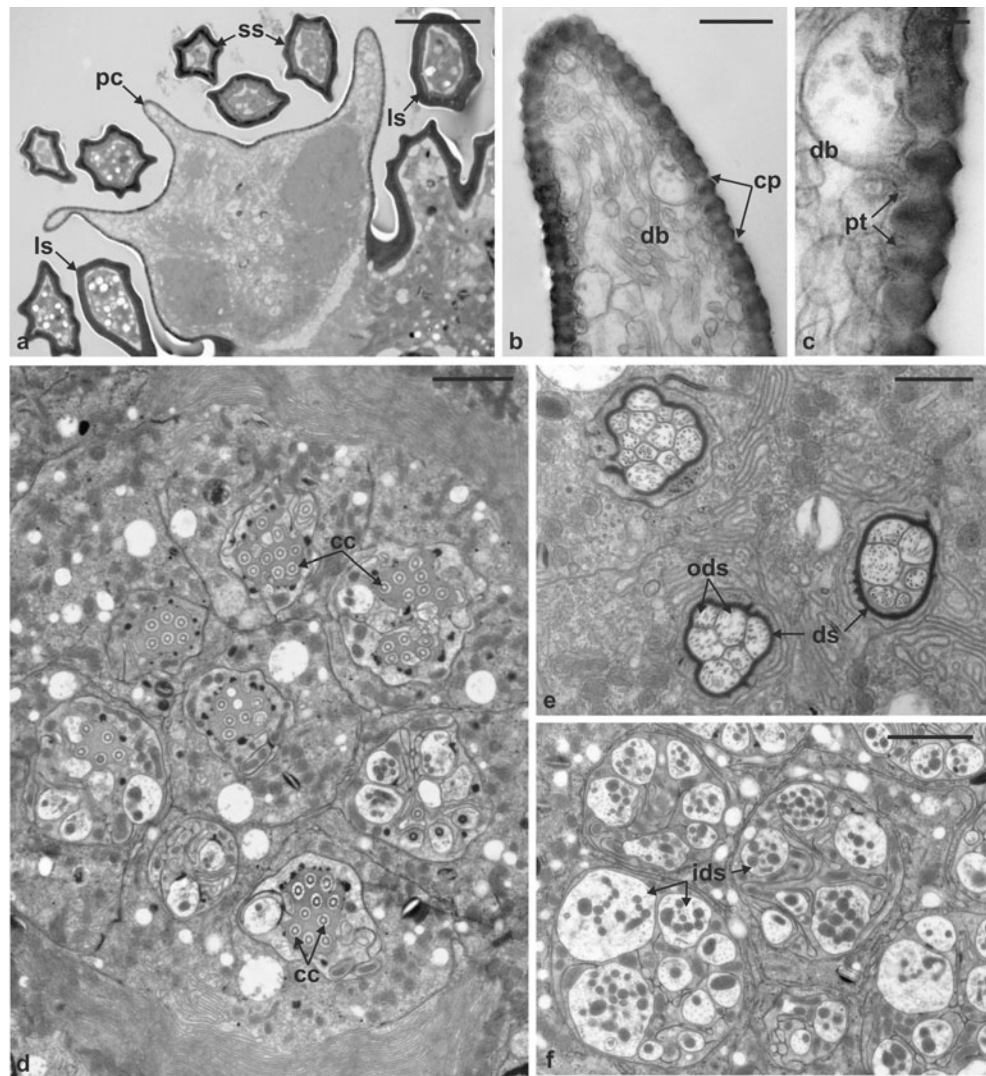

Fig. 4 TEM images of *Hyalesthes obsoletus* sensilla placoidea. **a** Longitudinal section of a sensilla placoidea. The porous cuticle (PC) develops into three arms, surrounded by the aporous long cuticular spines (LS) and short cuticular spines (SS). **b**, **c** Details of the porous cuticle at the level of the folded cuticle. A large number of dendritic branches (DB) completely fill the lumen of the process, running very close to the cuticular pores (CP), where pore tubules (PT) are found. **d** Cross-section of a sensillum placoideum below the sensillum socket, at the level of the sensory neuron ciliary constrictions (CC). The sensory neurons are grouped into eight different 'neuronal units'. Each unit is made up of two to 18 sensory neurons. **e** Cross-section of three neuronal units, showing the outer dendritic segments (ODS). Each bundle of the ODS is isolated from the others by a separated dendritic sheath (DS). **f** Cross-section showing three neuronal units at the inner dendritic segment (IDS) level. Scale bars: a: 5 μm; b: 500 nm; c: 100 nm; d, f: 2 μm; e: 1 μm

Sex-specific differences between behavioral responses of *H. obsoletu*s to plant VOCs are reflected by the different ecological niches that males and females occupy. Indeed, *H. obsoletus* males are found in the canopy of nettle, probably for reasons of mate searching (Mazzoni et al., 2010). On the other hand, females lay their eggs in the soil near the roots (Sforza et al., 1999), and so they feed more frequently on basal stems and find hiding places in the organic mulch (Riolo personal observation). The *H. obsoletus* males might use nettle VOCs to avoid adverse effects of intraspecific and interspecific competition, as has been reported for other planthoppers that live and feed in the canopy layer (Ferrenberg and Denno, 2003; Matsumura and Suzuki, 2003).

Hedge bindweed has not been reported as an *H. obsoletus* plant host in Italian agroecosystems, where it frequently grows amongst nettle. Further GC-EAD and behavioral assays are needed to investigate the deterrent activity of hedge bindweed VOCs towards *H. obsoletus* males.

Gas Chromatography and Electroantennography Detection The odor bouquets emitted from the shoots of nettle and chaste tree are considerably different in their compositions and quantities, and in the ratios of the compounds released. Nevertheless, there were 17 VOCs that overlapped between the 41 and 26 compounds that we identified here in the headspace from nettle and chaste tree, respectively. Similarly, of the 11 and 8 compounds, respectively, that elicited GC-EAG responses in both *H. obsoletus* males and females, six were present in both the nettle and chaste tree extracts. This represents the first successful application of a GC-EAG experimental approach to a planthopper species.

This subset of six compounds that elicited antennal responses and were present in the headspace of both nettle and chaste tree included: one aromatic compound (methyl salicylate), one monoterpene (±)-linalool, and four sesquiterpenes [(*E*)-β-caryophyllene, (*E*)-β-farnesene, (*Z*,*E*)-α-farnesene, (*E*,*E*)-α-farnesene]. These are all ubiquitous plant

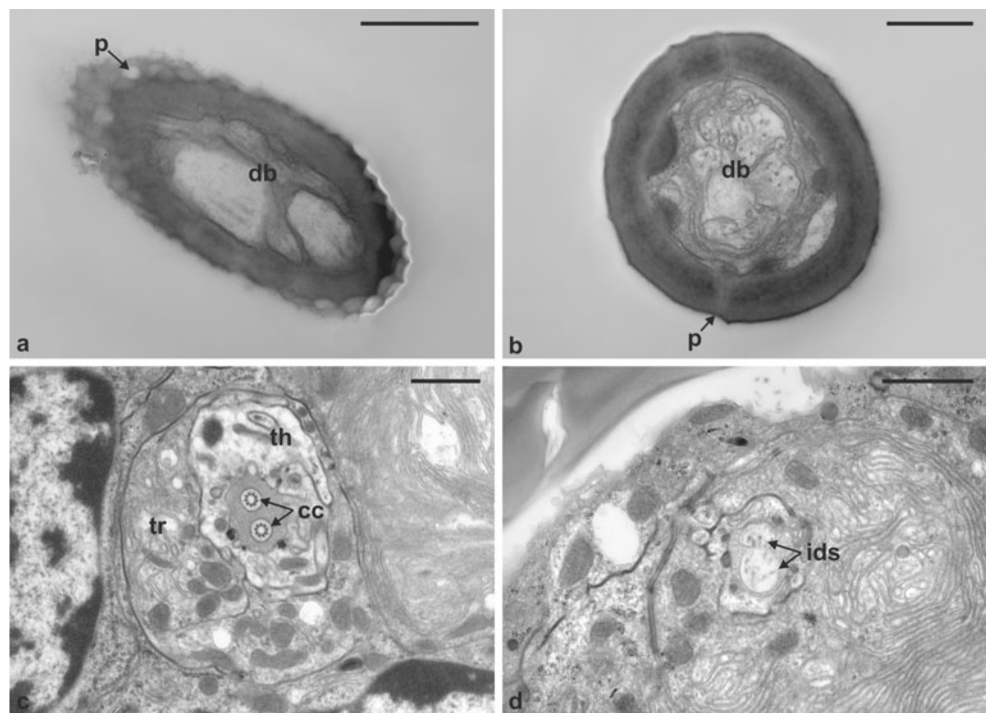

Fig. 5 TEM images of *Hyalesthes obsoletus* sensilla trichoidea. **a**, **b** Cross-section of the cuticular shaft of a sensillum trichoideum, showing the pores (P) and the dendritic branches (DB) within the sensillum lumen. **c**, **d** Cross-sections below the sensillum trichoideum socket: the two sensory neurons are pictured at the level of the ciliary constrictions (CC) and inner dendritic segment (IDS). TH, thecogen cell; TR, trichogen cell. Scale bars: a: 10 µm; b: 2 µm; c: 100 nm; d: 200 nm

volatiles that are known to mediate host recognition in several insect species (Visser, 1986; Bruce et al., 2005). With (±)-linalool, we note that it was not possible to distinguish between its enantiomers.

All of these compounds have also been detected in head-space volatiles from grapevine (Tasin et al., 2005; Cha et al., 2008). One of the mechanisms that mediates the shift in host range of an insect might be the sharing of a subset of compounds that are important for attraction (Tasin et al., 2010). Therefore, as for the majority of polyphagous herbivores, *H. obsoletus* might rely on the absolute and relative amounts of such ubiquitous plant volatiles during the process of finding a host plant (Bruce et al., 2005; Anfora et al., 2009; Cha et al., 2011). They might, thus, be driven towards grapevine by the same group of compounds, and transmit the stolbur phytoplasma during their feeding probing.

On the other hand, among the EAG-active compounds, the terpenoids 4,8-dimethyl-1,(*E*)-3,7-nonatriene and (*Z*)-jasmone were extracted only from the nettle headspace, while the monoterpene 1,8-cineole was present only in chaste tree. In contrast, (*Z*)-3-hexen-1-ol, benzothiazole, and (*Z*)-3-hexenyl-acetate were identified in both nettle and chaste tree extracts, but elicited antenna responses only in the case of nettle; these compounds were, thus, potentially under the *H. obsoletus* perception threshold with chaste tree. A reversed result was seen for methyl benzoate. (*Z*)-3-hexen-1-ol, (±)-linalool, and methyl benzoate have been shown to elicit antennal responses in another planthopper species, the brown rice planthopper *N. lugens*, where the females were more responsive than the males (Youn, 2002). Furthermore, as host plants are often selected by gravid females, it is particularly intriguing that only the females show sensitivity to (*Z*)-3-hexenyl acetate and 4,8-dimethyl-1,(*E*)-3,7-nonatriene, which were abundant in the nettle. Variations in the sensitivities of the antennal receptors and the specificities between males and females might explain this sex-biased plant-volatile attraction in some insects (Fraser et al., 2003).

The GC-EAG technique was chosen as a suitable and powerful tool for the identification of active compounds in a complex odor blend, where it can often reveal the biological importance of secondary compounds in a mixture (Riffell et al., 2009). Compounds that elicited electrophysiological responses in *H. obsoletus* antennae are likely to have roles in the behavior of this insect. However, further studies need to investigate whether the EAG active compounds indeed affect *H. obsoletus* behavior and also play a role in the field.

Functional Anatomy Despite differences in the responses to these plant volatiles shown by *H. obsoletus* male and female individuals, the antennal structures investigated did not show any sexual dimorphism. Similar results have been reported for the carrot psyllid, *Trioza apicalis* Foerster (Hemiptera: Triozidae); the male and female antennae of this psyllid have the same sensilla, but they respond to different volatiles (Kristoffersen et al., 2006, 2008). In *H. obsoletus*, we found two different types of olfactory sensilla. The first type are the sensilla placoidea, and these have been reported to be common (although with some structural variations) within the Fulgoromorpha (Lewis and Marshall, 1970; Marshall and Lewis, 1971; Aljunid and Anderson,

1983; Bourgoin and Deiss, 1994). In *H. obsoletus*, we found ca. 30 sensilla placoidea scattered on the pedicel surface. Within the Fulgoromorpha, the number of sensilla placoidea ranges between 17 with *N. lugens* (Aljunid and Anderson, 1983) and 200 with *P. candelaria* (Lewis and Marshall, 1970). The organization of the porous cuticle varies from simple, flat plates (as in Tettigometridae; Bourgoin, 1985) to elaborate structures (Marshall and Lewis, 1971; Bourgoin and Deiss, 1994). This large variation appears not to be common, and it has been reported almost exclusively for this insect group, together with most of these sensilla being at the pedicel level.

Sensilla placoidea in *H. obsoletus* are innervated by up to 80 sensory neurons, which makes them one of the highest innervated olfactory sensilla described to date. Other exceptionally innervated sensilla have been reported by Slifer and Sekhon (1961), Behan and Ryan (1978), Aljunid and Anderson (1983) and Isidoro et al. (2001).

Another relevant feature is the presence of bundles of dendrites that are separated by their own dendritic sheath. Within the Fulgoromorpha, TEM-based ultrastructural details of the olfactory sensilla are known only in *P. candelaria* (Marshall and Lewis, 1971) and *N. lugens* (Aljunid and Anderson, 1983). In both of these species, groups of neurons forming 'neuronal units' (*sensu* Jez and McIver, 1980) were described, although in *N. lugens* there was no dendritic sheath. In more recent studies, the occurrence of neuronal units has also been seen in other hemipterans, belonging to Homoptera (Rossi Stacconi and Romani, 2011) and Heteroptera (Romani and Rossi Stacconi, 2009). The occurrence of dendritic bundles within a single sensillum that is related to the potential fusion of earlier separated structures into a more complex sensory element has been hypothesized in Fulgoromorpha (Lewis and Marshall, 1970; Aljunid and Anderson, 1983).

The second type of antennal sensilla in *H. obsoletus* is represented by the sensilla trichoidea, which show structural features that are linked to an olfactory function. Aljunid and Anderson (1983) reported that in *N. lugens* there are three different types of sensilla trichoidea, one of which has a putative olfactory function.

The occurrence of at least two different types of olfactory sensilla in *H. obsoletus* promotes the hypothesis that they can be tuned to different volatiles coming from either the host plant or from conspecifics, although there is yet no evidence of sex pheromones available for these planthoppers. In a relatively recent study, Kristoffersen et al. (2008) showed that the primary olfactory centers in Homoptera are not organized into distinct glomeruli, as has been seen in neuroanatomy studies carried out in psyllids and aphids (Hemiptera: Sternorrhyncha). We hypothesize that the antennal lobe organization in *H. obsoletus* in particular, and in the Fulgoromorpha in general, is characterized by a glomerular structure, which would reflect the large number of olfactory receptor neurons on the antennae.

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Reinvestigation of the Sex Pheromone of the Wild Silkmoth *Bombyx mandarina*: The Effects of Bombykal and Bombykyl Acetate

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Abstract Sex pheromone investigations of the domesticated silkmoth, *Bombyx mori* (Lepidoptera: Bombycidae), helped elucidate the molecular and physiological fundamentals of chemical communication in moths, yet little is known about pheromone evolution in bombycid species. Therefore, we reexamined the sex pheromone communication in the wild silkmoth, *Bombyx mandarina*, which is considered ancestral to *B. mori*. Our investigations revealed that (a) *B. mandarina* females produce (*E,Z*)-10,12-hexadecadienol (bombykol), but not (*E,Z*)-10,12-hexadecadienal (bombykal) or (*E,Z*)-10,12-hexadecadienyl acetate (bombykyl acetate), which are pheromone components in other bombycid moths; (b) antennae of male *B. mandarina* respond strongly to bombykol as well as to bombykal and bombykyl acetate; and (c) bombykal and bombykyl acetate strongly inhibit attraction of *B. mandarina* males to bombykol in the field. The present study clarifies the evolution of pheromone communication in bombycid moths.

Keywords Silkworm · Bombycidae · Bombykol · Receptor · Bombykal · Bombykyl acetate, pheromone evolution

Introduction

The silkworm, *Bombyx mori* (Lepidoptera: Bombycidae: Bombycinae), has been a model insect for studying the biosynthesis and reception of female-produced sex pheromones in moths (Nakagawa et al., 2005; Matsumoto, 2010; Sakurai et al., 2011). Female moths of *B. mori* produce two compounds in their pheromone gland (PG), (*E,Z*)-10,12-hexadecadienol (bombykol; E10,Z12-16:OH) (Butenandt et al., 1959) and (*E,Z*)-10,12-hexadecadienal (bombykal; E10,Z12-16:Ald) (Kaissling et al., 1978), at a ratio of approximately 11:1 (Kaissling et al., 1978). Previous studies demonstrated that bombykol alone is sufficient to elicit complete conspecific male mating behavior (Butenandt et al., 1959), and that bombykal has an antagonistic effect on the male response to bombykol (Kaissling et al., 1978; Daimon et al., 2012). Thus, a longstanding mystery is why *B. mori* females produce bombykal in addition to bombykol.

Recently, we found that antennae of *B. mori* moths respond strongly to (*E,Z*)-10,12-hexadecadienyl acetate (bombykyl acetate; E10,Z12-16:OAc), in addition to bombykol and bombykal (Daimon et al., 2012). Bombykyl acetate also is a female sex pheromone component in *Rondotia menciana* (Bombycinae) (Dai et al., 1988) and *Trilocha varians* (Bombycinae) (Daimon et al., 2012). Like bombykal, bombykyl acetate antagonizes the response of *B. mori* males to bombykol (Daimon et al., 2012).

Bombyx mandarina is the wild ancestor of *B. mori* (Goldsmith et al., 2004; Xia et al., 2009), and often is found in mulberry fields in Japan, Korea, and China. In the laboratory, *B. mandarina* and *B. mori* readily mate (Kuwahara et

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al., 1983), and the hybrids are fertile and develop normally. *Bombyx mandarina* females produce bombykol as a sex pheromone, and this compound alone is sufficient to attract *B. mandarina* males (Kuwahara and My-Yen, 1979; Kuwahara et al., 1983; Kuwahara, 1984). Thus, the pheromone communication systems of *B. mandarina* and *B. mori* have long been assumed to be undifferentiated. However, previous studies had not thoroughly investigated whether bombykal is produced in the PGs of *B. mandarina*. Moreover, to our knowledge, no studies have examined whether bombykal elicits antennal responses in *B. mandarina* males or affects their mating behavior. Accordingly, we reexamined the pheromone components of *B. mandarina* females and the antennal responses of males to the pheromone components used in bombycid moths, i.e., bombykol, bombykal, and bombykyl acetate. We also performed field trapping experiments using synthetic lures to determine whether bombykal and/or bombykyl acetate affects the attraction of *B. mandarina* males to bombykol.

Methods and Materials

Insects A laboratory colony of *B. mandarina* was maintained at the University of Tokyo and National Institute of Agrobiological Sciences, Japan. The larvae were individually reared on fresh mulberry leaves in plastic Petri dishes (90 mm diam) from hatching to pupation at 25 °C under a 12:12, L:D photoperiod.

Chemical Analysis Bombykol was separated from racemic Δ10,12-16:OH (Shin-Etsu Chemical Co., Tokyo, Japan) by Dr. S. Matsuyama (Tsukuba University). Authentic standards of bombykal and bombykyl acetate were synthesized as described (Daimon et al., 2012). The purity of each standard was checked by gas chromatography (GC) (isomeric purity >99.5 %). For extracting the sex pheromones of *B. mandarina*, the PG was excised from 1-d-old virgin females at 2–4 hr into the photophase, and immersed in hexane for 20 min at room temperature. Pheromone extracts were analyzed by a GC-mass spectrometry (QP2010 plus GC–MS; Shimadzu, Kyoto, Japan) with a DB-35MS column (0.25 mm i.d. × 30 m, J&W Scientific). The injector and ion source were maintained at 230°C, and the ionization voltage was 70 eV. Helium (1.0 ml/min) was used as the carrier gas. The column oven temperature was held at 120 °C for the first 2 min, then increased at 12 °C/min to 180 °C and finally at 5 °C/min to 240 °C.

GC-Electroantennographic Detector (EAD) Analysis Antennal responses to compounds in crude pheromone extract (1 female equivalent) and to synthetic bombykol, bombykal, or bombykyl acetate (200 ng each) were analyzed by a GC-EAD using a DB-Wax column (0.25 mm i.d. × 30 m, J&W Scientific), as described previously (Fujii et al., 2010).

Field Trapping Experiments Three series of field trapping experiments were conducted in mulberry fields in Tokyo, Japan, as described below. Green delta traps (Sankei Chemical, Tokyo, Japan) were hung singly on mulberry trees at a height of approximately 1.0 m, with at least 5-m spacing between traps, and at least 15-m spacing between replicates. Lures were made by impregnating rubber septa (5.3-mm OD; Sigma-Aldrich, Milwaukee, WI, USA) with different mixtures of synthetic compounds dissolved in 200 μl of hexane. No antioxidants were included in the present study. A single lure was placed at the center of each trap, and traps were left in the field throughout the test period in the same positions.

In trial A (18–25 October, 2010), the attraction of *B. mandarina* males to various combinations of bombykol, bombykal, and bombykyl acetate was tested in mulberry fields at the campus of the University of Tokyo (Nishitokyo City; 35.74°N, 139.54°E) and Tokyo University of Agriculture and Technologies (Fuchu City; 35.68°N, 139.49°E). Five combinations of compounds were tested in quintuplicate at each site, and the data were pooled for statistical analysis. In Trial B (8–19 Nov 2010, Nishitokyo City) and Trial C (8–19 Nov 2010, Fuchu City), the dose-dependent effect of bombykal and bombykyl acetate on the attraction of *B. mandarina* male moths to bombykol was investigated. Five compound combinations were tested in quintuplicate in each trial. Only treatments that trapped moths were included in the statistical analyses. Trapping data were transformed to $\log_{10}(x+1)$, and subjected to analysis of variance (ANOVA) followed by Tukey's test.

Results

Absence of Bombykal in the PG of Bombyx mandarina GC-MS analyses showed that PG extracts of *B. mandarina* contained bombykol (compound I in Fig. 1; base ion, *m/z* 67; molecular ion, *m/z* 238; diagnostic ion, *m/z* 220; retention time (Rt)= 13.04 min). However, neither bombykal nor bombykyl acetate, which were monitored by the fragment ion *m/z* 67, were detected in the PG extract (Fig. 1). The entire experiment, including the sample preparation and the subsequent GC-MS analysis, was repeated several times using different batches of *B. mandarina*, but bombykal and bombykyl acetate were always below the level of detectability, indicating that *B. mandarina* females produce only bombykol.

Antennal Responses of Bombyx mandarina GC-EAD analyses of the crude PG extracts from female *B. mandarina* showed that bombykol reproducibly elicited strong responses from

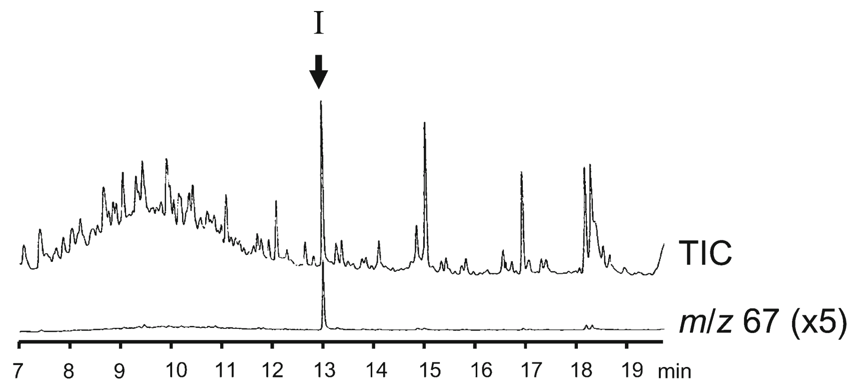

Fig. 1 A crude PG extract of *Bombyx mandarina* (0.5 female equivalent) was analyzed by GC-MS with a DB-35MS column. Total ion chromatogram (TIC) and a selected ion (*m*/*z* 67) chromatogram are shown. Bombykol (peak I; Rt=13.04 min) was detected in the PG extract, but not bombykal (Rt=12.22 min) or bombykyl acetate (Rt=14.95 min)

antennae of conspecific males (Fig. 2a; *N*=5). However, GC-EAD analyses using a mixture of synthetic bombykol, bombykal, and bombykyl acetate revealed that all three compounds elicited strong responses from males' antennae (Fig. 2b; *N*=11) but not from females' antennae (*N*=4; data not shown).

Field Attraction of Bombyx mandarina to Synthetic Lures In Trial A (Fig. 3a) we examined whether bombykal and bombykyl acetate affected the attraction of *B. mandarina* males to bombykol. As reported previously (Kuwahara et al., 1983), bombykol alone was sufficient to attract males. In contrast, no or few males were attracted by lures containing bombykal or bombykyl acetate alone. Instead, these compounds hindered trap catches by bombykol; catches of *B. mandarina* males were drastically reduced when bombykal or bombykyl acetate was blended with bombykol at a ratio of 1:1 (Fig. 3a). We next evaluated the potency of the inhibitory effects of bombykal and bombykyl acetate using lures containing 100 μg of bombykol plus different amounts of bombykal or bombykyl acetate (10, 25, 50, or 100 μg). A small amount of bombykal or bombykyl acetate was sufficient to significantly reduce the trap catches (Fig. 3b and c); e.g., the number of trapped males was reduced by 77 % by addition of 10 μg of bombykol, and by 94 % by addition of 10 μg of bombykyl acetate.

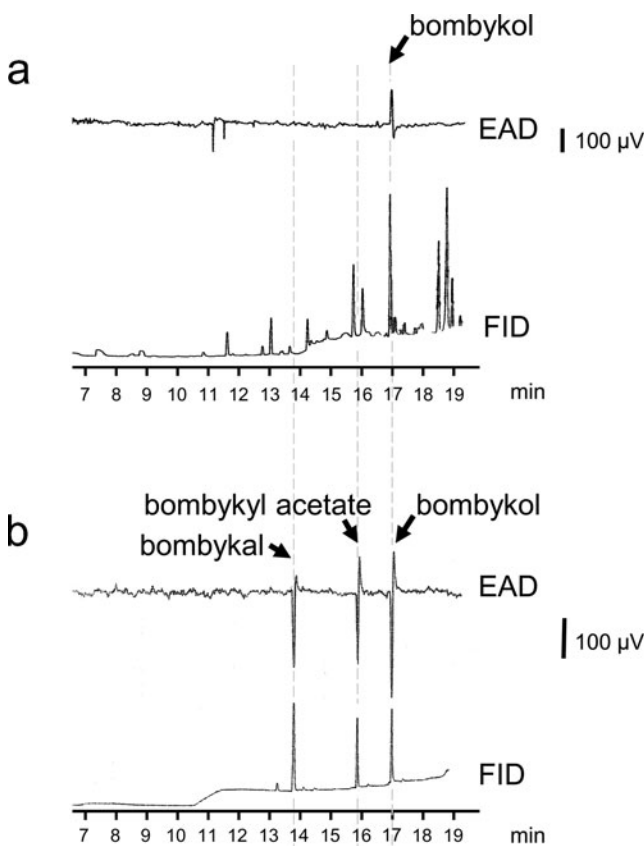

Fig. 2 GC-FID-EAD analyses of (**a**) a crude PG extract of *Bombyx mandarina* (1 female equivalent) or (**b**) a mixture of authentic bombykol, bombykal, and bombykyl acetate (200 ng each). The GC was equipped with a DB-Wax column. Antennae were excised from 1-day-old *B. mandarina* males. Similar results were obtained in several independent experiments (*N*=5 for a and 11 for b)

Discussion

In the present study, we analyzed the female sex pheromone components of *B. mandarina*, and investigated the antennal and behavioral responses of males to bombycid pheromone components. We found that: (a) *B. mandarina* females produce bombykol but not bombykal or bombykyl acetate in the PG; (b) only the antennae of *B. mandarina* males respond to bombykol, bombykal, and bombykyl acetate; and (c) bombykal and bombykyl acetate act as potent inhibitors toward *B. mandarina* males.

To date, the female-produced sex pheromones of four moth species belonging to the subfamily Bombycinae have been investigated (Table 1): *B. mori* (Butenandt et al., 1959; Kaissling et al., 1978), *B. mandarina* (Kuwahara and My-Yen, 1979), *R. menciana* (Dai et al., 1988), and *T. varians* (Daimon et al., 2012). The female sex pheromones of these species are comprised of bombykol and/or its derivatives (Table 1), suggesting that the diversity of pheromones in *B. mori* and its allied species has occurred primarily through the conversion of the terminal functional group. The sex pheromone of *B. mori* and *B. mandarina* is bombykol, but *B. mori* females produce a small amount of bombykal as

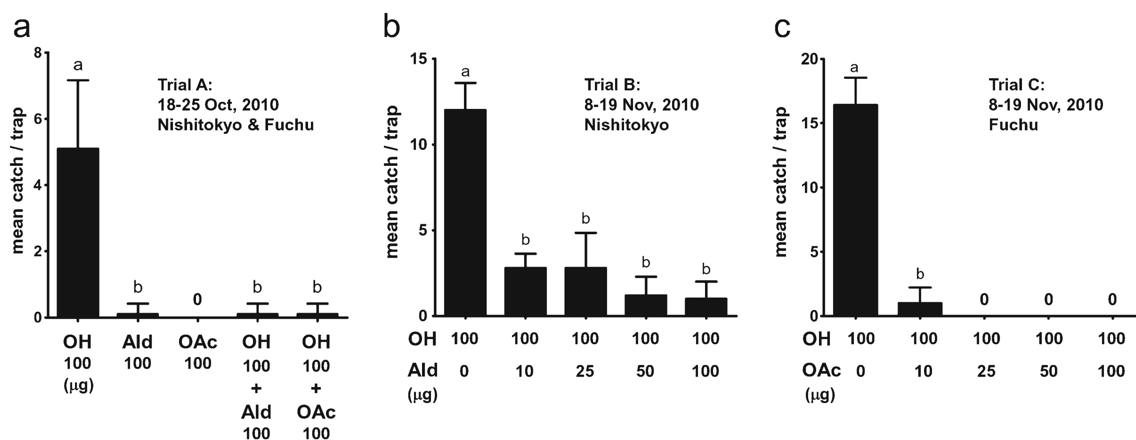

Fig. 3 **a** Field trapping of *Bombyx mandarina* males using lures containing one or two synthetic compounds. OH, Ald, and OAc indicate bombykol, bombykal, and bombykyl acetate, respectively. Values under the compound name indicate the amount of the compound applied to the lure septa (μg). Bars indicate the mean number of trapped males per trap (mean±SEM, $N=10$). Bars with different letters are statistically different ($P<0.01$, Tukey's test). **b** and **c** Dose-dependency of the effect of bombykal (**b**) or bombykyl acetate (**c**) on the attraction of *B. mandarina* male moths to bombykol. Values under the compound name indicate the amount of the compound applied to the lure septa (μg). Five traps were used for each kind of lure. Bars indicate the mean number of trapped males per trap (mean ± SEM, $N=5$). Bars with different letters are statistically different ($P<0.01$, Tukey's test)

well, which inhibits the wing fluttering response of *B. mori* males to bombykol (Kaissling et al., 1978; Daimon et al., 2012). Production of bombykal in *B. mori* might be due to the loss of absolute control of the oxidation of bombykol (Matsumoto et al., 2007; Matsumoto, 2010).

Non-production of bombykal and bombykyl acetate by female *B. mandarina* (Fig. 1), and the strong aversive response of *B. mandarina* males to these compounds (Fig. 3), may have contributed to pre-reproductive isolation from sympatric species that use bombykal or bombykyl acetate as sex pheromone components. Indeed, we frequently observed that lures containing bombykal attracted a large number of males of the sphingid moth *Neogurelca himachala*—a diurnal flyer like *B. mandarina*—in the field trapping experiments (data not shown). Therefore, as an avoidance to attracting undesired sympatric species, *B. mandarina* females might have undergone strong selective pressure not to produce bombykal, even in small amounts. From this standpoint, the absence of bombykal in the PG of *B. mandarina* suggests the possibility that the oxidation of bombykol is more strictly suppressed in *B. mandarina* than in *B. mori*, a fully domesticated insect. The domestication process over the long history of sericulture has led *B. mori* to lose various traits that are important to survival in the field, including the ability to fly. Therefore, the presence of a small amount of bombykal in *B. mori* could be explained as one such consequence of domestication. From this standpoint, it is intriguing that some *B. mori* strains do not produce bombykal (Daimon et al., unpublished data).

Table 1 Female sex pheromone and male responses in the subfamily Bombycinae

Species	Sex pheromone (ratio)	Male antennal response[b] (behavioral effect)			Reference
		bombykol	bombykal	bombykyl acetate	
Bombyx mori	bombykol[a]	+ (attractant)	+ (antagonist)	+ (antagonist)	Butenandt et al., 1959; Kaissling et al., 1978; Daimon et al., 2012
Bombyx mandarina	bombykol	+ (attractant)	+ (antagonist)	+ (antagonist)	Kuwahara and My-Yen, 1979; This study
Rondotia menciana	bombykyl acetate	-	+	+ (attractant)	Dai et al., 1988; Daimon et al., unpublished
Trilocha varians	bombykal and bombykyl acetate (1: 2.3)	-	+ (attractant)	+ (attractant)	Daimon et al., 2012

[a] *B. mori* is reported to produce a small amount of bombykal in addition to bombykol (Kaissling et al., 1978)

[b] +, responded; -, not responded

While the antennal responses to bombykal and bombykyl acetate have been observed in the males of all four species tested (*B. mori*, *B. mandarina*, *R. menciana*, and *T. varians*) (Dai et al., 1988; Daimon et al., 2012; see Table 1), responses to bombykol were observed only in *B. mori* and *B. mandarina*. These results suggest a "pheromone shift" from bombykal and/or bombykyl acetate to bombykol, or in the opposite direction, occurred in the subfamily Bombycinae. Elucidating the evolution of sex pheromones in Bombycinae will require the investigation of additional species in the genus *Bombyx*, such as *B. huttoni*, *B. horsfieldii*, and *B. rotundapex*, mulberry feeders occurring in South Central and Southeast Asia (Moore, 1858; Zolotuhin and Witt, 2009), as well as species in other genera included in the Bombycinae (Lemaire and Minet, 1998; Zolotuhin and Witt, 2009).

Bombykol and bombykal interact with specific pheromone receptors in the olfactory sensillae of males: *Bombyx mori* odorant receptor 1 (BmOR1) and BmOR3 are specifically tuned to bombykol and bombykal, respectively (Sakurai et al., 2004; Nakagawa et al., 2005; Sakurai et al., 2011). Nevertheless, a receptor for bombykyl acetate has not been identified in *B. mori*. Whether *B. mori* has another receptor specifically tuned to bombykyl acetate, and whether such a receptor is found in other bombycid moths should be clarified.

In summary, our study clarifies that the sex pheromone of *B. mandarina* is comprised of a single component, bombykol. Further, our results suggest that bombykal and bombykyl acetate, bombykol derivatives commonly used as pheromone components in other bombycids, work as potent inhibitors against *B. mandarina*, thus shedding light on the evolution of sex pheromone communication systems in bombycid moths.

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Analyzing Diurnal and Age-Related Pheromone Emission of the Olive Fruit Fly, *Bactrocera oleae* by Sequential SPME-GCMS Analysis

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Abstract The olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae), uses 1,7-dioxaspiro[5.5]undecane ("olean"), produced primarily by females, as a sex pheromone. We used sequential solid phase microextraction—gas chromatography mass spectrometry (SPME-GCMS) analysis to show that female olive flies release about 1000 ng of pheromone at the onset of scotophase for several weeks, while males release about 1/100 as much during the first week after eclosion. The present research demonstrates details of employing SPME-GCMS with the partially known pheromone system of the olive fruit fly as a model for pheromone identification and diurnal release patterns in insects, especially fruit flies. The sequential SPME-GCMS method will readily allow detection and semi-quantification of semiochemicals released by insects in minute amounts throughout the diurnal cycle.

Keywords Olive fruit fly · *Bactrocera oleae* · Sequential SPME-GCMS analysis · Autosampler · Pheromone chirality · Diptera · Tephritidae

Introduction

The olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae), is a key pest of olives in the Mediterranean basin, as well as in California and Mexico (Estes et al., 2012). Evidence for the existence of a pheromone system in the olive fruit fly was presented in the early 1970's by Haniotakis (1974). Interestingly, in contrast to most tephritid fruit flies analyzed to date (Carpita et al., 2012), the attractant pheromone is produced mainly by female flies (Haniotakis 1974). Based on dissections of olive fruit fly rectal glands, Baker et al. (1980), Baker and Herbert (1987) and Mazomenos and Haniotakis (1981) described a volatile pheromone blend produced by females that is attractive to males. Mazomenos and Pomonis (1982) characterized the main chemical component of the male and female pheromone as 1,7-dioxaspiro[5.5] undecane ("olean"). These authors claimed that males reared as larvae on an artificial diet failed to synthesize olean after one generation, whereas males reared as larvae in the laboratory on olive fruits produced pheromone. In a later study, Mazomenos (1984) described the age-related oscillations in rectal gland pheromone production of virgin laboratory-reared female flies. Olean enantiomer composition from females is reportedly racemic (Haniotakis et al. 1986), but, to our knowledge, the chirality of male-produced olean remains unknown.

Once the main pheromone component of the female fly was synthesized and tested in the field (Mazomenos and Haniotakis, 1985), several companies and institutions engaged in the development of mass-trapping techniques and strategies to control this fly (e.g., Broumas et al., 2002; Noce et al., 2009). During this time, work on the olive fruit fly pheromone and biological activity of olean was almost completely halted. However, recently Carpita et al. (2012) detected (*Z*)-9-tricosene as a new male-emitted pheromone in the olive fruit fly, and described the age-related glandular production of olean, which peaks with gonad-maturation and ceases by 11 days after eclosion.

Solid phase microextraction (SPME) has been a sensitive, clean method for pheromone isolation and analysis for

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almost two decades (Frérot et al. 1997). However, until recently *in vivo* hourly collection and analysis of insect emissions by SPME has been technically difficult and time-consuming, particularly in flies (Robacker et al. 2009, Alfaro et al. 2011). Lately, we demonstrated that by combining two commercially available techniques, SPME and automatic injection into a gas chromatography–mass spectrometry (GCMS) system, it is possible to characterize diurnal volatile emissions of living insects (Levi-Zada et al., 2011). We showed that this analytical method facilitates the study and characterization of moth pheromones emitted in minute amounts with time, which is not easily studied by classical methods such as gland extraction and airborne volatile collection. The present study utilizes the sequential SPME-GCMS method to investigate the pheromone emission pattern of an insect belonging to another insect order and family, Diptera: Tephritidae. Since olean is a known pheromone component of the olive fruit fly, we used the automatic sampling and analysis system to monitor the diurnal pheromone emission from virgin female olive fruit flies, and to investigate the age-related pheromone emission patterns from both sexes of *B. oleae* virgins. The enantiomeric composition of olean has not been fully described, probably because of low amounts of male-emitted pheromone (Mazomenos and Pomonis 1982), and inadequate technology. Thus, another goal of this study was to elucidate the chirality of the pheromone from both sexes as a function of their age.

Methods and Materials

Chemicals Racemic olean (Alfa Aesar) and (*S*)-olean 90 % ee (a gift from Prof. K. Mori) were used as standards.

Insect Source Wild flies were procured from infested olive fruits, collected during the autumn (November-December, 2011). Fruits were collected in the field, placed inside plastic containers with a layer of sand in the bottom, incubated in the laboratory at 25 °C, and illuminated at a 12 L: 12D photoperiod with fluorescent lights turned off coinciding with the natural scotophase. Olive fruit fly larvae abandon the fruit and pupate in the sand. Pupae were collected, and flies were allowed to emerge inside an insect cage. Immediately after emergence, flies were sorted by sex to preclude mating, and maintained as small groups in insect cages with food and water until the appropriate age for pheromone analysis. Due to lack of synchrony in the emergence, groups of flies included a range of ages between 3 and 5 d.

Diurnal Patterns of Pheromone Emission by Sequential SPME-GCMS Analyses Analyses were performed with an Agilent 6890 N GC interfaced with an Agilent 5973 MS detector equipped with a nonpolar RTX-5SilMS (Restek, Bellefonte, PA, USA) column (30×0.25 mm i.d.×0.25 µm film) at 60 °C for 2 min, then programmed to 240 °C at 20°/min, and held isothermally for 20 min. Column helium flow was 1.5 ml/min, and the inlet temperature of the GCMS instrument was 230 °C with an injection time of 6 min in splitless mode. For SPME injections, a 0.75 mm i.d. glass inlet liner was used, but for liquid injections it was replaced with a 4 mm i.d. liner. The GCMS instrument was equipped with a commercial auto-sampler (MPS2-Twister, Gerstel, M. Snir Technological Services Ltd, K. Ata, Israel) holding an SPME syringe with a 100 µm polydimethylsiloxane fiber (Supelco, Bellefonte, PA, USA) for headspace analyses or a 10 µl syringe for liquid analyses. The fiber was baked in the Gerstel Needle heater unit for at least 10 min at 240 °C prior to use. The SPME fiber of the auto-sampler was programmed (Maestro software, Gerstel) to collect volatiles emitted by females every 2 hr, and then inject samples directly into the GCMS instrument repeatedly for several days. Individual female flies were placed inside cylindrical 20 ml glass vials (75 mm height× 20 mm i.d.) with septum caps. A small cotton wick impregnated with a 10 % sucrose solution was placed inside the vial as a source of liquid and food, and a bended strip of paper provided a resting surface for the fly. To prevent saturation of the pheromone, vials were ventilated via a 16-gauge syringe needle inserted in the septum. The auto sampler was located close to a window, thus allowing flies to experience the natural photoperiod. On the day of experiments, flies were placed before noon in glass vials in the autosampler tray. Sampling of volatiles inside each vial proceeded throughout the day and night continuously for a few days until the fly showed signs of stress, weakness or deterioration.

Quantification of Chiral Pheromone Emission of Male and Female Olive Flies as Affected by Age GCMS analyses were performed by an Agilent 7890A GC interfaced with an Agilent 5975 C MS detector and a flame ionization detector (FID). In this instrument, the flow of the column was split between each of the detectors equally by an Agilent purged two-way effluent splitter, enabling simultaneous qualitative and quantitative analyses. For chiral analysis, an Rt-βDEX (Restek) column (30×0.25 mm i.d.×0.25 µm film) was installed, and kept isothermally at 125 °C with a helium flow of 1 ml/min. In this machine, SPME injections were done manually using a 0.75 mm i.d. glass inlet liner, and liquid injections were done with an Agilent G4513A auto-sampler using a 4 mm i.d. liner. The 100 µm polydimethylsiloxane fiber (Supelco) was baked in the split/splitless inlet 10 min prior to use. Analyses were performed in splitless mode with the split valve opened after 6 min.

Individual female flies at the appropriate age were placed in glass vials as described earlier. For each age group,

volatiles were collected from an individual female fly. Male flies at the appropriate age were placed in groups in glass vials as described earlier for females. However, in contrast to the females, and due to the observed low emission quantities of olean by males, 5–12 males were included in each vial without ventilation. For each age class, we collected emissions from at least 3 replicates.

Calibration Curves In order to compare the amount of olean emitted in a collection vial and the SPME sampling, solutions of 1 µl of 25, 50, 100, 500, and 1000 ng racemic olean were placed in 20 ml vials under the same conditions used for flies (N=3). These analyses started at once, and the SPME fiber sampled the volatiles for 1 hr. The MS peak areas were converted to nanograms using a calibration curve of synthetic racemic olean. For this purpose, a series of 1-µl solutions containing 0.05, 0.1, 1, 10, 25, 50, and 100 ng olean in *n*-hexane (Merck) were injected into the GCMS (N=3). The MS responses of the two calibration curves (not presented here) were incorporated into Fig. 1. In addition, the effect of sampling time on the GCMS peak areas was tested. This was accomplished using 500 ng of olean as above, and collecting the vapors for 1 hr and 2 hr, and by comparing the MS peak area of the two runs. All SPME samplings were performed in a temperature-controlled room at 25 °C.

Results

Emission of Olean by Individual Female Flies During Several Days We examined the continuous diurnal changes in pheromone emission in females of different ages during several days. Figure 2 shows two examples of automatic SPME collections from a 9-d-old and a 17-d-old female. Females release the pheromone mostly during the scotophase (emphasized in Fig. 1 by the gray background), but the pattern of emission is not uniform even for the same female. The changes show that the female starts to increase production of pheromone close to the onset of scotophase. Pheromone production and accumulation peaks sometime in scotophase. Afterwards, pheromone emission declines or is completely halted, as previously reported (Mazomenos, 1989), never reaching zero values. The absolute amount of natural pheromone in the vial was calibrated by the relationship between the actual amount of synthetic olean introduced into the vial and the amount that reached the partition equilibrium with the SPME fiber (Fig. 1).

The calibration curve shows that over the range of 0–1000 ng of olean dispensed into the vial, the SPME fiber samples only a small part (1/21 or 4.8 %) of this amount. The maximal amount of olean that was sampled by the fiber

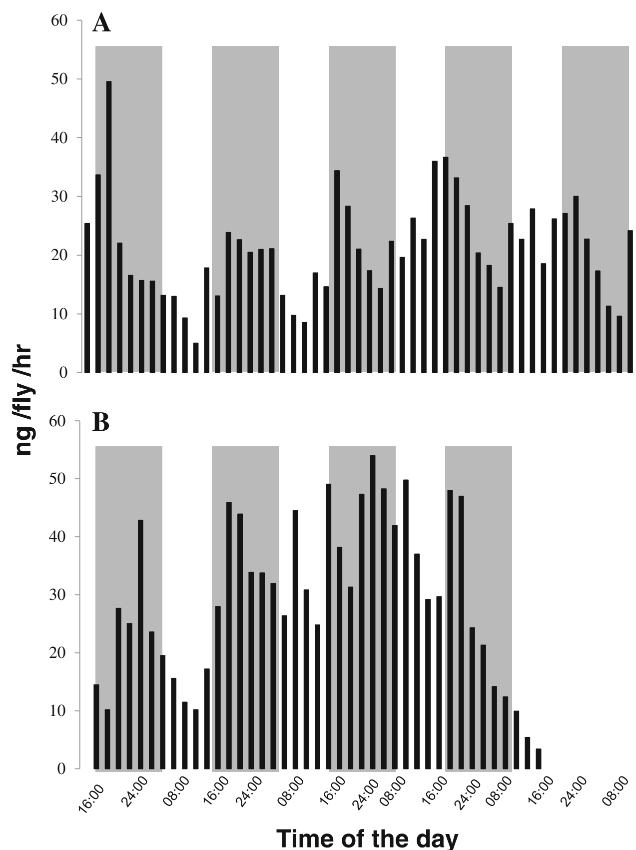

Fig. 1 Diurnal changes in pheromone emission during 5 d for a 9-d-old female **a**, and of a 17-d-old female **b**. The gray background columns illustrate the approximate period of the scotophase for each day. Y-axis shows the amounts sampled on a 100 % polydimethylsiloxane SPME fiber in each GC-MS run

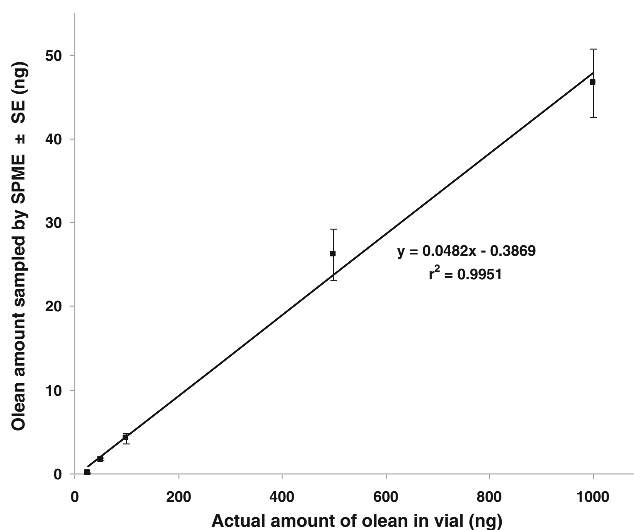

Fig. 2 The correlation between the amount of synthetic racemic olean sampled on 100 % polydimethylsiloxane fiber and the actual amount of olean dispensed in 20 ml vial (±SE). The experiment was performed under the same conditions as used with flies (N=3)

in the two examples was approximately 50 ng. This quantity corresponds to approximately 1050 ng, at equilibrium between olean in the vial and in the fiber (Fig. 1). The "leaking" rate of olean for 36 hr was assessed by SPME sampling of 1000 ng of synthetic olean, initially placed inside the vessel, keeping the same conditions as those used for living flies. A steady decline of olean in the vessel was observed, halving the initial absorbed amount in approximately 10 hr (Fig. 3). In most cases, the increase of olean concentration in the vial was observed shortly before the scotophase or early in the night (Fig. 2).

The effect of the time of fiber and headspace equilibrium was tested as well, and no difference was observed in the amount of olean sampled by SPME during 1 hr as compared to 2 hr when 500 ng olean was introduced to the vial in the two analyses.

Stereochemistry and Age-Pattern of Olean Emission by Female and Male Olive Flies The chiral column employed resulted in baseline separation of olean enantiomers, with the *S* enantiomer eluting first. Both sexes release racemic olean, but males release the pheromone only during the first week after adult eclosion, while females released significant amounts of olean each day throughout the 5 weeks of the study with maximal release in the first 18 days (Fig. 4). Moreover, females were observed to release about 100 times more olean than males during the period of maximal emission by the two sexes (Fig. 4). Based on our calibration curve between SPME equilibrium and dispensed amount of pheromone, males produce an average of 15 ng of each olean enantiomer per hour, which is at the detection threshold-limit of our system.

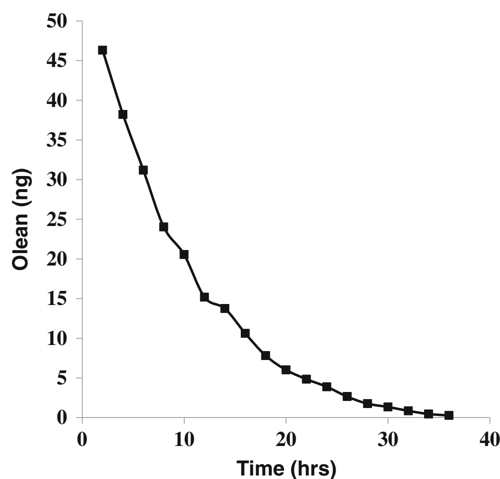

Fig. 3 Decline of olean inside 20 ml vial with a hole to allow gas interchange over 36 hr as monitored by sequential SPME GCMS analysis. Initially, 1000 ng olean was placed inside the 20 ml vial under the same conditions as those used with live flies

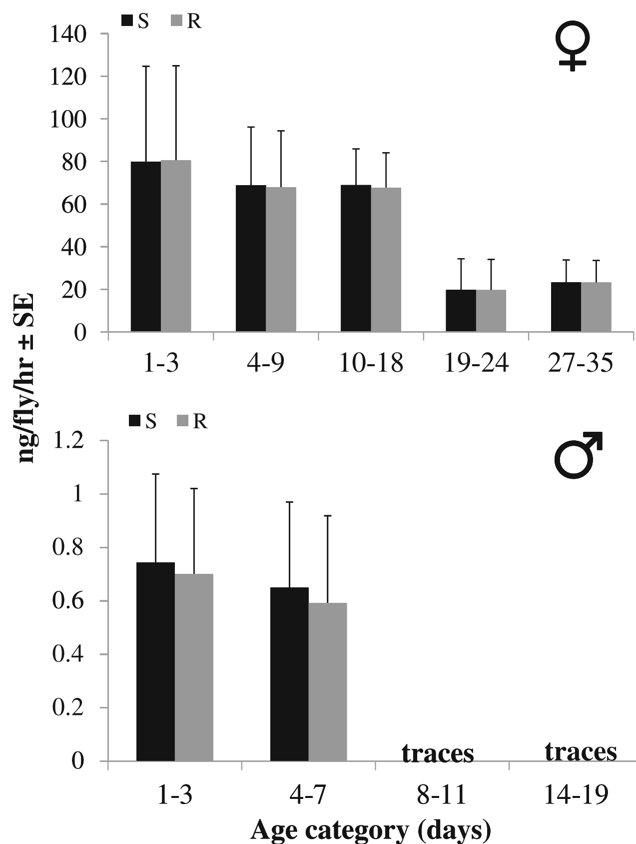

Fig. 4 The average amount of (*S*)-olean and (*R*)-olean released by one individual female or male fly that was sampled with a 100 % polydimethylsiloxane SPME fiber during 1 hr

Discussion

The sequential SPME-GCMS analysis method enables evaluation of diurnal pheromone emission rates that in the past were tedious or impossible to determine. This method, based on a SPME syringe connected to an autosampler, produces an exceptionally flat and noise-free baseline because there is no solvent peak that can mask analytes, the method is non-destructive, and little sample preparation and handling is required. SPME is a non-exhaustive extraction technique in which only a small portion of the target analyte is removed from the sample matrix, and therefore, quantitative analysis is not a trivial procedure, and could be affected by many factors such as sampling time and temperature (Bartelt and Zilkowski 1999; Ouyang and Pawliszyn 2008). Different types of SPME fibers are not uniformly sensitive to all compounds, and therefore, relative GC peak areas for a SPME sample do not properly reflect the true proportions of the components in the headspace. However, in this study only one pheromone component was involved, and the goal was to evaluate the diurnal pheromone emission rate from both live females and males, and its correlation with age of the flies. The simplicity of the method as

compared to tedious airborne collections and individual analyses of each sample makes the sequential SPME-GCMS analysis the method of choice. The main advantage of the system is the small number of insects that are required, the high sensitivity of the GCMS instrument, and the automatic programmed analysis of emitted volatiles.

In SPME, equilibriums are established among the concentrations of an analyte in the headspace above the sample, and in the polymer coating of the fused silica fiber. Ouyang and Pawliszyn (2008) showed that the amount of analyte sampled by the coating at equilibrium is directly related to its concentration in the sample. The amount of analyte sampled by the fiber is related to the sample volume, only when the volume is small. A smaller vial will allow a limited amount of pheromone emitted by flies to reach a higher concentration in the headspace compared to a larger vial. The same vial, at the same conditions, was used for all the analyses, and was kept as small as possible but still large enough to enable the flies to move freely. In addition, when the sampling time is long enough, the extraction reached equilibrium. Bartelt (1997) found that most compounds with a Kovats retention index of <1300 on a nonpolar GC column equilibrated with the fiber in 30 min or less. The Linear Retention Index (LRI) of olean on our non-polar column is 1145, thus, the sampling time of 1 hr that was used is appropriate to achieve equilibrium.

Baker et al. (1980) extracted 5 pheromone glands, of about 1-wk-old females, for the pheromone identification, and estimated that every female contained about 300 ng pheromone. Mazomenos (1984) needed to extract 30 pheromone glands of females in order to analyze the pheromone, and to study the effect of age on the pheromone production of virgin laboratory female flies. He found a cyclical production recurring at about 10-d intervals with peaks of 300 ng in 1-wk-old females and of 240 ng in 17-19-d-old females. Our findings, reflecting the pheromone emitted by live females, show that the females produce and emit the pheromone continuously over a number of days, apparently increasing production just before or after nightfall. The SPME fiber collected an amount of 66–80 ng/female/h between the ages of 0–18 days, which corresponds to 1470–1780 ng being released in 1 hr by one female. The differences between earlier work and our results are related to the fact that Baker et al. (1980) and Mazomenos (1984) assessed the static amount of pheromone from gland extracts of females at a certain time and age while our analysis reflects the continuous emission of the pheromone by live females, reflecting the real pheromonal activity of the olive fruit fly. The fact that, in our system, it takes more than 30 hr for synthetic olean to completely leak-out from the vial, suggests that the inability of the fly-vial system to reach zero pheromone levels is probably related to both the slow leaking of olean from the vial and the renewed emission of olean by females. However, our results indicate that females produce and release much higher amounts of pheromone than were previously reported.

The amount of pheromone released by the male is much smaller than that of the female, in agreement with the results of Mazomenos and Pomonis (1982). Our results show an average release of 15 ng/male/h of racemic olean, while Mazomenos and Pomonis (1982) measured the content in gland extracts and found varying amounts depending on the period of the year in which wild males were collected.

In the present study we showed for the first time, that SPME sequential analysis enables the evaluation of the pheromonal emission behavior of live fruit flies. The diurnal changes in the pheromone emission could help in identifying new pheromones of insects, not only of flies and moths. The diurnal cycle provides a record of potential pheromone components, thus enhancing the identification process even before conducting any bioassay.

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Characterization of Epoxytrienes Derived from (3*Z*,6*Z*,9*Z*)-1,3,6,9-Tetraenes, Sex Pheromone Components of Arctiid Moths and Related Compounds

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Abstract *Cis*-9,10-epoxy-(3*Z*,6*Z*)-1,3,6-henicosatriene has been identified from a pheromone gland of arctiid species, such as *Hyphantria cunea*. Since the diversity of lepidopteran species suggests that structurally related compounds of the 9,10-epoxide are also utilized as a sex pheromone components, epoxytrienes derived from (3*Z*,6*Z*,9*Z*)-1,3,6,9-tetraenes with a C_{19}–C_{21} chain were systematically synthesized and characterized. While 1,2-epoxy-3,6,9-triene was not obtained, peracid oxidation of each tetraene produced a mixture of three *cis*-epoxides (3,4-epoxy-1,6,9-triene, 6,7-epoxy-1,3,9-triene, and 9,10-epoxy-1,3,6-triene), which were separable by LC as well as GC. Detailed inspection of the mass spectra of the C_{19}–C_{21} epoxides indicated the following diagnostic ions for determining the chemical structures: *m/z* 79, M-70, and M-41 for the 3,4-epoxytrienes; *m/z* 79, 95, 109, and 149 for the 6,7-epoxytrienes; and *m/z* 79, 106, 120, M-121, and M-107 for the 9,10-epoxytrienes. Resolution of two enantiomers of each C_{21} epoxytriene was accomplished by HPLC equipped with a chiral column, and analysis of the pheromone extracted from virgin females of *H. cunea* revealed the 9*S*,10*R* configuration of the natural epoxytriene as the same configuration of C_{21} 9,10-epoxydiene, a main pheromone component of this species. GC-EAD analysis of the optically pure epoxides showed that the antennae of male *H. cunea* were stimulated more strongly (>100 times) by the (9*S*,10*R*)-isomers than the antipodes.

Keywords Female sex pheromone · Lepidoptera · Epoxytrienes · GC-MS · Diagnostic fragment ions · Resolution by chiral HPLC · Fall webworm

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Introduction

Sex pheromones, which have been identified from female moths of about 630 species to date (Ando, 2012; El-sayed, 2012), are classified mainly into two groups (Types I and II) according to their chemical structures (Ando et al., 2004). Type I pheromones, which are composed of fatty alcohols with a C_{10}–C_{18} straight chain and derivatives, have been widely identified from insects in many families. Each component is produced in a pheromone gland by *de novo* synthesis *via* a saturated fatty acyl intermediate. On the other hand, Type II pheromones are composed of unsaturated hydrocarbons and epoxy derivatives, which universally have (*Z*)-double bond(s) or a *cis*-epoxy ring at 6,9- or 3,6,9-positions in a C_{17}–C_{23} straight chain; these compounds are biosynthesized from linoleic and linolenic acids (Ando et al., 2004; Jurenka, 2004). In addition to 6,9-dienes and 3,6,9-trienes, novel polyenes with a further modified structure including an extra double bond at the 1-, 4-, or 11-position are known (Ando et al., 2004). These positions are conjugated to the original unsaturated structure, and we recently found other novel polyenes with a double bond at the 12-position from several emerald moth species in the geometrid subfamily, Geometrinae (Yamakawa et al., 2009, 2011). Many species in Geometrinae are forest defoliators, but no sex pheromones in this insect group have been studied. While the Type II pheromones have been identified from insects in rather limited groups, such as Geometridae, Arctiidae, Lymantriidae, and Noctuidae, these families include many species. The species diversity indicates that the knowledge of Type II pheromones remains fragmentary, and several insects are expected to utilize unknown compounds for their reproductive communication.

Type II pheromones with an epoxy ring are biosynthesized from unsaturated hydrocarbons by action of epoxidases vested with reaction selectivity to one specific

double bond. In the case of monoepoxides derived from 3,6,9-trienes, all three kinds of epoxydienes (3,4-epoxy-6,9-dienes, 6,7-epoxy-3,9-dienes, and 9,10-epoxy-3,6-dienes) have been found in female moths, indicating that lepidopteran insects in highly evolved groups developed the ability to oxidize each double bond. However, despite four kinds of epoxytrienes being possibly produced from a 1,3,6,9-tetraene, only 9,10-epoxy-1,3,6-triene has been identified as a natural pheromone component from two species in Arctiidae, the fall webworm (*Hyphantria cunea* Drury) (Tóth et al., 1989) and the mulberry tiger moth [*Lemyra (= Spilosoma) imparilis* Butler] (Ando et al., 2004). Females of these two species also produce 9,10-epoxy-3,6-diene, and the two 9,10-epoxides are expected to be biosynthesized from the corresponding 3,6,9-triene and 1,3,6,9-tetraene by a common epoxidase with reaction specificity to the double bonds at the 9-position (Kiyota et al., 2011). Formation of the 1,3,6,9-tetraene is the first key step for the biosynthesis of the epoxytriene. It is highly probable that some species secrete 3,4-epoxy-1,6,9-trienes or 6,7-epoxy-1,3,9-trienes for species-specific communication because the epoxytrienes can be biosynthesized by a combination of desaturation at the terminal position and 3,4- or 6,7-epoxidation. Therefore, we systematically synthesized the epoxytrienes with a C_{19}–C_{21} chain, and analyzed them by GC-MS in order to accumulate spectral data. Diagnostic fragment ions of each epoxytriene could be utilized in future pheromone studies. Next, since the epoxytrienes include chiral centers, each synthetic epoxytriene was analyzed by chiral HPLC. Furthermore, based on the results of enantiomeric separation, we examined the stereochemistry of the natural pheromone of *H. cunea*. By field evaluation of synthetic stereoisomes, 9*S*,10*R* configuration has been tentatively assigned for two epoxy components (Senda et al., 1991; Su et al., 2008), but configuration of the components secreted by the females has never been examined. This study successfully revealed the absolute configuration and optical purity of the natural components included in the pheromone glands.

Methods and Materials

Analytical Instruments ^{1}H and ^{13}C NMR spectra were recorded by a Jeol Delta 2 Fourier transform spectrometer (JEOL Ltd., Tokyo, Japan) at 399.8 and 100.5 MHz, respectively, for $CDCl_3$ solutions containing TMS as an internal standard. ^{1}H-^{1}H COSY, HSQC, and HMBC spectra also were measured with the same spectrometer, using the usual pulse sequences and parameters. GC-MS was conducted in EI mode (70 eV) with an HP5973 mass spectrometer system (Hewlett-Packard) equipped with a cool-on-column injector, and a DB-23 column (0.25 mm ID×30 m, 0.25 µm film, J & W Scientific, Folsom, CA, USA). The column temperature program was 50 °C for 2 min, 10 °C/min to 160 °C, and 4 °C/min to 220 °C. The carrier gas was helium. Medium-pressure liquid chromatography (MPLC) and high performance liquid chromatography (HPLC) employ a system composed of a pump (PU-980, Jasco, Tokyo, Japan), a refractive index (RI) detector (RI-98SCOPE, Labo System, Tokyo, Japan), a UV detector (UV-970, Jasco), and an integrator (807-IT, Jasco). MPLC with a Lobar column (Merck Lichroprep Si 60, 10 mm ID×240 mm, 40–60 µm) was eluted with 0.5 % tetrahydrofuran (THF) in hexane at a flow rate of 3.0 ml/min, and HPLC with an Inertsil ODS column (4.6 mm ID×25 cm, Senshu-kagaku, Tokyo, Japan) was eluted with 3.5 % water in MeOH at a flow rate of 1.0 ml/min. Resolution of the enantiomers was examined with four chiral HPLC columns (Chiralpak AY-H, Chiralpak AS-H, Chiralpak AD, and Chiralcel OJ-R, 4.6 mm ID×25 cm; Daicel Chemical Industry, Osaka, Japan). The former three normal-phase columns were eluted with 0.1 % 2-propanol in hexane at a flow rate of 0.5 ml/min. The last reversed-phase column was eluted with 10 % water in MeOH.

Abbreviations of Chemicals The chemical structures of polyunsaturated hydrocarbons and the epoxy derivatives are abbreviated as follows: Z = (*Z*)-double bond, epo = *cis*-epoxy ring, number before the hyphen = position of the double bond or epoxy ring, number after the hyphen = carbon number of the straight chain, and H = compound without terminal functional group. For example, *cis*-9,10-epoxy-(3*Z*,6*Z*)-1,3,6-henicosatriene is abbreviated as 1,Z3,Z6,epo9-21:H.

Epoxidation of (3Z,6Z,9Z)-1,3,6,9-tetraenes The tetraenes had been synthesized in our previous study (Yamamoto et al., 2008). *m*-Chloroperoxybenzoic acid (MCPBA, Tokyo Kasei Kogyo Co., 70 % pure, 490 mg, 2.0 mmol) was added to a solution of 1,Z3,Z6,Z9-21:H (470 mg, 1.8 mmol) in dry CH_2C1_2 (30 ml), and stirred at 0 °C for 1 h. After further stirring at room temperature for 2 h, the reaction mixture was washed with a saturated aqueous solution of $NaHCO_3$ (20 ml×2), and dried with Na_2SO_4. Silica gel column chromatography gave a mixture of the three *cis*-monoepoxy derivatives (240 mg, 48 % yield), which were separated by MPLC as shown in Fig. 1a. NMR analysis of the separated epoxytrienes confirmed that 1,epo3,Z6,Z9-21:H (Fr. 1, RT 21.7 min), 1,Z3,epo6,Z9-21:H (Fr. 2, RT 25.7 min), and 1,Z3,Z6,epo9-21:H (Fr. 3, RT 29.2 min) were produced in about 3:9:10. Other C_{19} and C_{20} tetraenes were treated with MCPBA in a similar manner to obtain mixtures of the corresponding epoxytrienes.

Insects and Pheromone Extraction Larvae of *H. cunea* were continuously reared on a semisynthetic diet [Insecta LF(S), Nippon-Nosan-Kogyo Co, Yokohama, Japan] after being

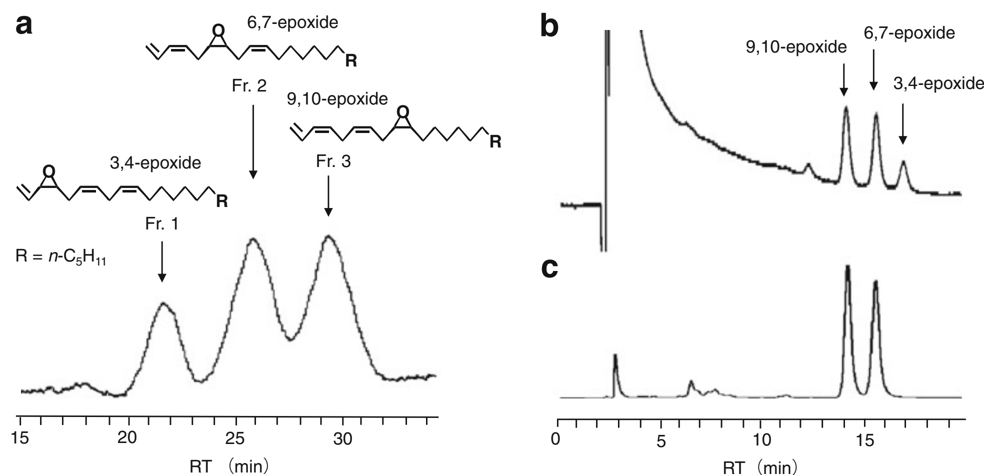

Fig. 1 Analyses of a mixture of the epoxytrienes derived from (3*Z*,6*Z*,9*Z*)-1,3,6,9-henicosatetraene by MPLC (**a**) and HPLC (**b** and **c**). **a**, Chromatogram on a Lobar column (10 mm ID×24 cm) recorded by an RI detector using 0.5 % THF in hexane (3.0 ml/min) as eluent; **b**, chromatogram on an ODS column (4.6 mm ID×25 cm) recorded by an RI detector using 3.5 % water in MeOH (1.0 ml/min) as eluent; and **c**, chromatogram recorded by a UV detector (235 nm) with the same HPLC condition

collected from mulberry plants in Ibaraki Prefecture, and sexed at the pupal stage. Insects were maintained under a 16:8 L-D cycle at 25 °C. The ovipositor tips of 25 females, including pheromone glands, were excised and immersed in hexane for 30 min to yield a crude pheromone extract. The females produced (9*Z*,12*Z*)-9,12-octadecadienal, (9*Z*,12*Z*,15*Z*)-9,12,15-octadecatrienal, *Z*3,*Z*6,epo9-21:H, and 1,*Z*3,*Z*6,epo9-21:H in a ratio of about 206 5:4:10:2, respectively (Kiyota et al. 2011). The extract was injected into an ODS column, and the epoxytriene (RT 14.1 min) and epoxydiene (RT 16.9 min) were separately recovered by monitoring with UV 215 nm (Ando et al., 2004). After HPLC isolation, each epoxy pheromone component was analyzed by a chiral HPLC. Male moths were used for measurements of electroantennographic (EAG) activity of both enantiomers, which were resolved from a racemic mixture by chiral HPLC.

GC Combined with an EAG Detector (GC-EAD) An HP-5890 Series II gas chromatograph (Hewlatt-packard, Wilmington, DE, USA) was equipped with a DB-23 column (0.25 mm ID, 30 m length, 0.25 μm film thickness, J & W Scientific, Folsom, CA, USA). The column temperature was programmed for 50 °C for 2 min, 10 °C/min to 160 °C, and 4 °C/min to 220 °C. The effluent from the column was split into two lines, which led to a flame ionization detector (FID) and EAD at a ratio of 1:1 (Inomata et al., 2005).

Results

NMR and HPLC Analyses of Epoxytrienes The chemical structures of three C_{21} epoxytrienes, which were separated by MPLC as Frs. 1–3, were elucidated by NMR analysis. Table 1 shows ^{1}H and ^{13}C NMR assignments confirmed by two-dimensional experiments. The spectrum of each epoxytriene includes signals of olefinic protons at the terminal double bond, indicating that MCPBA scarcely attacked the terminal double bond, and the 1,2-epoxy derivative was not produced. Since the compound in Fr. 1 showed a proton signal of the epoxy ring (δ 3.44 ppm) coupled with an olefinic proton at the 2-position (δ 5.77 ppm), it was determined to be 1,epo3,Z6,Z9-21:H. The compound in Fr. 2 with an allylic proton (δ 2.05 ppm), and the compound in Fr. 3 with a double-allylic proton (δ ~2.95 ppm) were determined to be 1,Z3,epo6,Z9-21:H and 1,Z3,Z6,epo9-21:H, respectively. The ^{13}C NMR spectrum of each epoxytriene showed similarity to the corresponding epoxydiene derived from a (3*Z*,6*Z*,9*Z*)-3,6,9-triene, Z3,Z6,Z9-21:H (Ando et al., 1993). Namely, carbons from the 5-position to the 11-position in 1,epo3,Z6,Z9-21:H resonated at almost the same chemical shifts as those in epo3,Z6,Z9-21:H (26.2, 124.2, 130.8, 25.8, 127.2, 130.7, and 27.3 ppm). 1,Z3,epo6,Z9-21:H showed quite similar C-6 – C-11 signals to those of Z3,epo6,Z9-21:H (56.5, 56.5, 26.1, 123.7, 132.9, and 27.5 ppm), and 1,Z3,Z6,epo9-21:H also showed similar C-6 – C-11 signals to those of Z3,Z6,epo9-21:H (130.8, 124.2, 26.3, 56.4, 57.2, and 27.8 ppm). Furthermore, C-1 – C-4 signals of 1,Z3,Z6,epo9-21:H were detected at chemical shifts similar to those of the parent tetraene, 1,Z3,Z6,Z9-21:H (117.4, 132.0, 129.3, and 130.4 ppm) (Yamamoto et al., 2008). Each epoxytriene derived from C_{19} and C_{20} tetraenes exhibited almost the same NMR spectra as those of the corresponding C_{21} epoxytriene except for the number of methylene protons resonating at about δ 1.26 ppm and methylene carbons resonating at about δ 29.5 ppm.

Table 1 ^{1}H and ^{13}C NMR assignments of monoepoxides derived from (3*Z*,6*Z*,9*Z*)-1,3,6,9- henicosatetraene

	Chemical shift (δ, ppm)					
	^{1}H NMR			^{13}C NMR		
Position	1,epo3,Z6,Z9-21:H [a]	1,Z3,epo6,Z9-21:H [b]	1,Z3,Z6,epo9-21:H [c]	1,epo3,Z6,Z9-21:H	1,Z3,epo6,Z9-21:H	1,Z3,Z6,epo9-21:H
1	5.38, 5.49	5.17, 5.26	5.13, 5.22	120.6	118.4	117.7
2	5.77	6.62	6.65	132.2	131.7 [d]	131.9
3	3.44	6.14	6.03	57.1	131.5 [d]	129.6
4	3.11	~5.5	~5.4	58.1	126.3	129.87 [f]
5	2.23, 2.45	2.39, 2.55	~2.95	26.2	26.7	26.28 [g]
6	~5.4	~2.98	~5.5	123.8	56.2 [e]	129.91 [f]
7	~5.4	~2.98	~5.5	131.1	56.5 [e]	125.0
8	2.79	2.24, 2.43	2.24, 2.41	25.8	26.2	26.30 [g]
9	~5.4	~5.5	~2.95	127.2	123.5	56.4
10	~5.4	~5.4	~2.95	130.7	133.0	57.2
11	2.04	2.05	~1.5	27.3	27.5	27.8
12	~1.26	~1.26	~1.26	~29.5	~29.5	26.6
13–18	~1.26	~1.26	~1.26	~29.5	~29.5	~29.5
19	~1.26	~1.26	~1.26	31.9	31.9	31.9
20	~1.26	~1.26	~1.26	22.7	22.7	22.7
21	0.88	0.88	0.88	14.1	14.1	14.1

[a] $J_{1,2}$=17, 10.5 Hz, $J_{2,3}$=7 Hz, $J_{3,4}$=4.5 Hz, $J_{4,5}$=7 Hz, $J_{5,5}$=15 Hz, $J_{5,6}$, $J_{7,8}$, $J_{8,9}$, $J_{10,11}$, $J_{11,12}$=7 Hz, $J_{20,21}$=6.5 Hz

[b] $J_{1,2}$=17, 10 Hz, $J_{2,3}$=11 Hz, $J_{3,4}$=11 Hz, $J_{4,5}$=7 Hz, $J_{5,5}$=15 Hz, $J_{5,6}$, $J_{7,8}$=7 Hz, $J_{8,8}$=15 Hz, $J_{8,9}$, $J_{10,11}$, $J_{11,12}$=7 Hz, $J_{20,21}$=6.5 Hz

[c] $J_{1,2}$=17, 10 Hz, $J_{2,3}$=11 Hz, $J_{3,4}$=11 Hz, $J_{7,8}$=5.5 Hz, $J_{8,8}$=14.5 Hz, $J_{8,9}$=5.5 Hz, $J_{20,21}$=6.5 Hz

[d-g] Chemical shift values may be reversed

When the mixture of epoxytrienes derived from 1,Z3,Z6, Z9-21:H was analyzed by an RI detector of HPLC equipped with an ODS column, three peaks were detected similarly to the case of MPLC analysis, but the peak areas were about 10:9:3 (RTs 14.1, 15.6, and 17.0 min, Fig. 1b). This result indicated opposite elution orders from a normal-phase MPLC and reversed-phase HPLC columns. Injection of each pure sample confirmed that 1,Z3,Z6,epo9-21:H, 1,Z3, epo6,Z9-21:H, and 1,epo3,Z6,Z9-21:H eluted from the ODS column in this order. When the mixture of three epoxytrienes was injected to the HPLC column and analyzed by a UV detector operated at 235 nm, only two peaks of the epoxides with a conjugated dienyl structure (RTs 14.1 and 15.6 min, Fig. 1c) were detected.

GC-MS Analysis of Epoxytrienes Figure 2 shows the EI mass spectra of three C_{21} epoxytrienes. The spectral patterns of the three positional isomers were clearly different. Table 2 shows RT values and the relative intensities of M^+ and some characteristic fragment ions of the synthetic epoxytrienes with a C_{19}–C_{21} chain. From a polar GC column (DB-23), three positional isomers are eluted in the same order as from an MPLC column. Each 3,4-epoxide eluted fastest among the three positional isomers because it lacked a conjugated dienyl structure. While the 3,4-epoxides and 6,7-epoxides produced a base peak of $[H(CH=CH)_3]^+$ at *m/z* 79, the larger ion of $[H_2(CH=CH)_4]^+$ at *m/z* 106 was observed as a base peak for the 9,10-epoxides. This table revealed that these positional isomers are also distinguishable by the following diagnostic ions, i.e., ions at *m/z* M-70 and M-41 of the 3,4-epoxides, *m/z* 95, 109, and 149 of the 6,7-epoxides, and *m/z* 106 and 120 of 9,10-epoxides. The relative intensities of the ions at *m/z* M-121 and M-107 of the 9,10-epoxides were small, but these ions were also diagnostically detected. Figure 3 indicates the fragmentation for the diagnostic ions, and these ions of the epoxytrienes are compatible with the diagnostic ions of the epoxydienes derived from 3,6,9-trienes. Namely, the following ions are characteristic in the mass spectra of the epoxydienes: ions at *m/z* M-72 of 3,4-epoxides; *m/z* 97 and 111 of 6,7-epoxides; and *m/z* 108, 122, M-121, and M-107 of 9,10-epoxides (Ando et al., 1993).

Resolution of Epoxytrienes by Chiral HPLC Enatiomeric separation of the C_{21} epoxytrienes was examined with three normal-phase and one reversed-phase HPLC columns. Table 3 shows RT values of each enantiomer, the resolution (*Rs*), and the separation factor (α). While the resolution of 3,4-epoxytriene was insufficient, separated peak tops of the two enantiomers were observed by the analysis with an AD column. In

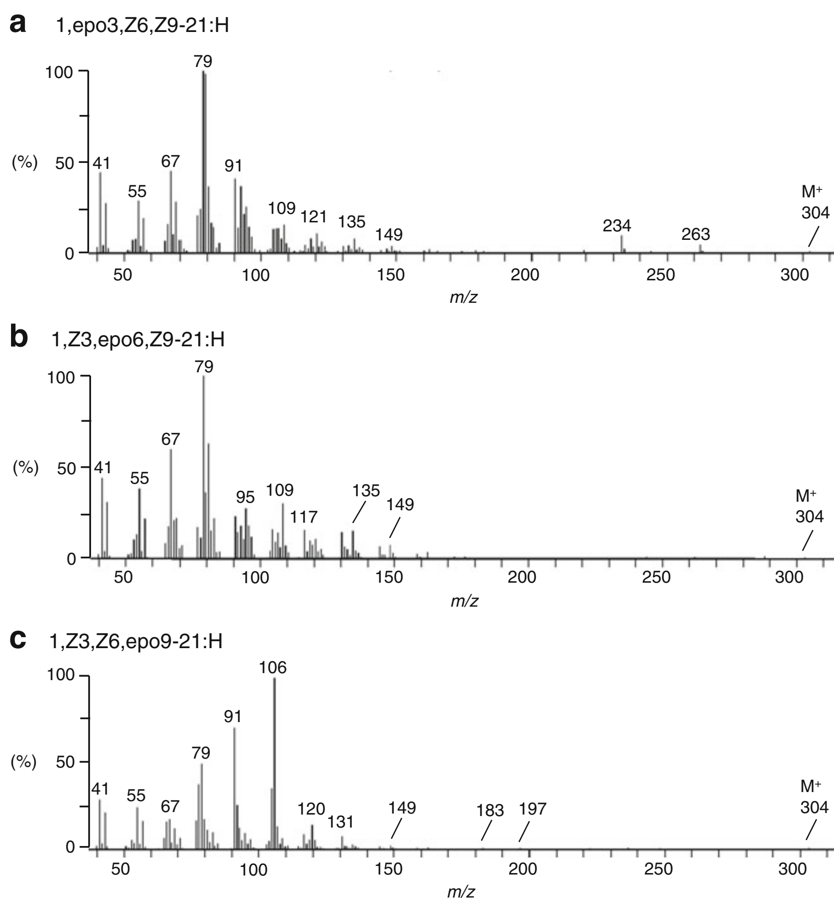

Fig. 2 Mass spectra of epoxytrienes derived from (3*Z*,6*Z*,9*Z*)-1,3,6,9-henicosatetraene. **a**, 1,epo3,Z6,Z9-21:H; **b**, 1,Z3,epo6,Z9-21:H; and **c**, 1,Z3,Z6,epo9-21:H

the case of 6,7- and 9,10-epoxytrienes, an AY-H column completely separated two enantiomers. For the resolution of the 6,7-epoxytriene, AD and OJ-R columns also were available. The absolute configuration of each enantiomer was determined by

Table 2 GC-MS data of synthetic monoepoxides derived from C_{19}–C_{21} (3*Z*,6*Z*,9*Z*)-1,3,6,9-tetraenes measured in EI mode (70 eV)

	RT [a]		Relative intensity of fragment ions, *m/z* (%)											M^+	
	(min)	KI	79	91	95	106	109	120	149	M-121	M-107	M-70	M-41	*m/z*	(%)
3,4-Epoxide															
1,epo3,Z6,Z9-19:H	17.30	2444	100	46	23	13	8	5	3	0	0	15	4	276	1
1,epo3,Z6,Z9-20:H	20.00	2551	100	33	23	8	14	2	3	1	1	8	4	290	1
1,epo3,Z6,Z9-21:H [b]	22.73	2651	100	41	22	14	16	4	3	0	0	9	4	304	1
6,7-Epoxide															
1,Z3,epo6,Z9-19:H	18.87	2559	100	26	24	9	27	6	6	0	0	0	1	276	1
1,Z3,epo6,Z9-20:H	21.57	2661	100	21	25	7	27	6	6	1	0	0	1	290	1
1,Z3,epo6,Z9-21:H [b]	24.41	2756	100	32	28	9	31	8	8	0	0	1	1	304	2
9,10-Epoxide															
1,Z3,Z6,epo9-19:H	19.28	2587	54	83	8	100	5	13	1	1	1	0	0	276	1
1,Z3,Z6,epo9-20:H	21.98	2688	54	82	8	100	5	13	1	2	1	0	0	290	1
1,Z3,Z6,epo9-21:H [b]	24.82	2782	54	73	10	100	6	14	2	2	1	1	0	304	2

[a] Each compound was analyzed by GC-MS equipped with a DB-23 column (0.25 mm ID×30 m). The column temperature program was 50 °C for 2 min, 10 °C/min to 160 °C, and 4 °C/min to 220 °C

[b] C_{21} epoxytrienes showed the following chromatographic behaviors in the analyses with an HP-5 column (0.25 mm ID×30 m): 1,epo3,Z6,Z9-21:H, RT 27.47 min (KI 2212); 1,Z3,epo6,Z9-21:H, RT 28.70 min (KI 2271); 1,Z3,Z6,epo9-21:H, RT 28.95 min (KI 2283). The column temperature program was the same as the analyses with DB-23 column

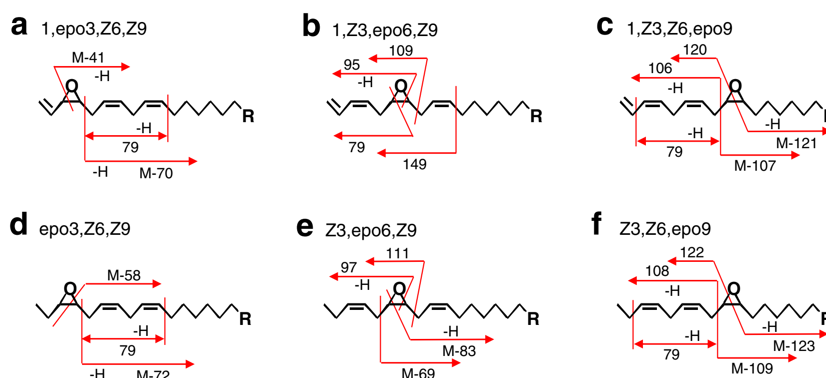

Fig. 3 Diagnostic fragment ions for the GC-MS analysis of epoxytrienes derived from (3*Z*,6*Z*,9*Z*)-1,3,6,9-tetraenes (**a**–**c**) and epoxydienes derived from (3*Z*,6*Z*,9*Z*)-3,6,9-trienes (**d**–**f**)

comparing the authentic samples after hydrogenation. Namely, an enantiomer of 1,epo3,Z6,Z9-21:H isolated by a chiral column was hydrogenated over Pd-C to yield optically active 3,4-epoxyhenicosane (epo3-21:H), whose chromatographic behavior on a chiral HPLC column (Chiralcel OJ-R) was compared with the authentic epoxide produced by hydrogenation of optically active 3,4-epoxydiene (epo3,Z6,Z9-21:H) (Qin et al., 1997). Configuration of isolated enantiomers of 1,Z3, epo6,Z9-21:H and 1,Z3,Z6,epo9-21:H also was determined in a similar manner.

Stereochemistry of Hyphantria cunea Pheromone Components Two enantiomers of 9,10-epoxydiene (Z3,Z6,epo9-21:H) were also separable on a chiral HPLC column (Chiralpak AY-H) without overlapping to the enantiomers of 1,Z3,Z6, epo9-21:H (Fig. 4a). The epoxydiene less than 1 µg was detectable by a UV detector (215 nm), and the epoxytriene with a conjugated dienyl structure was more sensitively detected than the epoxydiene. Each natural pheromone component of *H. cunea* females, which had been isolated by HPLC fractionation, was analyzed by the chiral HPLC. The natural epoxytriene showed only one peak of the (9*S*,10*R*)-isomer (Fig. 4b), indicating high optical purity of the minor pheromone component. While the peak area of the natural epoxydiene was small, this compound co-eluted with the (9*S*,10*R*)-enantiomer (Fig. 4c), indicating that the females exclusively produced the (9*S*,10*R*)-isomer as a major pheromone component.

Antennal Responses to Optically Active Epoxy Pheromone Components By GC-EAD equipped with an *H. cunea* male antenna, the EAG activity of each enantiomer of 1,Z3,Z6, epo9-21:H and Z3,Z6,epo9-21:H was measured at some different doses. Figure 5 contains the result. In both epoxy components, the activities of (9*S*,10*R*)-isomers at a 0.1-µg dose were higher than those of (9*R*,10*S*)-isomers at a 10-µg dose. The antenna was stimulated more strongly (>100 times) by enantiomers with the same configuration as the natural pheromone components than the antipodes.

Discussion

Three kinds of epoxytrienes with a C_{19}–C_{21} chain were prepared by MCPBA oxidation of 1,3,6,9-tetraenes, which

Table 3 Enantiomeric separation of monoepoxides derived from (3*Z*,6*Z*,9*Z*)-1,3,6,9-heniosatetraene on chiral HPLC columns[a]

	1,epo3,Z6,Z9-21:H				1,Z3,epo6,Z9-21:H				1,Z3,Z6,epo9-21:H			
	RT (min)				RT (min)				RT (min)			
Column	Fr. 1 3*S*,4*R*	Fr. 2 3*R*,4*S*	*Rs*	α	Fr. 1 6*S*,7*R*	Fr. 2 6*R*,7*S*	*Rs*	α	Fr. 1 9*R*,10*S*	Fr. 2 9*S*,10*R*	*Rs*	α
Normal-phase[b]												
AY-H		14.73	0.00	1.00	20.61	23.40	1.55	1.20	19.98	22.80	2.13	1.21
AS-H	9.40	9.58	0.50	1.06		11.77	0.00	1.00	12.91	13.49	0.93	1.10
AD	10.63	10.98	0.63	1.08	15.61	17.67	1.59	1.24	16.27	16.73	0.50	1.05
Reversed-phase[c]												
OJ-R		8.32	0.00	1.00	8.59	9.52	1.42	1.22		9.10	0.00	1.00

[a] Analyses of 1,epo3,Z6,Z9-21:H by an RI detector and others by a UV detector (235 nm). *Rs*, resolution; α, separation factor

[b] Chiralpak AY-H, AS-H, and AD columns (4.6 mm ID×25 cm); eluent, 0.1 % 2-propanol in hexane (0.5 ml/min)

[c] Chiralcel OJ-R column (4.6 mm ID×25 cm); eluent, 10 % water in MeOH (0.5 ml/min)

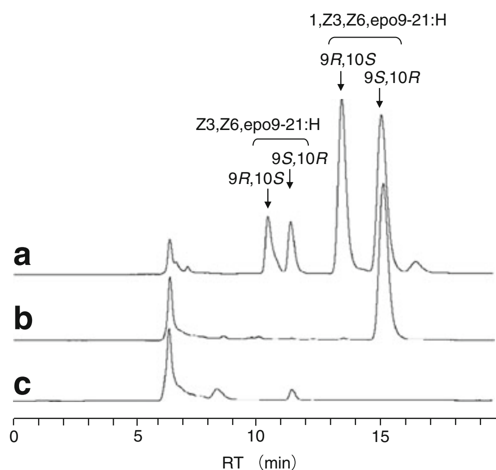

Fig. 4 Determination of the absolute configuration of the 9,10-epoxides, sex pheromone components produced by *Hyphantria cunea* females, by chiral HPLC analyses. Chromatograms on a Chiralpak AY-H column (4.6 mm ID×25 cm) were recorded by a UV detector (215 nm) using 0.1 % 2-propanol (0.5 ml/min) as the eluent: **a**, synthetic racemic mixtures of 9,10-epoxytriene (1,Z3,Z6,epo9-21:H, 1 μg) and 9,10-epoxydiene (Z3,Z6,epo9-21:H, 10 μg); **b**, the epoxytriene isolated from a pheromone gland extract (25 females); and **c**, the epoxydiene isolated from the same pheromone gland extract

had been synthesized from 3-hexyn-1,6-diol (Yamamoto et al., 2008). While the epoxidation resulted in a mixture of the positional isomers (3,4-, 6,7-, and 9,10-epoxytrienes), each isomer was easily separated by MPLC with a normal-phase column or HPLC with a reversed-phase column, and the structures were confirmed by the NMR analysis. On the GC-MS analysis, each positional isomer also was separately eluted from a capillary column and showed some characteristic fragment ions, which could be utilized to distinguish other isomers. Since GC-MS with high sensitivity is the best instrument for the chemical analysis of lepidopteran sex pheromones, diagnostic ions have been observed for many kinds of components (Ando and Yamakawa, 2011), particularly Type II pheromones, such as epoxydienes (Ando et al., 1993), epoxymonoenes (Ando et al., 1995), diepoxymonoenes (Yamazawa et al., 2001), and polyenes (Yamamoto et al., 2008). In addition to the GC-MS data of 9,10-epoxytrienes, the diagnostic fragment ions of 3,4- and 6,7-epoxytrienes listed in Table 2 will likely be utilized to find new compounds that are expected to be pheromones of female moths. On the other hand, the terminal double bond of each 1,3,6,9-tetraene was not attacked by MCPBA, and no 1,2-epoxytrienes were produced in this study. It is also expected that some species might develop the ability to oxidize the terminal double bond. Synthesis and GC-MS analysis of the 1,2-epoxytrienes are the next subjects on the extension of this study.

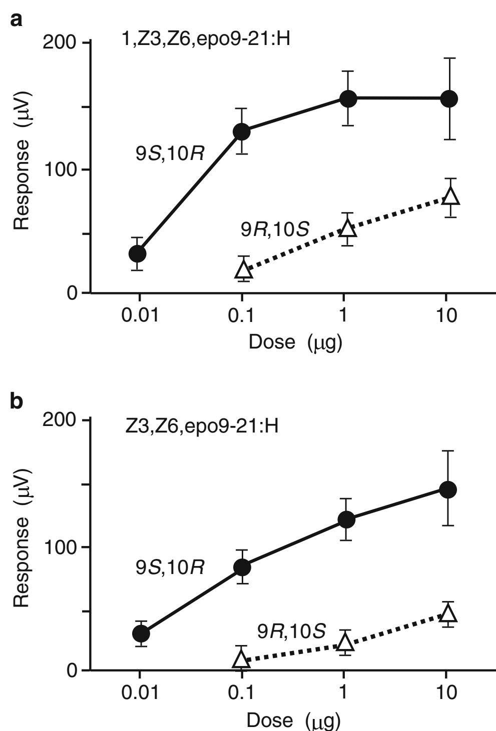

Fig. 5 EAG responses of male *Hyphantria cunea* antennae stimulated by optically active 9,10-epoxytriene (1,Z3,Z6,epo9-21:H, **a**) and 9,10-epoxydiene (Z3,Z6,epo9-21:H, **b**). The responses (mean ± SE) were calculated with data measured by GC-EAD using at least 4 antennae for each dose and each stereoisomer [●, (9*S*,10*R*)-isomers; Δ, (9*R*,10*S*)-isomers]

Resolution of two enantiomers of the C_{21} epoxytriene was accomplished by HPLC equipped with a chiral column. While no chiral columns have the ability to separate enantiomers of all three epoxytrienes universally, the resolution of each epoxytriene was accomplished by selecting the chiral HPLC column. We have achieved enantiomeric separation of epoxydienes and epoxymonoenes by chiral HPLC with normal-phase columns (Qin et al., 1997; Yamamoto et al., 1999) and a reversed-phase column (Pu et al., 1999). This experiment revealed that the configuration of the earlier eluting isomer of each epoxytriene was the same as that of the corresponding epoxydiene and epoxymonoene. The enantiomers of 3,4-epoxydienes with a C_{17}–C_{23} chain were well separated by normal-phase Chiralpak AS and reversed-phase Chiralcel OJ-R columns (Ando et al., 2004). In the case of the 3,4-epoxytriene, however, only one peak was observed on the analysis with those columns, indicating that the terminal double bond adversely affected resolution.

The chiral HPLC analysis of the pheromone extracted from virgin females of *H. cunea* revealed the absolute configuration of two natural pheromone components, C_{21} 9,10-epoxydiene (major component) and 9,10-epoxytriene (minor component). For the 9,10-epoxy compounds, a normal-phase AY-H column showed the best enatiomeric separation among four chiral columns examined. The analysis with the column revealed the 9*S*,10*R* configuration and high enantiomeric excess of both components (Fig. 4). The titer of the minor component in pheromone glands is low but well detected by a UV detector because the epoxytriene included a conjugated

dienyl structure. This study indicates that chiral HPLC is useful to determine the stereochemistry of epoxytrienes as well as the other epoxy components of Type II pheromones (Ando et al., 2004).

For monitoring *H. cunea* males in the field, lures including synthetic (9*S*,10*R*)-isomers of two epoxy components are commercialized (Senda et al., 1991). Antennae of male *H. cunea* selectively responded to the (9*S*,10*R*)-isomers of two epoxy components. Although, to the best of our knowledge, detailed results on the field evaluation of (9*R*,10*S*)-isomers have not been published, our EAG experiments suggest the antipodes will be biological inactive. In addition to this arctiid pheromone, other novel pheromone components have been identified from lymantriid and geometrid species, i.e., 4,5-epoxy-6,9-nonadecadiene from *Bupalus piniaria* (Francke et al., 1998) and 11,12-epoxy-6,9-heniosadiene from *Orgyia postica* (Wakamura et al., 2001). The former epoxide might be biosynthesized from the corresponding 4,6,9-triene, and the latter, from the corresponding 6,9,11-triene. We are also interested in the positional isomers of these epoxy compounds and have plans to synthesize and characterize them. The results will be published elsewhere.

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Defensive Roles of (*E*)-2-Alkenals and Related Compounds in Heteroptera

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Abstract We examined whether shared volatiles found in various heteropteran species and developmental stages function to repel predators. The nymphal dorsal abdominal gland secretions of *Riptortus pedestris* (Heteroptera: Alydidae) and *Thasus acutangulus* (Heteroptera: Coreidae), and the metathoracic scent gland secretion of *Euschistus biformis* (Heteroptera: Pentatomidae) adults were identified by gas chromatography/mass spectrometry (GC/MS). (*E*)-2-Hexenal, 4-oxo-(*E*)-2-hexenal (4-OHE), and (*E*)-2-octenal were found in all three species and deemed likely candidates for repelling predators. In addition to (*E*)-2-alkenals, the adult *E. biformis* secreted (*E*)-2-hexenyl acetate, (*E*)-2-octenyl acetate, and four hydrocarbons. We evaluated the potential predator repellent properties of these compounds and compound blends against a generalist, cosmopolitan insect predator, the Chinese praying mantid (Mantodea: Mantidae: *Tenodera aridifolia sinensis*). Mantids that experienced (*E*)-2-hexenal, (*E*)-2-octenal, and (*E*)-2-octenyl acetate moved away from the site of interaction, while 4-OHE and (*E*)-2-hexenyl acetate did not affect mantid behavior. The compound blends did not have additive or synergistic repellency effects on predator behavior. Compound repellency was not related to compound volatility. Instead, the repellent effect is likely related to predator olfaction, and the affinity of each compound to receptors on the antennae. Our results also suggest the repellents might intensify the visual defensive signals of aposematism (*T. acutangulus* nymphs) and mimicry (*R. pedestris* nymphs) in heteropteran bugs.

Keywords Olfactory repellency · *Thasus acutangulus* · *Euschistus biformis* · *Riptortus pedestris* · *Riptortus clavatus* · (*E*)-2-hexenal · (*E*)-2-octenal · (*E*)-2-octenyl acetate · Aposematism · Mimickry · Soybean pest

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Introduction

Repellent chemicals discourage the receiver from further interaction with the signaler, for example, unwanted pollinators, predators, or mates. Repellents interact with the olfactory system eliciting a negative behavior in the receiver such as fleeing or bypassing prey, but do not often result in the death of the receiver (Bowers, 1993; Chapman, 1998; Ruxton et al., 2004). In predator–prey interactions, these compounds are often generalist signals emitted by the prey communicating information to a variety of predator species with varying sensory capabilities. Thus, anti-predator compounds are more likely to be similar across species and developmental stages. Predator repellent compounds may also synergize visual and auditory signals to promote long-term learning and memory retention of undesirable prey or mates (Rowe, 1999; Hebets and Papaj, 2005).

Aposematism and mimicry are frequent traits among Heteroptera, visually advertising toxicity or other unpleasant prey qualities to predators (Bowers, 1993; Chapman, 1998; Ruxton et al., 2004). These "true bugs" also possess a variety of defensive chemicals ranging from terpenes, phenolics, pregnanes, and cardiac glycosides to alkanes, aliphatic esters, and aldehydes in their nymphal and adult

stages (Aldrich, 1988; Krall et al., 1999; Eisner et al., 2005; Millar, 2005). Compounds shared across species and developmental stages are thought to function as either nonspecific irritants or specific toxins. In general, volatile irritating compounds are probably more effective against arthropod predators than vertebrate predators (e.g., birds), while toxic compounds are much more effective against birds and other vertebrates than against insects (Eisner, 1970; Pasteels et al, 1983; Aldrich, 1988). However, despite advances in chemical identification, the behavioral evidence supporting effects of heteropteran compounds on potential predators is still largely missing. The biological significance of mixtures of defensive secretions also is still poorly understood (Pasteels et al., 1983).

(*E*)-2-Alkenals are practically ubiquitous in Heteroptera, and are known to function as intra-specific signals such as aggregation and sex pheromones (Aldrich, 1988; Millar, 2005). Previously, we reported that 4-oxo-(*E*)-2-hexenal (4-OHE) in the giant mesquite bug, *Thasus neocalifornicus* (Heteroptera: Coreidae), functions as both an alarm pheromone and a deterrent to predators (Prudic et al., 2008). Here, we evaluated the repellent effects of (*E*)-2-hexenal, (*E*)-2-octenal, and 4-OHE. Many heteropteran bugs emit volatile compounds from specialized secretory glands when they are molested by a predator (Borges and Aldrich, 1992; Marques et al., 2007; Prudic et al., 2008). We first identified the shared volatile components of nymphs of *Riptortus pedestris* (Alydidae) and *Thasus acutangulus* (Coreidae), and adults of *Euschistus biformis* (Pentatomidae) by using gas chromatography/mass spectrometry (GC/MS). With this information, we performed predator bioassay experiments using a generalist, cosmopolitan predator, the Chinese mantid (Mantodea: Mantidae: *Tenodera aridifolia sinensis*), to examine how the individual compounds and compound blends affect predator behavior, and how predator response is related to compound volatility. Mantids are ambush predators common in habitats where heteropteran insects occur. These predators rely on visual and olfactory cues to locate and capture prey. Although mantids have limited or no color vision, they can detect high-contrast prey and can learn to avoid aposematic prey (Prudic et al., 2007). Thus, mantids are an appropriate predator model to understand the putative repellent role of volatile heteropteran exocrine compounds.

Methods and Materials

Insects Riptortus pedestris (F.) (= *R. clavatus* (Thunberg); Kikuhara 2005) (Alydidae) 5th-instar nymphs (13–14 mm; Fig. 1a) were collected on soybean, *Glycine max* (Fabaceae) in September, 2010, in the experimental field of Akita Prefectural University, Akita, Japan. This species is a well-known seed pest of soybean and other legumes in Japan (Yasunaga et al., 1993). The nymphs resemble ants in morphology and behavior, while the adults resemble wasps when they fly. The adult aggregation pheromone is a blend of (*E*)-2-hexenyl (*E*)-2-hexenoate, (*E*)-2-hexenyl (*E*)-3-hexenoate, and myristyl isobutyrate, which also attracts nymphs (Leal et al., 1995).

Thasus acutangulus (Stål) (Coreidae) 4th-instar nymphs (16–18 mm; Fig. 1b) were collected from their host plant, *Pithecellobium dulce* (Fabaceae) ("Guamuchil"), in June, 2007, on a natural site in Puente Beltran, Colima, Mexico. *Thasus acutangulus* is found in Mexico, Guatemala, Belize, El Salvador, Honduras, and Costa Rica (Aldrich and Blum, 1978; Brailovsky et al., 1994; Schaefer and Packauskas, 1997). The nymphs are aposematically colored with vivid orange, yellow, and black. When perturbed, they release a potent, foul-smelling secretion similar to other *Thasus* spp., accompanied by a vivid group visual display (Aldrich and Blum, 1978). The adults are much larger (32–34 mm for females and 34–40 mm for males, Brailovsky et al., 1994), and cryptically colored with dark reddish-brown resembling the bark of their host plant. Nymphs and adults often form large clusters feeding on the same trees. Despite conspicuous coloration of the nymphs, little is known regarding the chemical defenses of *T. acutangulus* at any life stage (but see Aldrich and Blum, 1978).

Adults of *Euschistus biformis* Stål (Pentatomidae) (11–12 mm; Fig. 1c) were collected on mullein, *Verbascum thapsus* (Scrophulariaceae), in October, 2008, on Mt. Lemmon (at 1800 m elevation), Tucson, Arizona, USA. This species was loosely aggregated with *Chlorochroa ligata* (Heteroptera: Pentatomidae) on the same plants.

Oothecae of Chinese praying mantids, *Tenodera aridifolia sinensis* (Mantodea: Mantidae) were purchased from Carolina Biological Supply (Burlington, NC, USA). The mantids were reared individually on a successive diet of fruit flies (*Drosophila melanogaster* and *D. hydei*) and crickets (*Achetus domesticus*) depending on mantid developmental stage.

Preparation and Extraction of Heteropteran Secretions Whole insects were extracted by dipping an individual into hexane containing 10 ng/μl 1-dodecene (Sigma-Aldrich, St. Louis, MO, USA) as an internal standard for 5 min (1 ml for *R. pedestris* and *E. biformis*, and 5 ml for *T. acutangulus* to accommodate its larger size; *R. pedestris*, N=8; *T. acutangulus*, N=3; *E. biformis*, N=5). To confirm that compounds originated from the dorsal abdominal scent glands (DAGs), we also made extractions of only these glands. For *R. pedestris*, DAG reservoirs shed with the exuviae (Borges and Aldrich, 1992) were collected from the exuviae of the 5th-instar nymphs within 24 h after they ecdysed into adults (N=3). The DAGs then were macerated in 1 ml of hexane, and extracted for 5 min. For *T. acutangulus*, the 4th-instar

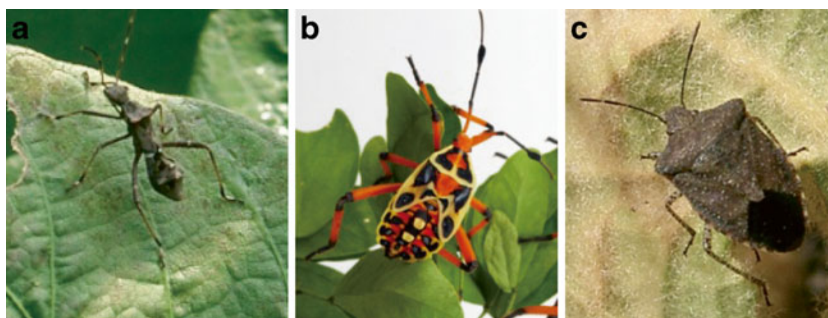

Fig. 1 (**a**) *Riptortus pedestris* nymph (**b**) *Thasus acutangulus* nymph (photo courtesy of Alex Wild) and, (**c**) *Euschistus biformis* adult

nymphal secretions were collected on a piece of filter paper from its DAGs after molestation with forceps. The filter paper was extracted in 1 ml of hexane for 5 min (N=3). For *E. biformis*, adults were placed into a plastic container, and then anesthetized under nitrogen gas to prevent premature discharge of the secretory compounds. The metathoracic scent gland (MTG) complex was removed by dissection, and extracted in 1 ml of hexane for 5 min (N=5). All extractions were performed immediately after insect collection, and the extract (1 μl) was analyzed by GC–MS for chemical identifications.

Isolation, Identification, and Quantification of Secretory Compounds GC/MS analysis was performed on an Agilent 6890N GC linked to an Agilent 5975B MS operated at 70 eV using an HP-5MS capillary column (Agilent Technologies, 30 m×0.25 mm i.d., 0.25 μm in film thickness) with helium carrier gas at 1.2 ml/min in splitless mode. The oven temperature was programmed from 50 °C (3 min) to 300 °C at 10 °C/min, and then held for 5 min. A PerkinElmer Turbo Mass also was used under the same conditions as the above with a DB-5MS capillary column (Agilent Technologies, 30 m×0.25 mm i.d., 0.25 μm in film thickness).

All compounds were identified by comparing their GC retention times and mass spectra of commercial or synthesized standards. (*E*)-2-Hexenal (Sigma-Aldrich), (*E*)-2-octenal (Sigma-Aldrich), and (*E*)-2-hexenyl acetate (Bedoukian Research, Dunbury, CT, USA) were commercially-available products. Undecane, dodecane, and tridecane were provided by Dr. Naoki Mori (Kyoto University, Japan), and 1-tridecene was provided by Dr. Leif Abrell (University of Arizona, USA). 4-OHE was synthesized by oxidative ring opening of 2-ethylfuran (Sigma-Aldrich) using aqueous *N*-bromosuccinimide (Sigma-Aldrich) according to Moreira and Millar (2005). ^{1}H NMR ($CDCl_3$): δ_H 9.76 (d, 1H, J=7.2 Hz), 6.87 (d, 1H, J=16.4 Hz), 6.76 (dd, 1H, J=16.4, 7.2 Hz), 2.72 (q, 2H, J=7.2 Hz), 1.14 (t, 3H, J=7.2 Hz); ^{13}C NMR ($CDCl_3$): δ_C 200.47, 193.52, 144.81, 137.37, 34.60, 7.62. (*E*)-2-Octenyl acetate was synthesized by DMAP-catalyzed esterification under solvent free condition described in Sakakura et al. (2007) with slight modification. Briefly, (*E*)-2-octenol (645 mg, 5.03 mmol, Bedoukian Research) and DMAP (3.2 mg, 0.262 mmol, Sigma-Aldrich) were mixed with acetic anhydride (574 mg, 5.63 mmol, Sigma-Aldrich), and then the mixture was stirred at 50 °C for 4 h. It was quenched with water (90 μl), and the product was extracted with dichloromethane (20 ml). The organic layer was washed with saturated aqueous $NaHCO_3$ (40 ml) and brine (40 ml), dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography eluting with hexane–ethyl acetate (97:3) to afford 758 mg of (*E*)-2-octenyl acetate (88 % yield). ^{1}H NMR ($CDCl_3$): δ_H 5.76 (m, 1H), 5.54 (m, 1H), 4.49 (d, 2H, J=6.7 Hz), 2.04 (s, 3H), 2.03 (q, 2H, J=6.9 Hz), 1.44–1.20 (m, 6H), 0.87 (t, 3H, J=7.0 Hz); ^{13}C NMR ($CDCl_3$): δ_C 171.09, 136.95, 123.88, 65.54, 32.43, 31.57, 28.76, 22.69, 21.22, 14.21. ^{1}H and ^{13}C NMR spectra were acquired on a Varian Unity 300 spectrometer (^{1}H at 300 MHz and ^{13}C at 75 MHz) in a $CDCl_3$ solution with tetramethylsilane (TMS) as an internal standard.

For quantitative analysis of the identified compounds, GC analysis was performed on an Agilent 6890 N gas chromatograph with a flame ionization detector (FID), using a DB-5MS capillary column (Agilent Technologies, 25 m× 0.32 mm i.d., 0.52 μm in film thickness) with the same conditions as those for GC/MS except for the velocity of helium carrier gas (2.0 ml/min). A Shimadzu GC 2010 with a DB-5MS capillary column (Agilent Technologies, 30 m× 0.25 mm i.d., 0.25 μm in film thickness) also was used for quantitative analysis using the same methods for GC/MS except for the velocity of helium carrier gas was lowered (1.0 ml/min). One microliter of the extracts with internal standard were analyzed by GC–FID; the quantity of (*E*)-2-alkenals and their related compounds per individual bug was determined by the relative ratio of the peak area to the internal standard.

Insect Predator Behavioral Assays of Individual Compounds and Compound Blends We used a modified air puff test (Leal et al., 1994) to evaluate the repellent effect of the different compounds found in the GC/MS experiments described above. For single compound bioassays, a small strip of filter paper was loaded with 2 μl of each compound [(*E*)-2-hexenal, 4-OHE, (*E*)-2-hexenyl acetate, (*E*)-2-octenal, or (*E*)-2-octenyl acetate]. For the compound blend assays, a mixture was prepared loading 2 μl of each compound onto

the same filter paper. The mixtures qualitatively resembling the bugs' secretion were: [(*E*)-2-hexenal +4-OHE + (*E*)-2-octenal] and [(*E*)-2-hexenal + 4-OHE + (*E*)-2-hexenyl acetate + (*E*)-2-octenal + (*E*)-2-octenyl acetate]. Each filter paper was placed into a glass Pasteur pipette with an 8 ml silicone bulb at the other end.

At the beginning of each trial, a single mantid was taken from its cage and placed at the center of the observation arena for several minutes (30×15 cm; N=8). The tip of the loaded pipette was placed 1 cm from the mantid head, and the silicone bulb was puffed three times. We recorded how far (cm) the mantid moved away from the pipette within a 15 sec interval as an indicator of repellent activity for each compound or blend. Each mantid was tested 8 times with 8 different treatments over the course of 1 d. First, the mantid experienced a control puff (hexane), and then the mantid was exposed to 7 treatments in random order (5 individual compounds, and 2 compound blends). The interval between trials was 1 h. Preliminary trials using (*E*)-2-octenal (repellent) and 4-OHE (non-repellent) in a random order at a 1 h interval showed that the previous treatment did not affect subsequent behavioral (N=3, data not shown). A new pipette and silicone bulb were used with each trial. All data were analyzed with Tukey-Kramer test using JMP 5.1.2 (SAS Institute, 2003).

Compound Air Puff Recovery Assay To examine whether the amount of the compounds used in predator bioassays were ecologically relevant, we measured the air puff recovery rate for each compound (Leal et al., 1994 with slight modifications). The air puff prepared at the same volume as the bioassay described above was absorbed by a small piece of glass wool in a glass vial. The glass wool was extracted immediately for 5 min in 1 ml of hexane containing 10 ng/μl of 1-dodecene as an internal standard. One μl of each extract was injected into the GC, and the absorbed amounts of the compounds by glass wool were quantified by GC–FID as described above. The recovery rate was calculated as a ratio of the absorbed amounts of the compounds by glass wool divided by the original amounts of the compounds (N=3 replicates/compounds). The density and the boiling point of each compound obtained from Chemical Abstracts are: (*E*)-2-hexenal (0.828 g/cm^3, 146.5 °C), 4-OHE (0.969 g/cm^3, 210.1 °C), (*E*)-2-hexenyl acetate (0.898 g/cm^3, 165.5 °C), (*E*)-2-octenal (0.832 g/cm^3, 190.1 °C) and (*E*)-2-octenyl acetate (0.892 g/cm^3, 216.8 °C).

Results

Chemical components of Riptortus pedestris, Thasus Acutangulus, and Euschistus biformis Secretions The three heteropteran species contained a variety of volatile compounds in their secretory glands (Table 1). The chemical profiles of whole insect extractions were consistent with the gland-specific extractions, thus the extracts obtained by whole insect extractions were used for quantitative analysis of the identified compounds. (*E*)-2-Hexenal, 4-OHE, and (*E*)-2-octenal were identified and quantified in all three species. The nymphs of *R. pedestris* secreted (*E*)-2-hexenal (mean±SD=6±8 μg/bug), 4-OHE (79±54 μg/bug), and (*E*)-2-octenal (39±20 μg/bug). The nymphs of *T. acutangulus* secreted (*E*)-2-hexenal (3±6 μg/bug), 4-OHE (169±158 μg/bug), and (*E*)-2-octenal (61±62 μg/bug). The adults of *E. biformis* had (*E*)-2-hexenal (77±43 μg/bug), 4-OHE (394±193 μg/bug), and (*E*)-2-octenal (25±12 μg/bug) together with six unique compounds not found in the other two species: (*E*)-2-hexenyl acetate (159±72 μg/bug), (*E*)-2-octenyl acetate (243±138 μg/bug), and four aliphatic hydrocarbons.

Repellent Activities of Each Compound Against an Insect Predator Of the compounds shared among species, (*E*)-2-hexenal and (*E*)-2-octenal showed repellent activities against the mantids compared to the control treatment, hexane (P<0.05; Fig. 2). When treated with these compounds, mantids turned in the opposite direction in relation to the air puff and moved rapidly away [(*E*)-2-hexenal, mean distance ±SD=10.3±3.5 cm; (*E*)-2-octenal, mean distance±SD=12.6±3.0 cm; hexane, mean distance±SD=2.0±1.7 cm]. Of the unique compounds found in *E. biformis*, (*E*)-2-octenyl acetate elicited a repellent response from the mantids [(*E*)-2-octenyl acetate, mean distance±SD=13.9±3.7 cm, P<0.05]. The other compounds were not significantly different between control and treatment [4-OHE, mean distance±SD=6.0±1.9 cm; (*E*)-2-hexenyl acetate, mean distance±SD=5.1±2.0 cm; P>0.05].

Both compound blends showed a strong repellent effect against mantids compared with the control treatment (P<0.05; Fig. 2); however, neither blend demonstrated additive or synergistic effects on predator behavior. The repellent effect of the blends could be explained by (*E*)-2-octenal alone (P>0.05) or (*E*)-2-octenal and (*E*)-2-octenyl acetate (P>0.05). No compound or compound blend killed the mantids at the test dosage.

The following amounts were recovered: (*E*)-2-hexenal (mean±SD=6.6±0.6 μg), 4-OHE (1.5±0.0 μg), (*E*)-2-octenal (1.1±0.07 μg), (*E*)-2-hexenyl acetate (2.7±0.3 μg), and (*E*)-2-octenyl acetate (1.0±0.07 μg). The amounts contained in the air puffs were the same or lower than the amount found in each bug. Recovery rates of the bioassay conditions showed a tendency to decrease in relation to the boiling point of the particular compound. (*E*)-2-Hexenal (0.40 %) had the highest recovery, followed by (*E*)-2-hexenyl acetate (0.15 %), 4-OHE (0.08 %), (*E*)-2-octenal (0.06 %), and (*E*)-2-octenyl acetate (0.05 %). Extremely small recovery rates similar to those reported here have been observed in previous experiments (Todd and Baker, 1983).

Table 1 Chemical composition of secretion of nymphs of *Riptortus pedestris* and *Thasus acutangulus*, and adult of *Euschistus biformis*

Compounds	Composition (%)[a]		
	R. pedestris (*N*=8)	*T. acutangulus* (*N*=3)	*E. biformis* (*N*=5)
(*E*)-2-Hexenal	1.8±2.8	0.6±0.01	4.9±0.6
4-Oxo-(*E*)-2-hexenal	59.8±3.1	49.9±0.2	11.5±1.5
(*E*)-2-Hexenyl acetate	n.d.[b]	n.d.	14.4±4.9
(*E*)-2-Octenal	38.4±3.0	49.5±0.2	1.9±1.3
Undecane	n.d.	n.d.	0.2±0.06
Dodecane	n.d.	n.d.	1.1±0.06
(*E*)-2-Octenyl acetate	n.d.	n.d.	18.3±1.2
1-Tridecene	n.d.	n.d.	0.2±0.03
Tridecane	n.d.	n.d.	47.5±2.8

[a]Percentages (means±SD) are based on GC peak area.

[b]n.d. = not detected.

Discussion

The (*E*)-2-alkenals and related acetates found in these three species are common in heteropterans (Aldrich, 1988; Moraes et al., 2008). (*E*)-2-Octenal has been previously described in the nymphal secretion of *R. pedestris* (Leal et al., 1995), but (*E*)-2-octenal is somewhat unusual secretory compound for a coreids, such as *T. neocalifornicus*, whose exocrine secretions are typically dominated by C-6 aldehydes (Gunawardena and Bandumathie, 1993; Prudic et al., 2008). (*E*)-2-Hexenal has not been reported for *R. pedestris*, but has been found in adults of the congener, *R. serripes* (Aldrich et al., 1993), and in *Euschistus* spp. (Aldrich et al., 1995). Interestingly, the chemical profile of *E. biformis* is more similar to individuals of *C. ligata* that were collected on the same host plants (data not shown) than other reported chemical profiles of *Euschistus* spp.

(*E*)-2-Hexenal, (*E*)-2-octenal, and (*E*)-2-octenyl acetate, repelled mantids (Fig. 2). (*E*)-2-Hexenal is also known to repel other predatory insects such as the fire ant, *Solenopsis saevissima*, and the harvester ant, *Pogonomyrmex barbatus*, but did not cause ant mortality (Blum, 1961). (*E*)-2-Octenal is a major component of *Leptocorisa oratorius* secretion and it is toxic and repellent against the crazy ant, *Anoplolepis longipes*, and the Angoumois grain moth, *Sitotroga cerealella* (Gunawardena and Bandumathie, 1993). Neither additive nor synergistic effects among (*E*)-2-alkenals and (*E*)-2-octenyl acetate were observed in this study. The repellent effects of compound blends are explained primarily by the presence of (*E*)-2-octenal or (*E*)-2-octenyl acetate. Tridecane and 1-tridecene found from *E. biformis* might enhance the repellent effects of (*E*)-2-alkenals and related acetates, although we did not include these components found in *E. biformis* in blends we tested. *n*-Alkanes such as undecane, dodecane, and tridecane show synergistic effects on toxicity and repellency when mixed with (*E*)-2-alkenals (Gunawardena and Herath, 1991). Also, we only tested the innate predator

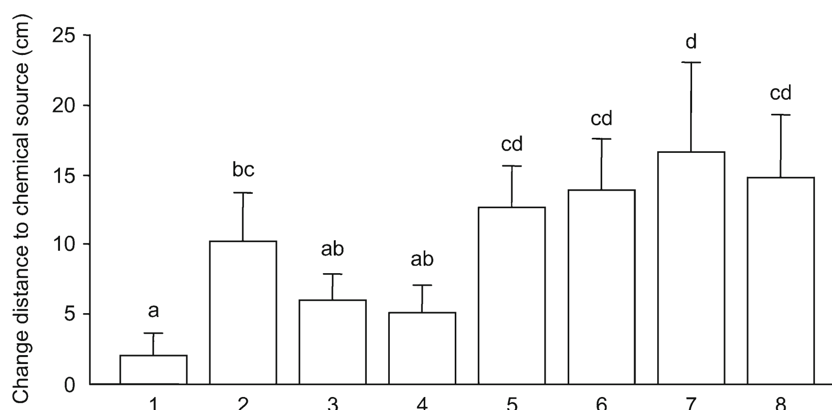

Fig. 2 Behavioral responses of Chinese preying mantids to heteropteran exocrine semiochemicals. All are shown by changes in distance (means±SD cm; *N*=8 mantids) after the exposure of each chemical source [1, hexane (control); 2, (*E*)-2-hexenal; 3, 4-oxo-(*E*)-2-hexenal; 4, (*E*)-2-hexenyl acetate; 5, (*E*)-2-octenal; 6, (*E*)-2-octenyl acetate; 7, mixture of (*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal and (*E*)-2-octenal (secretory components found from the nymphs of *Riptortus. pedestris* and *Thasus acutangulus*); 8, mixture of (*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, (*E*)-2-hexenyl acetate, (*E*)-2-octenal and (*E*)-2-octenyl acetate (secretory components found from the adult of *Euschistus biformis*)]. Bars with the same letter are not significantly different ($P>0.05$)

responses to these compounds and compound blends. Multi-component olfactory signals and redundancy should increase the rate of predator aversive learning and memory retention, and is predicted to positively interact with another signal modality such as color (Rowe, 1999; Hebets and Papaj, 2005). The species we investigated have other visual defensive line of defense, including warning coloration (*T. acutangulus*) and mimicry (*R. pedestris*). Thus, our results suggest the chemical defense by repellents might intensify visual aposematism and mimicry in heteropteran bugs; these possible interactions warrant future study.

Our results also indicate that the anti-predator repellent effects of the compounds tested herein are not directly related to their volatility, as measured by the recovery rate. The recovery rates of C-8 compounds were lower than that of (*E*)-2-hexenal; however, their effects on mantid behavior did not differ. Thus, the efficiency of C-6 and C-8 compounds at repelling predators might be due to the affinity between their chemical structures and the olfactory receptors of predacious insects. Future investigations should explore the sensory biology of this predator–prey interaction, how these semiochemicals alter the predator's behavior, and if there is a shared mechanism between distantly related predators.

Two non-repellents, (*E*)-2-hexenyl acetate and 4-OHE could have other biological activities. For example, (*E*)-2-hexenyl acetate is part of attractant pheromones in the milkweed bugs (Aldlich et al., 1999). Thus, this compound may play a role in intra-specific communication rather than chemical defense and inter-specific communication. 4-OHE is a volatile mutagen that reacts with DNA components such as deoxyguanosine and deoxycytidine (Kasai et al., 2005), and can potentially act as a non-specific toxin for all organisms due to its high reactivity. Previously, we reported that 4-OHE deterred mantids from consuming live coreid bugs and was toxic to them (Prudic et al., 2008). Thus, 4-OHE potentially contributes to the chemical defensive system of heteropterans as a feeding deterrent or toxin, not as a repellent.

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RAPID COMMUNICATION

A Water-Specific Aquaporin is Expressed in the Olfactory Organs of the Blowfly, *Phormia regina*

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Abstract The high sensitivity and selectivity of perireceptor events in insect olfactory organs requires the concerted action of odorant-binding proteins (OBPs), odorant receptors (ORs), and odorant-degrading enzymes (ODEs). Sensillum lymph in the sensillum cavity is a physiological saline that not only mediates the olfactory signaling pathway described above, but also protects the olfactory neurons against desiccation. The molecular mechanism of how water balance is maintained in the sensillum cavity still remains to be elucidated. Here, we characterize an aquaporin from the blowfly, *Phormia regina* (PregAQP1). PregAQP1 possesses six predicted transmembrane domains and two asparagine-proline-alanine (NPA) motifs, and belongs to the *Drosophila melanogaster* integral protein (DRIP) subfamily. Transcript levels were high in the maxillary palp and moderate in the antenna. PregAQP1 accumulated in accessory cells located underneath a long-grooved hair in the maxillary palp and also in a receptor neuron in a thick-walled sensillum in the antenna. Expression of PregAQP1 in *Xenopus* oocytes showed water permeability in a mercury-sensitive manner. These results suggest that PregAQP1 plays a role in the maintenance of the aqueous environment of olfactory organs.

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Keywords Aquaporin · Water permeability · Olfactory organs · *Phormia regina* · Forensic entomology

Introduction

The blowfly, *Phormia regina* is an important species for forensic entomology (Nabity et al. 2006). Olfactory organs in insects including the blowfly are able to detect minute quantities of semiochemicals in the air plume. The high sensitivity and selectivity of olfactory organs requires the concerted action of odorant-binding proteins (OBPs), odorant receptors (ORs), and odorant-degrading enzymes (ODEs). During the perireceptor events in the sensillum lymph, OBPs solubilize hydrophobic semiochemicals, transport, and then release them to ORs. The ORs then convert the signals to receptor potentials in the receptor neuron. After signal transduction, ODEs rapidly catalyze the metabolism of the functional group of the semiochemical to reset the olfactory signal (Leal 2005).

The sensillum lymph in the sensillum cavity is a physiological saline that not only mediates much of the olfactory signaling pathway described above but also protects the olfactory neurons from desiccation. The sensillum cavity is isolated with cuticle and membranes of the dendrite of olfactory receptor neurons and accessory cells. Segregation of the sensillum cavity from hemocoel maintains an uneven distribution of ionic solutes between the sensillum lymph and hemolymph that results in an electrically isolated condition (Keil and Steinbrecht 1987). Despite its complete isolation, the aqueous environment in the sensillum cavity must be maintained continuously.

Aquaporins (AQPs) are integral membrane channel proteins that play a specific role in transferring water molecules (Jung et al. 1994; Agre 2006; Campbell et al. 2008). We hypothesized that AQPs might be responsible for water

homeostasis in the sensillum cavity. To elucidate the molecular mechanism of water homeostasis in the sensillum cavity, we identified a cDNA encoding an AQP from *P. regina* (PregAQP1), detected its expression, localized the protein in the olfactory organs, and measured the ability of PregAQP1 to induce water permeability by using a *Xenopus* oocyte swelling assay.

Methods and Materials

cDNA Cloning Total RNA was extracted from the head of a day 0 adult blowfly using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA), and was used as template for cDNA synthesis with a SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) and SuperScript II (Life Technologies) as reverse transcriptase. The following degenerate and gene-specific primers were used for PCR amplification of cDNA fragments encoding PregAQP1; Aqp-1 (GCHINPAVT, conserved among *Aedes aegypti* (AF218314), *Haematobia irritans exiqua* (AAA96783), *Drosophila melanogaster* [(DRIP; ABA81817), and *Cicadella viridis* (AQPcic; X97159)]), 5′-GGNTGYCAYATHAAYCCNGCNGTNAC-3′; Aqp-2 (MNPARSFGPA), 5′-GCNGGNCCRAARCTNCGNGCNGGRTTC-3′; PregAQP1-1, 5′-GGTGTCTCTGAAGCAGTGGGTGGTAATG-3′; PregAQP1-2, 5′-CAGCTAAAGGAGCTGAGCCCTTGATATC-3′; PregAQP1-3, 5′-GAAAGTGTTAGCAACTTAGCCCAGGCGCG-3′. *Takara Taq* (Takara Bio, Otsu, Japan), KOD FX (Toyobo Biologics, Osaka, Japan), and Advantage GC2 Polymerase Mix (Clontech) were used as *Taq* DNA polymerases. PCR products were ligated into the *Eco*RV site of pBluescript II SK (+) (Agilent Technologies, Santa Clara, CA, USA) and sequenced. The full-length cDNA sequence was determined from 19 independent clones to eliminate PCR-derived sequence errors. The DNA and the deduced amino acid sequences were analyzed by CLC DNA Workbench 5.7 (CLC Bio, Aarhus N Denmark), and SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/), respectively. Phylogenetic analysis was performed by the neighbor-joining method with 1,000 bootstrap replicates using the software MEGA 5 (http://evolgen.biol.metro-u.ac.jp/MEGA/).

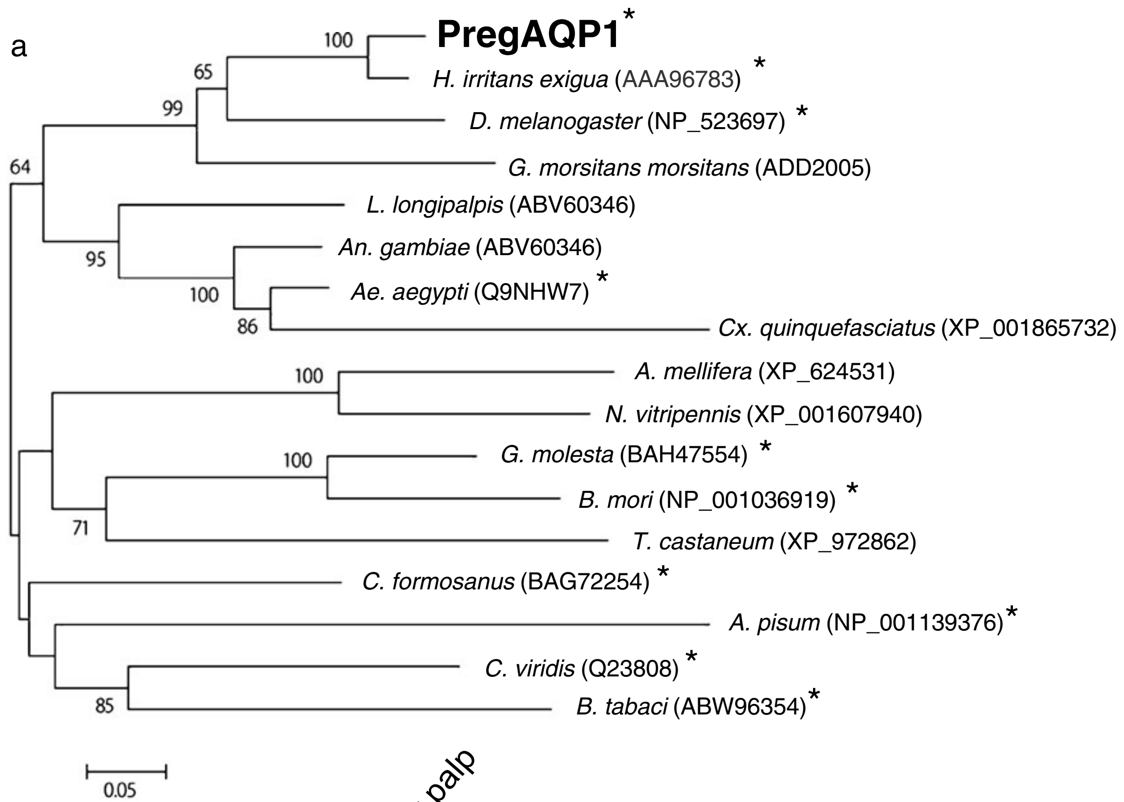
RT-PCR Total RNA was extracted from each experimental tissue at the day 0 adult stage and reverse-transcribed as described above. The following PCR program was carried out using PregAQP1-1 and PregAQP1-2 gene-specific primers and KOD + (Toyobo Biologics) as a *Taq* DNA polymerase: 94 °C for 2 min, 24 cycles of 94 °C for 15 s, 57 °C for 30 s, and 68 °C for 1 min. The 175 bp-long PCR product was identified following 1.5 % agarose gel electrophoresis. Expression of *actin* was used as an internal control (Ishida and Leal 2002).

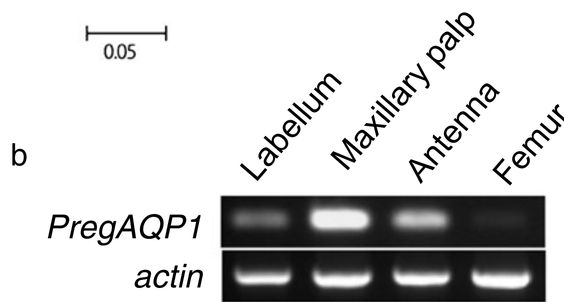
Immunohistochemistry Immunohistochemistry was performed as described previously (Ishida and Ozaki 2011). In brief, the antennae and maxillary palps were embedded in Tissue-Teck O.C.T. Compound (Sakura Finetek Japan, Tokyo, Japan), frozen, and cut into 15 μm thick slices. Anti-(AQP-Bom1 [DRIP]) serum (Azuma et al. 2012) and Alexa Fluor 594-conjugated anti-rabbit IgG (Life Technologies) at 1:1,000 dilution were used as primary and secondary antibodies, respectively. The stained tissues were observed on a FLUOVIEW FV1000 confocal laser-scanning microscope (Olympus, Tokyo, Japan).

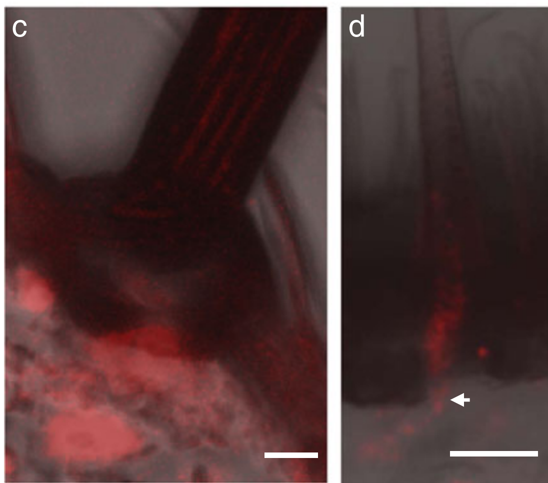
Xenopus Oocyte Permeability Assay PregAQP1 cDNA including sequences of the 5′-untranslated region were amplified by PCR using gene-specific primers PregAQP1-4 (5′-AAATCACAACAACCAAAGAGAATAAACCGC-3′) and PregAQP1-5 (5′-CATTTAAAAATCATAAGAATTAGCCTCATC-3′), and ligated into the *Eco*RV site of pBluescript II SK(+). After verification of the DNA sequence, the insert DNA was released by digestion with *Bam*HI and *Eco*RI, gel-purified, and ligated into *Bgl*II and *Eco*RI sites of pT7XbG2 vector. The constructed vector DNA was linearized by digestion using *Sac*I. The capped RNA (cRNA) was synthesized using a mMESSAGE mMACHINE T7 Kit (Life Technologies), and purified using a MEGAclear Kit (Life Technologies).

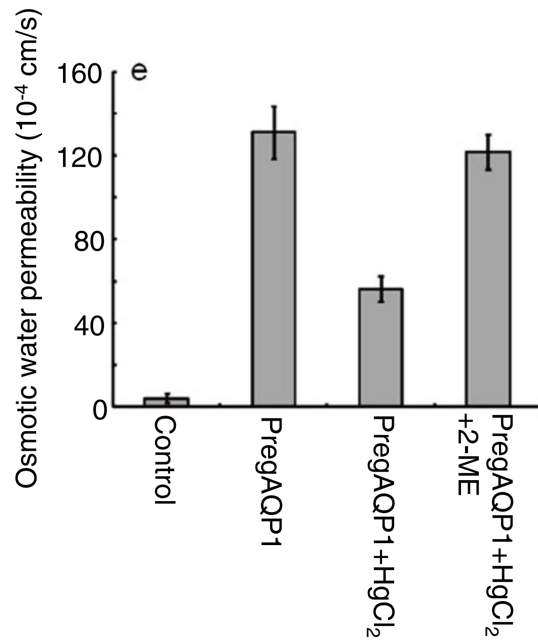
The *Xenopus* oocyte permeability assay for PregAQP1 was performed as described previously (Kataoka et al. 2009). In brief, 5 ng of the cRNA or water as a control were injected into

Fig. 1 Characterization of PregAQP1. **a** Phylogenetic analysis of insect aquaporins. PregAQP1 is in bold and larger font. Accession numbers of other insect aquaporins are in parentheses. The asterisks indicate AQP proteins with experimentally confirmed water permeability. Bootstrap values was shown at each node. The bar indicates 5 % divergence. **b** RT-PCR analysis of *PregAQP1* expression in an adult male. The expression of *actin* was used as an internal control. The expression of *PregAQP1* was the highest in maxillary palp and moderately expressed in antenna. **c** Localization of PregAQP1 in the maxillary palp. Immunoreactive material accumulated in the accessory cells beneath a longitudinally grooved hair. The bar indicates 5 μm. **d** Localization of PregAQP1 in the antenna. Immunoreactive material accumulated in a receptor neuron (indicated by the arrow) of a thick-walled sensillum. The bar indicates 5 μm. **e** The ability of recombinant PregAQP1 to facilitate water permeability in *Xenopus* oocytes. Swelling was measured after injection of PregAQP1 cRNA or water (control) into *Xenopus* oocytes. The effect of mercury chloride and 2-mercaptoethanol (2-ME), a known inhibitor and restoring reagent of water permeability of AQPs, respectively, also were tested. Swelling was observed in the oocytes injected with PregAQP1 cRNA and treated with 2-ME following mercury chloride treatment. On the other hand, water permeability was dramatically lower in water-treated control oocytes, and significantly lower in PregAQP1 cRNA-injected and mercury chloride-treated oocytes. The values shown represent the mean (n=5) ± standard deviation









a *Xenopus* oocyte. Following injection, the oocytes were incubated in the modified Barth's saline (MBS) containing 0.41 mM $CaCl_2$ at 15 °C for 3 d. At 3 d post injection, the oocytes were incubated in 3-fold diluted (with distilled water) MBS. Images of the swelling oocytes were taken using a CCD camera. The oocyte volume was calculated on the bases of the cross-sectional area of a single cell using Image J 1.46e (http://rsbweb.nih.gov/ij/). The osmotic water permeability coefficient ($Pf \times 10^{-4}$ cm/s) was calculated by using the following equation, where the oocyte surface area (S=0.045 cm^2), the initial

oocyte volume (V_0=9×10^{-4} cm^3), the molecular volume of water (V_w=18 cm^3/mol), the initial rate of the relative volume increase ($d(V/V_0)/dt$, in s^{-1}), and the osmotic gradient (Osm_{in}–Osm_{out}): $Pf=V_0 \times d(V/V_0)/dt/[S \times V_w \times (Osm_{in}-Osm_{out})]$.

Mercury chloride is a known inhibitor of the function of AQPs. Mercury chloride functions by binding cysteine residues within the channel aperture (Jung et al. 1994). To examine the effect of mercury chloride on water permeability, the oocytes were pre-incubated in MBS containing 1 mM mercury chloride for 10 min before the swelling assay. To confirm the reversible effect of the mercury chloride inhibition, the oocytes were rinsed with MBS containing 5 mM 2-mercaptoethanol (a known restoring reagent of the effect of mercury chloride), and subjected to the same swelling assay.

Results and Discussion

A 1202 bp-long cDNA containing 756 bp of an open reading frame was obtained from the blowfly, *P. regina* by RACE (Supplementary Material 1). The deduced protein has 251 amino acid residues, six predicted transmembrane domains, and two asparagine-proline-alanine (NPA) motifs, which are commonly observed in AQPs (Jung et al. 1994; Agre 2006). The protein showed 91 % and 74 % identity to *Haematobia irritans exigua* AQP (accession number, AAA96783) and *Drosophila melanogaster* integral protein (DRIP) (accession number, NP_523697), respectively. Based on the phylogenetic analysis, we classified the protein as belonging to insect DRIP subfamily (Fig. 1a). Thus, it was named PregAQP1 [DRIP] (accession number, AB713909).

RT-PCR was performed to localize *PregAQP1* expression in an adult male. Expression of *actin* as an internal control was detected from all experimental tissues. On basis of the intensity of the amplicon, *PregAQP1* was highly expressed in the maxillary palp and moderately expressed in the antenna (Fig. 1b). The level of *PregAQP1* expression appeared to be identical in a female (data not shown). To further localize PregAQP1 at the cellular level, immunohistochemisty was carried out using anti-(AQP-Bom1 [DRIP]) serum as the primary antibody. Immunoreactive material was detected in accessory cells beneath a longitudinally grooved hair on the maxillary palp (Fig. 1c). PregAQP1 also accumulated in a receptor neuron of a thick-walled sensillum on the antenna (Fig. 1d).

To characterize the potential function of PregAQP1, we injected *Xenopus* oocytes with *in vitro* transcribed cRNA encoding PregAQP1 and measured changes in the volume of the oocytes resulting from swelling. Injection of PregAQP1 cRNA gave an osmotic water permeability of 131.2±12.7×10^{-4} cm/s in low osmotic buffer. The oocyte did not show glycerol or urea uptake (data not shown). When the PregAQP1 cRNA-treated oocytes were incubated in buffer containing mercury chloride, a known inhibitor of AQP function, the osmotic water permeability decreased to 56.3±6.1×10^{-4} cm/s. This inhibition was restored by treatment with 2-mercaptoethanol (121.7±8.3×10^{-4} cm/s) (Fig. 1e).

Taken together, our experimental findings indicate that PregAQP1 belongs to the DRIP subfamily of AQPs. PregAQP1 is expressed in the accessory cells in the maxillary palp and the receptor neuron in the antenna, and likely plays a role in maintenance of the water environment. Analysis of genome sequences and morphological studies reveal that many AQPs exist and are expressed in different organs in insects (Campbell et al. 2008). Recently, we demonstrated that two water-specific AQPs, AQP-Bom1 [DRIP] and AQP-Bom3, are responsible for directional water transport in the cryptonephric system of the hindgut and Malpighian tubules in the silkworm, *Bombyx mori* (Azuma et al. 2012). We hypothesize that PregAQP1 [DRIP] and other unidentified AQP(s) maintain water homeostasis in a similar manner in olfactory organs.

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Field Evaluation of Larval Odor and Mixtures of Synthetic Pheromone Components for Attracting Migrating Sea Lampreys in Rivers

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Abstract The sea lamprey, *Petromyzon marinus*, is a harmful invader of the Laurentian Great Lakes. The odor emitted by larval lampreys resident to streams attracts migrating adults to high quality spawning habitats. Three components of the larval pheromone have been identified and tested in laboratory settings: petromyzonol sulfate, petromyzosterol disulfate, and petromyzonamine disulfate. Here, we report the first field test of six mixtures of synthetic versions of these pheromone components, and we compare lamprey responses to these with those elicited by the complete larval odor in a natural stream. Exposure to larval odor both increased upstream movement and attracted migrants into the portion of a channel containing the odor. No tested combination of synthetic pheromone components proved similarly attractive. These findings suggest the existence of unknown additional components of the pheromone that await discovery and are likely necessary if the pheromone is to be useful in management of this pest. Further, we hypothesize that the complete pheromone mixture is necessary to attract migrants into spawning habitat at the conclusion of the migration, whereas a partial pheromone may be effective at the transition from lake to stream when natural factors both dilute and alter the ratio of components from that actually emitted by sea lamprey larvae.

Keywords Sea lamprey · Larval pheromone · Migration · Petromyzonol sulfate · Petromyzonamine disulfate · Petromyzosterol disulfate · Vertebrate invasive pest

Introduction

At the conclusion of a terminal reproductive migration, the sea lamprey, *Petromyzon marinus*, responds to odor released by conspecific larvae by entering streams to spawn. The odor conveys a site-specific history of past reproductive success that guides the selection of a habitat able to support future offspring (Sorensen et al., 2003; Wagner et al., 2006, 2009). The success of this strategy has resulted in the sea lamprey becoming a significant pest in the Laurentian Great Lakes, where it has contributed to substantial changes to the native fish assemblage and the devastation of a valuable fishery (Smith, 1970; Smith and Tibbles, 1980; Madenjian et al., 2008). The sea lamprey's reliance on chemical-social information to locate spawning habitat and attract mates (Li et al., 2002) encouraged integrated pest management practitioners to pursue development of semiochemical-based redistribution and trapping strategies (Twohey et al., 2003). Sorensen et al. (2005) identified three sulfated steroid derivatives in larval odor and, based largely on small-scale laboratory tests of sea lamprey behavior, proposed that they constitute the principal components of a migratory pheromone (Sorensen and Hoye, 2007). These compounds are: 1) petromyzonol sulfate (PZS, Haslewood and Tokes, 1969); 2) petromyzosterol disulfate (PSDS, Hoye et al., 2007); and, 3) petromyzonamine disulfate (PADS, Hoye et al., 2007). Given the potential of these compounds for novel control of a vertebrate pest, it is of great importance to ascertain whether or not they are effective in the field and, if so, determine the most attractive combination.

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Although PZS was both discovered and tested first, it failed to reproduce the full attraction of larval odor, thus suggesting the presence of additional components (Li et al., 1995; Vrieze and Sorensen, 2001; Sorensen et al., 2003). Both PADS and PSDS were identified subsequently, and a mixture of all three components proved as attractive as larval odor in laboratory tests (Sorensen et al., 2005; Fine et al., 2006). The potency of these compounds is remarkable. PADS is released in the highest quantity and detected at the lowest threshold (10^{-13} M). It appears PADS and PSDS synergize the effect of PZS to yield high behavioral reactivity in the presence of river water, and a mixture of all three components may be necessary for efficacy in the field (Fine et al., 2006; Sorensen and Hoye, 2007).

This pheromone mixture has been hypothesized to mediate habitat selection at two points during the reproductive migration. First, as migrants move along the coast and search for a suitable spawning stream, the presence of pheromone may indicate rearing habitat quality and mediate localization of the river mouth (Teeter, 1980; Vrieze and Sorensen, 2001; Vrieze et al., 2010, 2011). Second, after the onset of the riverine migration, the presence of the pheromone may mediate habitat selection by guiding migrants into tributaries where the odor emitted by thriving larvae indicates recent reproductive success (Wagner et al., 2006, 2009). To date, neither hypothesis has been confirmed in the field by using synthetic pheromone components.

The process of pheromone identification requires careful testing in the laboratory, where the questions of whether an organism can detect (via olfactory recording) or respond to (via behavioral assay) a cue may be ascertained (Wyatt, 2003). In nature, animal choices typically integrate information of contrasting valence (opportunities and risks), acquired across multiple sensory modalities, and embedded within the full natural milieu of the principal life activities (foraging, reproduction, etc.). Thus, it is not surprising that many of the semiochemicals that exert strong influence on an organism's behavior in the laboratory have substantially less effect in the field (Magurran et al., 1996; Johnson and Li, 2010). This is particularly true when the deployed odor represents a partial pheromone (Shannon, 1949; Linn et al., 1987; Linn and Roelofs, 1989; Luehring et al., 2011). That migrating sea lampreys rely principally on olfaction to locate and enter rivers is certain (Vrieze et al. 2010). Three field tests (manipulated experiments) of the effect of sea lamprey larval odor on migratory movements have been reported and demonstrate a strong attraction. Application of the odor induced upstream movement in an *in situ* mesocosm (Bjerselius et al., 2000) and drew migrants into small streams (Wagner et al., 2006), or regions of the channel (Wagner et al., 2009), with high efficiency.

Here, we report a field experiment that tested the larval odor and three mixtures of synthetic pheromone components (PADS, PADS + PSDS, PADS + PSDS + PZS) at two concentrations (10^{-12} and 10^{-13} M) for ability to attract migrating sea lampreys into an active portion of a natural stream, per the methods of Wagner et al. (2009). Specifically, we addressed whether the synthetic components emitted at the estimated natural ratio of 10:3:1 (PADS:PSDS:PZS; Fine, 2006) were as attractive as the whole larval odor during the riverine portion of the migration.

Methods and Materials

On 47 nights from May 9–July 5 2007, we quantified the channel selection behavior of sexually immature migratory-phase sea lampreys exposed to three mixtures of synthetic migratory pheromone components (PADS, PADS + PSDS, PADS + PSDS + PZS) at two concentrations (10^{-13} M, 10^{-12} M), as well as to positive (larval odor) and negative (solvent) controls, in a fourth-order Michigan stream, per the approach of Wagner et al. (2009). Because male and female sea lampreys exhibit similar responses to the larval pheromone (Bjerselius et al., 2000; Wagner et al., 2006), we chose to use only females, due largely to their greater availability and use in related published studies.

Study Site The study site was located on the Ocqueoc River, MI , USA, in a lamprey-free reach, above low-head and electric barriers that precluded upstream migration of sea lampreys resident to Lake Huron. The site was a 150-m reach, composed entirely of pool and run habitat that varied from 8–14 m in width, with a maximum depth of 2 m in pools (Fig. 1). We monitored the movements of sea lampreys, equipped with Passive Integrated Transponder tags (PIT; 23 mm half-duplex tag, Oregon RFID, Portland, OR , USA) with pairs of PIT antennae placed at three locations in the stream: 1) one pair 20 m upstream of the release point, to determine the number of sea lampreys actively swimming upstream during each trial (release area, RA in Fig. 1); and 2) one pair each across the left and right halves of the stream, and on either side of a net barrier (test area, TA in Fig. 1), immediately below the pheromone release points, to quantify the number of sea lamprey entering the control and stimulus sides. Paired antennae were necessary to ensure detection and to identify the direction of movement (up- or down-stream). Control and stimulus odors were introduced into the stream, approximately 7 m upstream of the antennae in the test area, by laboratory-grade peristaltic pumps. The distance was determined with a series of fluorescein dye tests that determined the point upstream that the odor plumes intersected, approximately 1 m downstream of the mid-channel net divider.

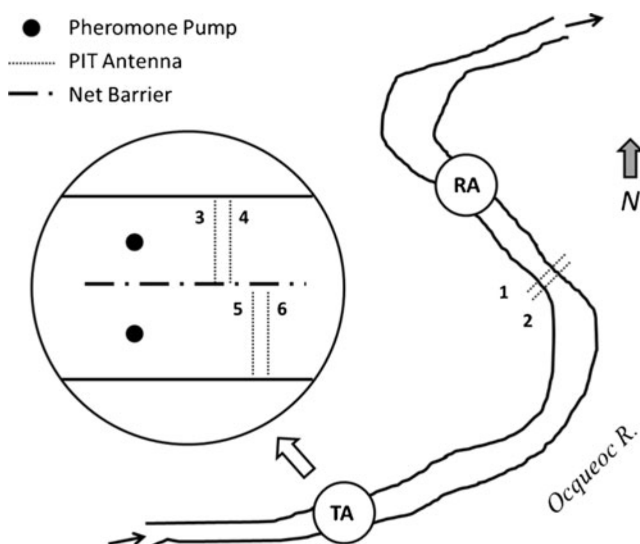

Fig. 1 A schematic of the Ocqueoc River field site. Sea lampreys were held in cages until released in the Release Area (RA). Upstream swimming was recorded with a series of Passive Integrated Transponder (PIT) antennae as the sea lampreys approached and passed the point of pheromone emission in the Test Area (TA)

Experimental Subjects Adult female, migratory-phase sea lampreys used in the study were captured live from several tributaries to Lakes Huron and Michigan in standard assessment traps operated by the U.S. Fish and Wildlife Service and the Department of Fisheries and Oceans-Canada. After capture, sea lampreys were transported and held at the Hammond Bay Biological Station near Millersburg, MI, USA [full transport and holding procedures reported in Wagner et al. (2009)]. Upon arrival at the station, sea lampreys were held in flow-through tanks receiving fresh Lake Huron water, at a rate sufficient to achieve 100 % exchange every 30 min. Water temperatures in the holding tanks naturally ranged from 5–18 °C across the season. Prior to use, each sea lamprey was measured (total length), weighed (wet weight), and implanted with a 23 mm PIT tag, placed intraperitoneally along the ventral midline in accordance with approved procedures (Michigan State University Institutional Animal Care and Use Committee, Permit # AUF/04-07-033-00). Subjects were monitored for 24 h post-surgery to ensure tag retention and general health. Only sea lampreys in excellent condition were tagged, and each sea lamprey was used once in a single trial.

Test Articles Test articles included: 1) larval odor extracted from the effluent of captive larvae held in flow-through tanks; 2) synthetic PADS, PSDS, and PZS, produced as dry ammonium salt dihydrate by Bridge Organics Co., Vicksburg, MI, USA (Fig. 2):, and3) laboratory-grade methanol, used as a solvent during the larval odor extraction and for dissolution of the synthetic compounds. Field application of the test articles was approved by the Michigan Department of Environmental Quality and the U.S. Environmental Protection Agency through Experimental Use Permit 75437-EUP-1.

Larval odor was collected in 2006 from a captive population of approximately 50,000 larval sea lampreys, held in thirty 500 l flow-through tanks partially filled with sand and receiving continuous water pumped from Lake Huron. Larvae were fed a diet of yeast (Polkinghorne et al. 2001). Larval odor was collected by shunting the effluent from the holding tanks into vertical columns containing 500 g of absorbent resin (Amberlite XAD7HP) and elution with methanol, according to Fine et al. (2006). We estimated the concentration of PADS in the extract by using purified natural compounds and mass spectrometry (Fine et al., 2006). The estimate was performed by Peter Sorensen and Thomas Hoye (University of Minnesota) via sub-contract; additional information about these data, including seasonal patterns in production rates, is reported in Fine (2006). The final extract (estimated PADS concentration of 5.4 $mg.l^{-1}$) was concentrated by rotovaporation, and stored at −80 °C until use (Sorensen and Hoye, 2007; Fine and Sorensen, 2010).

Pheromone components were synthesized in 2007. High pressure liquid chromatography (HPLC) and proton nuclear magnetic resonance (^{1}H NMR) analyses confirmed the structure of the compounds to be identical to those identified by Sorensen et al. (2005). This conclusion was independently verified through additional ^{1}H NMR conducted at the National High Magnetic Field Laboratory (Florida State University) and interpreted by Dr. Thomas R. Hoye (Dept. of Chemistry, University of Minnesota, co-author of the original description in Sorensen et al., 2005) and Dr. Dan Holmes (Dept. of Chemistry, Michigan State University). Finally, the purity was verified by Dr. Xiaodan Xi via UV HPLC, and evaporative light scattering detection HPLC analyses

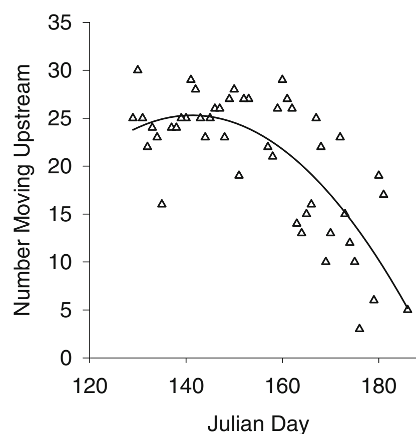

Fig. 2 The number of sea lampreys swimming upstream for each trial arranged by Julian date (all treatments represented) revealed a consistent seasonal pattern of reduced movement over time. The maximum possible value is 30 sea lampreys. The relationship was fit with a quadratic regression (r^2=0.56, P<0.001)

indicating that all batches were >95 % pure (e.g., Kibbey, 1995). All test articles were stored at −80 °C until used.

Effect of Test Articles on Movement To ascertain whether any of the test articles would attract migrating sea lampreys into the portion of a channel containing an odorant(s), we activated one half of the channel with a test article and the other half with a solvent control. We then monitored the upstream movements of responding sea lampreys. We tested a total of nine treatments: a) PADS at 10^{-12} M or 10^{-13} M vs. a methanol control, b) PADS + PSDS at 10^{-12} M or 10^{-13} M vs. a methanol control, c) PADS + PSDS + PZS at 10^{-12} M or 10^{-13} M vs. a methanol control, d) larval extract at 10^{-12} M or 10^{-13} M vs. a methanol control (N=5); and, e) a negative control, methanol vs. methanol, to evaluate any bias for one side of the channel, not related to the test articles.

Twelve hours prior to the start of a trial, we placed a group of 30 PIT-tagged female sea lampreys into either of two holding cages (15 lampreys each), located in the center of the stream 150 m downstream of the test area (Fig. 1). Two hours prior to sunset, we applied two test articles, separately, approximately 7 m upstream of the downstream end of the mid-channel net barrier (Fig. 1). Sea lampreys are nocturnal migrants; thus, we released them at sunset by removing the upstream gate on the holding cages and allowing them to exit at will. We monitored their movements (PIT antenna crossings) for 3 h. Prior to each trial, we tested the PIT reader stations and antennae with a dummy tag to ensure correct operation.

Approximately 4 h prior to test article application, we measured stream discharge ($m^3.sec^{-1}$) at the upstream end of the site by using the USGS midsection method, described by Gore (1996), with a Doppler flow meter (Flo-Mate Model 2000, Marsh-McBirney). We used the discharge estimate to calculate the amount of test article necessary to reach the desired concentration for a given trial when fully mixed into one half of the discharge. We created stock solutions (10 $mg.ml^{-1}$) of the synthetic components in a 50:50 (w/w) mixture of methanol and distilled water. At the site, the test article was mixed with a volume of river water, collected upstream of the test area, to bring the total volume of the mixture to 9 l. The final mixture was pumped into the river at the mid-point of the half-channel receiving the test article (treatment side, alternated between left and right channels on subsequent trials within a treatment) at a rate of 1.5 $l.h^{-1}$ by a laboratory-grade peristaltic pump (Masterflex 7553-70, Cole-Parmer) powered by a 12-volt battery (the use of battery-operated pumps ensured no interference with the PIT antennae). The opposite side (control) received a mixture of river water and methanol at the same rate. The amount of methanol used was equivalent to the amount in the test article mixture. During negative control trials (methanol vs. methanol), the mixture was formed by adding 5 ml of methanol to 8995 ml of river water.

Hypotheses and Data Analysis We completed 4 or 6 trials of each treatment (see Table 1 for total numbers of trials, sea lampreys released, and sea lampreys responding). We first examined upstream movement tendencies across treatments, defined here as the number of sea lampreys crossing the lower antennae. We hypothesized that increasing sexual maturation (prior to ovulation) affects the migration behavior of sea lampreys, and predicted that the proportion of released sea lampreys that actively migrated would decrease over time. We could not measure maturity (ovarian stage) by non-invasive techniques (Lewis and McMillan, 1965). Consequently, we evaluated changes in upstream movement (number of released sea lampreys that crossed the first set of antennae) over time (Julian date) with a quadratic regression, and examined the residuals for any relationship to water temperature. Julian date and temperature are part of a suite of factors that co-vary with maturation and season, each of which may mediate sea lamprey responses across a 47-day experiment. Thus, we predicted a global pattern (all treatments similarly affected) would be evident.

Because a seasonal pattern was evident (see *Results – Upstream Movement Tendencies*), we extracted the seasonal effect by evaluating the regression residuals, so as to ascertain whether any particular treatment increased or reduced upstream movement tendencies. The residuals were normally distributed (Shapiro-Wilk test, W=0.99, P=0.82). Consequently, we determined whether each odor treatment was related to changes in upstream movement with one-sample t-tests (α=0.05) of the mean untransformed residual value, with an expected mean of 0. A significant positive mean residual would indicate the odor treatment increased upstream movement, and *vice versa*.

To ascertain whether sea lampreys tended to select the side of the channel receiving a pheromone stimulus odor (vs. a methanol control), we pooled the results of trials within each treatment and compared the proportion that entered the stimulus vs. the control side of the stream. We tested preference for the treatment side using two-sample t-tests (α=0.05) on arcsin square-root transformed proportions, comparing each odor treatment to the negative control distribution.

Results

Upstream Movement Tendencies We released a total of 1410 PIT-tagged female sea lampreys across 47 trials, of which 70 % (985) moved upstream into the test area. Upstream movement tendency exhibited the expected seasonal attenuation beginning near Julian day 160 (9 June 2007, Fig. 2). A quadratic regression was fit to the movement data ($y=-0.01x^2+2.85x-175.8$, r^2=0.56, P<0.001) and was used to extract the seasonal pattern. The residuals were normally distributed (Shapiro-Wilk test,

Table 1 Female channel preferences and response rates to natural larval odor and synthetic components of larval odor

Treatment		*N*		Channel preference				Respondent rate	
Stimulus	Trials	Released	Responded	Stimulus	Control	*T*-Stat (DF)	*P* value	*T*-Stat (DF)	*P* value
Methanol Control	6	180	133	48.12	51.88	–	–	–	–
Larval Extract 1.0×10^{-12} M	5	150	117	70.94	29.06	2.583 (9)	0.015*	2.841(9)	<0.01*
Larval Extract 1.0×10^{-13} M	6	180	148	63.51	36.49	2.000 (10)	0.037*	1.070(10)	0.155
PADS 1.0×10^{-12} M	4	120	45	48.89	51.11	−0.105 (8)	0.459	−1.627(8)	0.315
PADS 1.0×10^{-13} M	6	180	144	53.47	46.53	0.447 (10)	0.332	0.946(10)	0.183
PADS + PSDS 1.3×10^{-12} M	4	120	50	52.00	48.00	−0.207 (8)	0.421	−0.916(8)	0.193
PADS + PSDS 1.3×10^{-13} M	6	180	138	62.32	37.68	0.863 (10)	0.204	0.444(10)	0.333
PADS + PSDS + PZS 1.4×10^{-12} M	4	120	65	49.23	50.77	0.111 (8)	0.457	0.424(8)	0.341
PADS + PSDS + PZS 1.4×10^{-13} M	6	180	145	49.65	50.35	−0.101 (10)	0.461	0.801(10)	0.221

The channel preference of females towards 8 treatments was evaluated independently for each treatment with a one-way two sample *t*-test, testing whether each treatment was significantly attractive when compared to the methanol control. The upstream response rate was affected by date but not temperature. The untransformed residual upstream movement (respondent rate) was calculated by subtracting the upstream movement for each trial above what was predicted via nonlinear regression after accounting for the date effect. Treatments were tested against the methanol control using a one-way two sample t-test, independently testing whether any treatments increased the response rate. Significant results (α=0.05) are marked with an asterisk. PZS = petromyzonol sulfate, PSDS = petromyzosterol disulfate, PADS = petromyzonamine disulfate

W=0.99, P=0.82) and unrelated to water temperature on the trial dates (Pearson correlation, r=−0.09, P=0.57). Only whole larval odor with a 10^{-12} M estimated concentration of PADS increased upstream movements ($t_{1,\ 4}$=5.29, P=0.006) with, on average, six additional sea lampreys venturing upstream per trial when exposed to the mixture (Fig. 3). No mixture of synthetic pheromone components increased upstream movement.

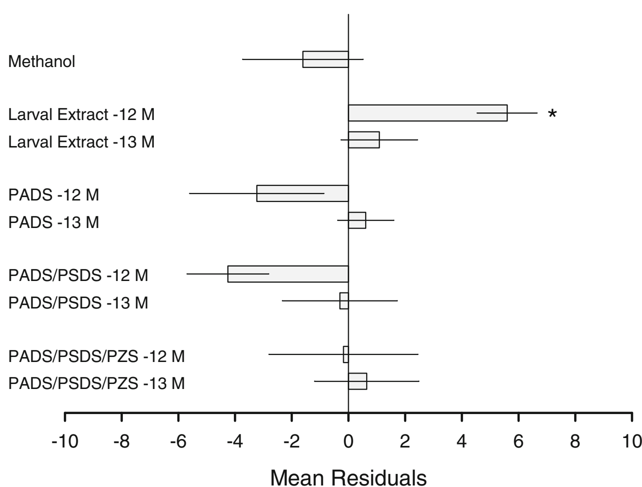

Fig. 3 Mean (± 1 SE) residual upstream movement by treatment after extraction of the seasonal pattern (quadratic regression reported in Fig. 2). A significant positive value indicates greater upstream movement than predicted by time of year was induced by addition of the treatment odor. PZS = petromyzonol sulfate, PSDS = petromyzosterol disulfate, PADS = petromyzonamine disulfate

Odor Preference Upstream migrating sea lampreys did not exhibit a preference for swimming on either side of the channel during the solvent control trials [$t_{1,5}$=0.15, P=0.89; 48 % (64) moved along the left-side, 52 % (69) moved along the right side] (Table 1). Sea lampreys were attracted to the stimulus side of the channel when exposed to larval extract with effective PADS concentrations of 10^{-12} (P=0.015) or 10^{-13} (P=0.037), whereas no combination or concentration of the synthetic compounds influenced channel preference.

Discussion

Sea lamprey responses to conspecific odors in the field were consistent with previously reported findings, only in that migrants were more likely to move upstream and were attracted into the portion of the channel activated with complete odor collected from captive larvae (Bjerselius et al., 2000; Wagner et al., 2006, 2009), confirming that sea lamprey larvae emit a strong attractant. However, our results show that three combinations, at two concentrations, of synthetic pheromone components, representing the ratios and quantities presumed present in streams with larvae, failed to attract migrants toward the release point in the field. The conditions of the field test were unlikely to cause an atypical response: the number and proportion of sea lampreys released and responding, respectively, were consistent with previous tests at this field site (Wagner et al., 2009), as was the positive response to whole larval odor observed during the experiment. Neither can the lack of

response to the synthetic pheromone be attributed to an insufficient stimulus, based on the evaluation of the synthetic compounds reported above.

Previous laboratory tests showed that sea lampreys responded to the identified pheromone components. However, the laboratory flume used to establish the activity of these pheromone components was designed to simulate, on a substantially smaller scale, the set of odors a migrating sea lamprey would encounter as it transitions from lake to stream (Vrieze and Sorensen, 2001; Fine et al., 2004; Fine and Sorensen, 2008). That mixture was predominantly lake water, with a small amount of river water (to simulate exposure to the river plume), with subsequent addition of purified compounds (80 % pure; Sorensen et al., 2005) or synthetic PADS (Fine and Sorensen, 2008). Thus, the findings from those tests are properly interpreted in light of what a migrant is likely to encounter in a river plume. A number of environmental and biological factors may substantially alter (vs. the 'true' odor emitted by larvae in streams) the nature of the odor encountered in the plume, presenting migrants with a partial odor that varies substantially in character over space and time. First, sea lampreys migrate in early spring, a time of year typified by frequent spring flooding and cold temperatures (Quinn, 1978). Temperature mediates larval feeding and metabolism (Sutton and Bowen, 1994), and colder temperatures should reduce the amount of pheromone produced. Compounding this effect, high mean flows and flooding will further reduce the intensity of the signal via dilution. Electrophysiological testing suggests that the compound consistently produced in high quantity, PADS, is also detected at the lowest threshold (Fine and Sorensen, 2008): these are attributes of a long-distance attractant, which may explain the strong laboratory response to PADS alone (Sorensen et al., 2005). Further, the release of PSDS appears directly tied to feeding, increasing in the surrounding waters immediately after a larva has fed (Fine and Sorensen, 2010), and likely varying with river water temperature and time of day. Finally, at least three additional species of lamprey native to the Great Lakes basin also produce components of the migratory pheromone, although the ratios of components in their emissions is unknown (Fine et al., 2004). If those ratios differed, the resulting odor would not represent that of the sea lamprey.

Under what conditions is a partial pheromone likely to invoke a response? The information contained in an olfactory signal is available to a receiver only when the form of the signal and the apparatus of the receiver are sufficiently attuned to overcome background noise (Endler, 1992). This suggests that receivers may discriminate against partial pheromones or mixtures that are off ratio, compared to the preferred blend. However, the turbulent dispersion of pheromone components in fluid environments can alter substantially both the character of a perceived odor (number of components received and their ratio) and its concentration, with increasing distance from the emitter (Cardé, 2005; Weissburg, 2011). Thus, the active space of a multi-component pheromone is likely inversely related to the strength of a receiver's allegiance to the preferred blend. It is, therefore, unsurprising that some receiver movement strategies are typified by attraction to distant and incomplete odors when not presented with the rare circumstance of a simultaneous choice between preferred and off-ratio blends (Evenden et al., 1999; Allison and Cardé, 2008; Trimble and Marshall, 2008; Luehring et al., 2011).

Because an animal's response to a pheromone is mediated both by factors that regulate perception of the signal and the context in which it was received (Tremaine et al., 2005; Atkinson and Joy, 2008), it seems reasonable that in the context of the sea lamprey's reproductive migration a partial odor would prove more active in river plumes than in upper-watershed spawning streams. As land-locked sea lampreys migrate, they undergo a predictable sequence of behaviors: 1) detachment from prey and searching for rivers in late winter or early spring (the lacustrine migration); 2) movement directed upstream into spawning reaches as waters warm (the riverine migration); and 3) stopping and spawning in suitable habitat, typically upstream of larval beds. As they move from lake to river, they should also encounter a predictable sequence of odors that mediate habitat selection at each of these phases by providing increasingly specific information (Fig. 4).

Fig. 4 The hypothetical relationship between distance from the spawning grounds, habitat type, and likely odors encountered by a migrating sea lamprey in the Great Lakes. The sequence anticipates the existence of a strategy whereby a migrant receives increasingly precise information about the location of larval habitat and mates as the migration progresses

Migrating sea lampreys are first attracted to river water (Vrieze and Sorensen, 2001). This signal likely indicates proximity to a river plume and triggers a set of search behaviors associated with the localization of a diffuse odor source (Vrieze et al., 2011). Whether or not the sea lamprey chooses to enter is thought to be mediated by the presence of larval odor (Teeter, 1980; Bjerselius et al., 2000), a subset of pheromone components (Sorensen et al., 2005; Fine and Sorensen, 2008), or the odor of native lampreys that have similar habitat requirements and also produce attractive odors (Fine et al., 2004). Finally, while migrating upstream, the complete odor produced by larval sea lampreys attracts migrants into tributaries with habitat that is currently supporting past generations and likely to support future ones as well (Wagner et al., 2006, 2009). The final localization of a spawning nest and acquisition of mates is mediated by a male-emitted sex pheromone (Li et al., 2002; Johnson et al., 2009). Thus, an incomplete or incorrect combination of larval pheromone components may prove attractive when present in river plumes, allowing migrants to choose a proper watershed, but may not be sufficient to induce entry into a particular spawning tributary.

In summary, we were unable to attract migratory-phase sea lampreys toward combinations of synthetic pheromone components in the field. Our results suggest the existence of other, unknown components of the migratory pheromone that complete the odor and mediate habitat selection immediately prior to spawning, but not necessarily at the transition from lake to stream. Alternatively, sea lampreys may exhibit a strong preference for a species-specific blend of the known components contained in the collected larval odor, but not represented precisely enough in our ratios of synthetic components. The notion of a single ratio seems unlikely given previous laboratory results and the likely large variations in component ratios, both as produced and perceived, that typify the migratory season. The synthetic odors, as tested here, are unlikely to prove efficacious in integrated pest management strategies that attempt to redirect sea lampreys after they have entered a watershed and are searching for a spawning site. Whether these chemicals will alter the animals' behavior at the transition from lake to stream, implied by previous laboratory results, needs to be tested by additional field tests.

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