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RAPID COMMUNICATION

p-Cresol: A Sex Pheromone Component Identified from the Estrous Urine of Mares

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Abstract Previously it was shown that *m*- and *p*-cresols in the urine of mares exhibits a temporally reproducible pattern that is dependent on ovarian activity and, thus, provides information about the timing of ovulation. New behavioral data demonstrate 1) that stallions spend significantly more time sniffing *p*-cresol as compared to *o*-, and *m*-cresols, and, 2) that the extent of stallions' erections differ significantly in response to different types of samples. The lowest erection level was recorded for the pure-water control, a moderate erection level was elicited by the urine of diestrous mares, and the highest erection level was elicited by urine of a diestrous mare containing synthetic *p*-cresol at a quantity equivalent to half of the amount of *p*-cresol found in the urine of estrous mares. Consequently, *p*-cresol is at least one of the components of a horse sex pheromone.

Keywords *Equs cabalus* · Horse · Estrus · Behavior · Erection

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Introduction

Like many other mammalian species, horses (*Equus caballus* L.) use olfactory communication to coordinate their reproductive and social activities (Kiley-Worthington, 1987). Behavioral observations indicate that the odor of a mare in heat is perceptible to a stallion from over half a kilometer (Ainslie and Ledbetter, 1980), implying that a sex pheromone is involved. Smearing of an estrous mare's urine onto castrated males, or non-estrous females, elicited mounting responses and copulation attempts by a stallion that previously had no interest in the untreated individuals (Wierzbowski and Hafez, 1961). This observation suggests that urine collected from a mare during the estrous stage contains a chemical signal or signals for stallions. It was found that two compounds, *m*- and *p*-cresol, occurred at significantly higher amounts in estrous urine samples prepared when the mares were in heat, as compared to those collected during diestrous periods. In addition, the amounts of these cresol was positively correlated with the ovulation time, and reached maximum a day before the breaking of the follicle (Mozūraitis et al., 2012).

The aim of the present study was to determine the behavioral function of cresols in horses. We present data herein demonstrating that an increase of *p*-cresol in the estrous urine of a mare stimulates sex behavior in stallions.

Methods and Materials

Study Subjects and Urine Collection Five mares were observed for two natural estrous cycles. Urine samples were

collected daily from each individual during the estrous periods when a mare was receptive to a stallion, and once during the diestrous period when a mare was clearly non-receptive. An ultrasound technique (5 MHz scanner 480 VET; Pie Medical, Maastricht, Netherlands) was used to determine the exact time of ovulation. After collection, urine was poured into glass jars, and samples were placed in a freezer at −20°C. The samples selected for analysis were placed on dry ice in a thermostat box, and transported to the laboratory for analysis. The work was carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

Determination of Absolute Amounts of m- and p-Cresols The absolute amounts of *m*- and *p*-cresols at diestrous and estrous (1 day before ovulation) periods were determined by three subsequent solvent extractions of 1 ml of the thawed urine sample. Two ml of pentane (HPLC grade, Carlo Erba Reagents, Val de Reuil, France) were used in each extraction step. After concentration to 0.5 ml, five µg of pentadecane (Fluka, Buchs, Switzerland) were added to the urine sample as an internal standard.

Analyses were performed using a Varian 3,400 gas chromatograph (GC) coupled with a Finnigan SSQ 7,000 mass spectrometer (Termo-Finnigan, San Jose, CA, USA). A DB-Wax capillary column (30 m, i.d. 0.25 mm, film thickness 0.25 µm; J&W Scientific, Folsom, CA, USA) was used with a temperature program from 40°C (2 min) to 230°C at 6°C/min, with a 27-min final hold. The injector temperature was 225°C splitless for 30 sec, helium was the carrier gas with an inlet pressure of 70 kPa, and electron ionization mass spectra were collected at 70 eV with the ion source at 150°C. The *m*- and *p*-cresols were identified by comparison of their mass spectral data, and GC-retention times with those of authentic synthetic standards of *o*-, *m*-, and *p*-cresols (>99 % purity, Fluka, Buchs, Switzerland).

Behavioral Tests Behavioral tests were conducted at the Vilnius stud-farm, Riešė, Vilnius district, Lithuania. Response of stallions to *o*-, *m*-, and *p*-cresols were tested using two-choice tests presenting *o*-cresol vs. *m*- cresol, *o*-cresol vs. *p*-cresol, and *m*-cresol vs. *p*-cresol. The two stimuli were presented simultaneously for 1 min, and the time spent by each stallion sniffing the samples was recorded. Each cresol was dissolved in hexane (0.1 µg/µl; HPLC grade, Carlo Erba Reagents, Val de Reuil, France), and 10 µl of solution were transferred onto 2 g of sterilized cotton. After evaporation of hexane, the cotton was humidified with 2.5 ml of distilled water. The experiments included control cotton wads treated with 2.5 ml of water.

In the second behavioral experiment, the level of erection of the stallion was estimated during a 30 min period when the stallion was exposed to a cotton wad treated with 2.5 ml of 1) distilled water, 2) diestrous urine, or 3) 0.1 mg of *p*-cresol added to 2.5 ml diestrous urine. The level of erection was estimated by grading the responses into four categories: the lowest category of 0 points was defined as when the penis was not protruded; next two categories of 1 and 2 points were assigned when the penis was protruded 5–10, and 10–20 cm, respectively; and the highest level of 3 points was assigned when the penis protruded more than 20 cm and became rigid. The highest level of erection observed for each stallion during the test period was taken as a final response, and the number of points was assigned accordingly. The sum of points was then calculated for each stimulus.

Statistical Analysis The data from the response of stallions to *o*-, *m*-, and *p*-cresols tests were analyzed with the mixed-model repeated measures ANOVA, followed by *post-hoc* HSD Tukey test for unequal number of replicates using the computer program package Statistica, version 10. The data regarding stallions' extent of erection in response to different sample types were subjected to nonparametric Friedman's ANOVA followed by Quade test (Conover, 1999) using the computer program package StatXact, version 9.

Results and Discussion

Extraction of cresols by pentane from the urine samples revealed that *p*-cresol occurs at 133±35 µg and 11±4 µg in 1 ml of estrous and diestrous urine samples, respectively. Small amounts (1.2±0.8 µg) of *m*-cresol were detected in 1 ml of estrous urine samples as well, while in diestrous urine the amounts of this compound were below quantification limits.

Data from the two-choice tests revealed that the stallions spent significantly more time sniffing *p*-cresol as compared with *m*-, and *o*-cresols. There were no preferences observed between *o*-, and *m*- cresols, or between the two water samples (Table 1).

The extent of stallions' erections differed significantly by sample type over the three trials (Fig. 1). The lowest level of

Table 1 Preference of stimuli by stallions during the two-choice tests

	Time (sec) spent on a stimulus during 60 sec testing period							
	Water / water		*o*-cresol / *m*-cresol		*o*-cresol / *p*-cresol		*m*-cresol / *p*-cresol	
Average	19.6	18.3	13.5	11.3	10.0	20.6	9.7	18.2
SD	7.3	8.6	6.3	6.4	5.2	11.3	5.6	9.3
P	0.99		0.97		< 0.001*		0.007*	
n	12		18		17		15	

SD represents standard deviation; when $P<0.05$ the difference between the two means is statistically significant and is marked by asterisk, the mixed-model repeated measures ANOVA $F_{12,26}=56.10$, $P<0.001$ followed by post-hoc HSD Tukey test for unequal number of replicates; n represents the number of replicates

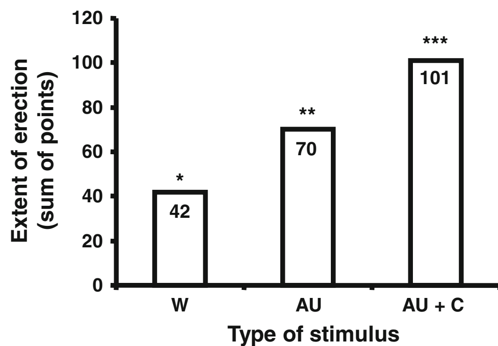

Fig. 1 Extent of stallions' erections evoked by three types of stimuli. W represents water; AU stands for 2.5 ml of diestrous urine; AU + C means 0.1 mg of *p*-cresol added to 2.5 ml of diestrous urine, number in the column indicates extent of stallions' erection expressed as sum of the points; Friedman's ANOVA $P<0.001$, $df=37.24$, columns bearing different number of asterisk marks differ with statistical significance according to nonparametric Quade test (difference between W and AU is significant at $P=0.007$, QT=8.37; between AU and AU + C, $P=0.003$, QT=10.35; and between W and AU + C, $P<0.001$, QT=45.29); number of replicates is 25

response was recorded to pure water; therefore, this was considered as the background level. Exposing the stallions to the diestrous urine of a mare significantly increased their erection level as compared with the background level. Furthermore, addition of synthetic *p*-cresol to the diestrous urine at half of the amount of *p*-cresol found in the estrous urine samples significantly increased the extent of stallions' erections.

The presence of *p*-cresol in the urine and feces samples of both mares and stallions was reported by Kimura, 2001; however, behavioral data to confirm the activity of this compound were not presented. Cresols have germicidal properties (Morris et al., 1979); consequently these compounds could act as antiseptics, protecting the genitals of both sexes during sexually active periods. Antimicrobial activity of *p*-cresol would explain the increase in concentration of cresols in the urine of both sexes during sexually active periods.

In summary, our results show that the amounts of *p*-cresol in the estrous urine of mares just before ovulation is ten times higher as compared with those for diestrous urine, and that the addition of this compound to diestrous urine significantly stimulates stallions' erections. Thus, *p*-cresol is at least one of the components of a horse sex pheromone.

Pheromonal activity of *p*-cresol has been reported in a number of insect species (El-Sayed, 2012). Our data provide one more linkage to a few known and intriguing similarities between insect and mammal pheromones (Goodwin et al., 2006).

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The Male-produced Sex Pheromone of the True Bug, *Phthia picta*, is an Unusual Hydrocarbon

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Abstract *Phthia picta* is part of a complex of true bugs (Heteroptera) in Brazil that attack tomatoes, being particularly damaging because nymphs and adults feed on both leaves and fruit. Gas chromatography (GC) of aeration extracts of adult males vs. females revealed the presence of a male-specific compound. GC-electroantennographic detector experiments indicated that the antennae of females are highly sensitive to this male-specific compound. GC-mass spectrometry and GC-FTIR analyses suggested a methyl branched hydrocarbon structure for this compound. After synthesis of three different proposed structures, the natural product was indentified as 5,9,17-trimethylhenicosane, which was strongly attractive to females in Y-tube olfactometer bioassays. Analysis of dissected body parts of adults revealed that the pheromone is produced in the lateral accessory glands of the metathoracic scent gland of males only.

Keywords *Phthia picta* · Coreidae · 5,9,17-Trimethylhenicosane · Metathoracic scent gland · Heteroptera

Introduction

Tomatoes (*Lycopersicon esculentum* Mill) are an important food commodity in Brazil and elsewhere for which quality is fundamental. *Phthia picta* (Heteroptera: Coreidae) is a particularly damaging tomato pest because nymphs and adults feed on both leaves and fruit. The piercing-sucking mode of feeding exhibited by true bugs directly damages fruit, and opens fruit to attack by other insects, fungi, and microorganisms, hastening deterioration and causing great economic losses (Silva and Carvalho, 2001; Silva et al., 2001).

Phthia picta occurs in California, Texas, Florida, Mexico, the Caribbean, and South America, including most regions of Brazil. The bug feeds on wild solanaceous and cucurbit host plants, and cultivated tomatoes are the preferred crop host. Nymphs and adults were described by Serantes (1973), and Silva et al. (2001) described some morphologic characteristics of the egg, the five nymphal instars, and adults. *Phthia picta* adults are elongated (males: 14.2 mm, and females: 15.5 mm long) black insects with yellow or orange pronotal margins and a traverse stripe.

In this work, we identified and synthesized the male-produced sex pheromone of *P. picta*, and tested its attractiveness to adults in an olfactometer. In addition, we determined the site of pheromone accumulation. Identification of this pheromone may eventually be useful for integrated pest management in tomatoes, one of the crops most heavily treated with pesticides in the country, and the discovery reveals a new and novel type of pheromone molecule for the Heteroptera.

Methods and Materials

Insects Phthia picta adults and nymphs were collected in commercial plantations of tomato in Uraí, Paraná, Brazil. Insects were sexed and maintained separately under controlled conditions (25 °C, 70 % relative humidity and a

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14:10 hL:D), and fed exclusively on tomatoes (Silva and Carvalho, 2001).

Collection of Volatiles Groups of 8males and females, separated by sex, were placed in glass aeration chambers (35 cm high × 3.5 cm outside diam). Emitted volatiles were trapped on 0.8 g of Super Q (Alltech, Deerfield, IL, USA) columns daily for 24 h over 20 consecutive days as previously reported (Zarbin et al., 2003a). A humidified and charcoal filtered air flow (1 $L.min^{-1}$) was maintained through the apparatus. Volatiles were eluted from Super Q with 600 µl of double-distilled hexane, and adsorbent traps were changed after each 10 collections. The daily extracts were not combined, and each was concentrated to 100 µl under an argon stream prior to analyses (Zarbin et al., 1999; Fonseca et al., 2010).

Extracts were analyzed by gas chromatography–mass spectrometry (GC–MS) using a Shimadzu GC (model 2010) coupled to a Shimadzu QP 2010 Ultra MS operated in the electron impact ionization mode (70 eV). The GC was operated in the splitless mode, and was equipped with a DB-5 (0.25, 0.25 mm × 30 m, J&W Scientific, Folsom, CA, USA) capillary column. The column oven was maintained at 50 °C for 3 min, increased to 250 °C at 7 °C/min, and then kept at this temperature for 5 min.

Kovats indices (Kovats, 1965) of analytes were calculated with reference to *n*-alkanes standards for determination of relative retention indexes.

Gas Chromatography-Infrared Spectroscopy (GC-FTIR) Infrared spectra were recorded using a DiscovIR-GC Spectra Analysis GC coupled to a Shimadzu GC (model 2010). The GC was operated in the splitless mode, and was equipped with a DB-5 capillary column and conditions as above, with helium as carrier gas. A liquid nitrogen-cooled photoconductive mercury-cadmium-telluride (MCT) detector was used with FT-IR resolution of 8 cm^{-1}.

GC-Electroantennogram Recordings (GC-EAD) A Shimadzu 2010 GC was coupled to a Syntech EC-03-300 model electroantenographic detector system (Hilversum, The Netherlands). GC-EAD experiments were performed with antennae from males and females (Cortés et al., 2010). A GC equipped with an RTX-5 column (0.25 × 0.25 mm × 30 m; J&W Scientific) with a splitless injector was used in these analyses. The oven was programmed as above, except starting at 100 °C. The EAD branch passed through a heated conduit (250 °C) with an humidified air flow (300 $ml.min^{-1}$) directed over the insect antennal preparation. The antenna was fixed between two stainless steel electrodes (Syntech probe) using conductive gel (Signa gel, Parker Labs, NJ, USA). Signals were registered and analyzed with Syntech GC-EAD32 software (version 4.6).

Extraction of Metathoracic Gland Secretion An adult *P. picta* was pinned dorsal-side-up through the prothorax in a Petri dish, and submerged in tap water. The dissection process (using small surgical scissors and sharpened forceps) consisted of removing the wings, cutting the lateral margins of the abdomen anteriorly up to the metathorax, and transversely cutting the anterior margin of the scutellum. The tergal cuticle was pulled back, and the viscera were removed. The scent gland complex, located in the ventral metathoracic region, could then be reached and removed by cutting laterally through the meso- and metathorax, turning the preparation over, and cutting transversely between the meta- and prothorax. The gland reservoir and the lateral accessory glands (Aldrich et al., 1978; Favaro et al., 2011) were removed separately, dried with tissue paper, immersed in 20 µl of analytical grade hexane, and each extract was stored at−20 °C until analysis. Three metathoracic scent glands (MSG) extracts were prepared for each sex.

Olfactometer Bioassay Responses of *P. picta* to volatiles from each sex were tested in a binary choice Y-tube olfactometer using humidified, charcoal filtered air at a rate of 4 L/min (Zarbin et al., 2007). Hydrochloric acid and ammonium hydroxide were mixed to visualize the plume distribution inside the system (Boyer et al., 1997). The olfactometer consisted of a Y-shaped glass tube 4 cm diam with a 40-cm long main tube and two 20-cm long arms. Odor sources consisted of 2×2 cm pieces of filter paper loaded with 20 µl of a 1000-ppm hexane solution of the standard synthetic molecule or hexane (control), placed in the base of the olfactometer arms. One male or female was introduced into the base of the main tube of the olfactometer, and behavior was observed for 20 min. Insects that walked upwind and made direct contact with the filter paper containing the odor source within 20 min were recorded as a positive response. An insect that did not walk upwind to either odor source within 20 min was recorded as no response. Insects that did not make a choice were excluded from statistical analysis. We tested 50 individuals, and each individual was tested once only, representing one replicate experiment. The Y-tube was cleaned with alcohol after 4 insects were tested, left to dry for 5 min, and the positions of the olfactometer arms were inverted between odor sources to avoid any positional bias. Statistics for the bioassays was performed with binomial test in BioEstat 3.0 software.

Synthesis High-grade reagents and solvents were used in the syntheses. Crude products were purified by flash chromatography on silica gel (230–400 mesh). ^{1}H- and ^{13}C-NMR spectra were recorded using a Bruker ARX-200 spectrometer (200 and 50 MHz, respectively) in a $CDCl_3$ solution. Chemical shifts are expressed in ppm relative to $CDCl_3$ (7.27 and 77.23 ppm for ^{1}H and ^{13}C NMR, respectively).

The infrared (IR) spectra were measured as films using a Bomem B100 spectrometer. GC-MS data for synthetic intermediates were obtained as described above.

Syntheses of 5,9,12,16-Tetramethyleicosane (7)

2-Cyclopropylhexan-2-ol **(2)** A solution of the Grignard reagent was prepared by slow, continuous addition of 1-bromobutane (10 mmol, 1.1 ml) to a stirred suspension in dry diethyl ether (5 ml) of magnesium turnings (11 mmol, 0.266 g) previously activated with iodine. To this a solution of cyclopropylmethylketone **1** (12 mmol, 1.2 ml) in dry diethyl ether (5 ml) was added gradually, and the mixture was stirred overnight at room temperature. The reaction was quenched by the addition of ice and saturated NH_4Cl solution. The ether layer was separated, and the aqueous layer was extracted with diethyl ether. The combined ether solutions were washed with brine, dried over anhydrous Na_2SO_4, and concentrated by rotary evaporation under reduced pressure. The crude product was purified by column chromatography on silica gel (hexane – ethyl acetate 8:2) yielding 1.36 g (95 %). IR υ_{max}, cm^{-1}: 3460, 2932, 1465, 1390, 1114, 1014. ^1H NMR (200 MHz, $CDCl_3$): δ 0.35 (m, 4H), 0.92 (m, 3H), 1.09 (s, 3H), 1.36 (m, 4H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 0.05, 0.32, 13.80, 20.73, 23.00, 25.33, 25.87, 42.72, 70.96. MS: *m/z* (%): 127 (5), 114 (5), 109 (2), 101 (6), 95 (2), 91 (1), 85 (100), 81 (2), 77 (1), 71 (15), 67 (10), 58 (5), 57 (10), 43 (100), 41 (10).

1-Bromo-4-methyloct-3-ene **(3)** In a portion, 48 % hydrobromic acid (4 ml) was added to stirred and ice-cooled **2** (5.8 mmol, 1 g), and the mixture was stirred for 15 min at 0 °C. Distilled water was added, the organic layer was separated, and the aqueous layer was extracted with hexane. The combined organic solutions were washed with saturated $NaHCO_3$ solution and brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The crude product was purified by column chromatography on silica gel (hexane) yielding 1.4 g (83 %). IR υ_{max}, cm^{-1}: 2920, 2859, 1663, 1451, 1238, 840, 642. ^1H NMR (200 MHz, $CDCl_3$): δ 0.90 (t, 3H), 1.34 (m, 4H), 1.62 (s, 3H), 1.99 (t, 2H), 2.57 (q, 2H), 3.34 (t, 2H), 5.12 (ddq, 1H, *J* 1.2, 7.0, 7.6). ^{13}C NMR (50 MHz, $CDCl_3$): δ 13.97, 16.21, 22.37, 30.06, 31.66, 32.94, 39.29, 120.69, 121.18, 139.09. MS: *m/z* (%): 204 (10), 162 (35), 149 (1), 135 (1), 125 (1), 121 (1), 111 (1), 95 (15), 83 (70), 79 (20), 69 (100), 55 (100), 51 (5), 41 (65).

5,9,12,16-Tetramethylicosa-5,15-diene-9,12-diol **(5)** As described above, compound **3** (20 mmol, 4.1 g) was converted into **5** (2.93 g, 80 % yield) using magnesium turnings (24 mmol, 0.58 g). To this, a solution of hexane-2,5-dione **(4)** (5 mmol, 0.58 g) in dry diethyl ether (2 ml) was gradually added . IR υ_{max}, cm^{-1}: 3270, 3054, 2963, 2926, 2855, 1671, 1451, 1380, 1085. ^1H NMR (200 MHz, $CDCl_3$): δ 0.89 (m, 6H), 1.19 (s, 6H), 1.32 (m, 8H), 1.52 (m, 8H), 1.64 (m, 6H), 2.00 (m, 8H), 5.13 (ddq, 2H, *J* 1.2, 7.0, 7.6). ^{13}C NMR (50 MHz, $CDCl_3$): δ 13.99, 15.79, 22.68, 26.90, 30.24, 35.59, 39.45, 41.87, 72.67, 124.01, 124.74, 135.94. MS: *m/z* (%): 346 (10), 331 (10), 281 (30), 253 (10), 223 (20), 207 (60), 164 (100), 135 (15), 123 (20), 107 (75), 95 (40), 69 (25), 57 (5), 44 (100).

5,9,12,16-Tetramethyleicosane-9,12-diol **(6)** Compound **5** (2.72 mmol, 1.0 g) in hexane (10 mL) was hydrogenated over palladium on charcoal (10 % Pd, 5.0 mg) at room temperature and 25 psi in a Parr apparatus for 3.0 h. The mixture was filtered, and the filtrate was evaporated at reduced pressure to afford 0.9 g (2.45 mmol) of **6**, in 90 % yield. IR υ_{max}, cm^{-1}: 3276, 2963, 2926, 2861, 1460, 1386. ^1H NMR (200 MHz, $CDCl_3$): δ 0.9 (m, 12H), 1.12 (m, 3H), 1.20 (s, 6H), 1.30 (m, 15H), 1.43 (m, 4H), 1.55(s, 14H), 1.88 (m, 3 H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 14.14, 19.63, 21.30, 22.81, 26.78, 29.25, 32.58, 35.36, 36.56, 37.43, 42.36, 72.56. MS: *m/z* (%): 337 (6), 319 (1), 281 (1), 249 (1), 243 (1), 236 (1), 225 (100), 171 (12), 150 (9), 137 (11), 123 (17), 109 (26), 97 (36), 95 (26), 83 (21), 69 (36), 55 (21), 43 (33).

5,9,12,16-Tetramethyleicosane **(7)** Compound **6** (0.88 g, 2.37 mmol) and *p*-toluenesulfonic acid (*p*-TSA) (5 mg) in benzene (10 ml) were heated to reflux for 3.0 h. The mixture was cooled to room temperature, and extracted with distilled water. The organic phase were washed with saturated $NaHCO_3$ solution and brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. This was hydrogenated without purification, as described above, for the synthesis of **6**. The mixture was filtered, and the filtrate was evaporated at reduced pressure. The crude product was purified by column chromatography on silica gel (hexane) yielding 0.2 g (25 %) in two stages. IR υ_{max}, cm^{-1}: 2957, 2928, 2861, 1467, 1374, 726. ^1H NMR (200 MHz, $CDCl_3$): δ 0.86 (m, 18H), 1.07 (m, 4H), 1.26 (m, 28H). ^{13}C NMR (50MHz, $CDCl_3$): δ 14.14, 19.70, 23.05, 24.47, 29.36, 32.75, 33.06, 33.10, 34.35, 34.43, 36.77, 36.85, 37.30, 37.43. MS: *m/z* (%): 338 (1), 323 (1), 309 (1), 295 (1), 281 (2), 253 (2), 239 (1), 225 (2), 211 (13), 183 (9), 169 (9), 155 (26), 141 (19), 127 (24), 113 (32), 99 (47), 85 (91), 71 (90), 57 (100), 43 (47).

Syntheses of 5,9-Dimethyldocosane (11)

5,9-Dimethyldocos-5-en-9-ol **(9)** As described above for the synthesis of **2**, bromide **3** (5.85 mmol, 1.2 g) was converted into **9** (1.24 g, 66 % yield) using magnesium turnings

(6 mmol, 0.145 g). To this, a solution of 2-pentadecanone **(8)** (5.83 mmol, 1.32 g) in dry diethyl ether (3 ml) was gradually added . IR υ_{max}, cm^{-1}: 3287, 3043, 2963, 2932, 2861, 1671, 1460, 1375, 1090. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.91 (m, 6H), 1.19 (s, 3H), 1.28 (s, 26H), 1.48 (m, 4H), 1.63 (s, 2H), 1.69 (m, 1H), 2.03 (m, 4H), 5.15 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 13.97, 14.08, 15.85, 22.33, 22.56, 22.66, 23.38, 23.94, 26.85, 29.33, 29.64, 30.16, 30.24, 31.90, 41.56, 41.86, 41.93, 72.83, 124.07, 124.80, 135.59, 135.85. MS: *m/z* (%): 334 (6), 277 (6), 249 (7), 207 (8), 165 (5), 151 (58), 137 (5), 123 (11), 109 (34), 95 (100), 81 (47), 69 (68), 55 (39), 43 (28).

5,9-Dimethyldocos-9-ol **(10)** Applying the same method to **6**, compound **9** (1.1 g, 3.4 mmol) was converted into **10** (1.07 g, 3.28 mmol, 97 % yield). ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.88 (m, 9H), 1.15 (s, 3H), 1.26 (s, 30H), 1.40 (m, 6H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 14.11, 19.66, 21.00, 21.30, 22.66, 22.99, 23.89, 26.96, 29.33, 29.64, 30.23, 31.90, 32.69, 36.72, 37.62, 41.88, 42.16, 72.82. MS: *m/z* (%): 336 (2), 227 (76), 207 (6), 171 (56), 153 (56), 139 (8), 125 (18), 110 (62), 97 (100), 83 (177), 69 (81), 55 (70), 43 (71).

5,9-Dimethyldocosane **(11)** As described above for the synthesis of **7**, compound **10** (0.535 g, 1.64 mmol) was converted into **11** (0.1616 g, 0.52 mmol, 32 % yield). IR υ_{max}, cm^{-1}: 2958, 2917, 1469, 1378, 728. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.85 (m, 12H), 1.06 (m, 2H), 1.26 (s, 32H), 1.60 (s, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 14.11, 19.70, 19.75, 22.70, 23.05, 24.45, 27.08, 29.35, 30.03, 31.92, 32.74, 36.75, 36.83, 37.06, 37.13, 37.37, 37.42. MS: *m/z* (%): 338 (1), 323 (1), 309 (1), 295 (1), 281 (7), 253 (3), 239 (1), 225 (2), 210 (7), 183 (5), 169 (5), 155 (20), 141 (10), 127 (15), 113 (23), 99 (39), 85 (85), 71 (81), 57 (100), 43 (51).

Syntheses of 5,9,17-Trimethylhenicosane (17)

2-Cyclopropyl-6-methyldec-5-en-2-ol (12) A Grignard reagent solution was prepared by addition of a solution of **3** (2.0 g, 9.75 mmol) in dry diethyl ether (3 ml) to a stirred suspension of magnesium (0.243 g, 10 mmol) in dry diethyl ether (2 ml). To this, a solution of **1** (1.15 ml, 12 mmol) in diethyl ether (5 ml) was gradually added, and the mixture was stirred overnight at room temperature. The reaction was quenched by addition of ice and saturated NH_4Cl solution. The ether layer was separated, and the aqueous layer was extracted with diethyl ether. The combined ether solutions were washed with brine, dried over anhydrous Na_2SO_4, and concentrated by rotary evaporation under reduced pressure. The crude product was purified by column chromatography on silica gel (hexane – ethyl acetate 8:2) yielding 1.36 g (85 %)). IR υ_{max}, cm^{-1}: 3469, 3087, 2917, 1672, 1452, 1372, 1101, 1061, 901, 820. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.35 (m, 4H), 0.9 (m, 4H), 1.11 (s, 3H), 1.13 (s, 1H), 1.31 (m, 4H), 1.57 (m, 2H), 1.61 (s, 2H), 1.96 (q, *J* 6.61Hz, 2H,), 2.12 (q, *J* 8.03Hz, 2H), 5.15 (tq, *J* 7.06 Hz *J* 1.34 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 0.40, 0.51, 14.01, 16.17, 20.84, 22.28, 22.67, 25.87, 30.54, 39.53, 43.12, 71.51, 123.98, 124.94, 135.84, 135.95. MS: *m/z* (%): 192 (8), 177 (3), 163 (5), 149 (5), 135 (65), 125 (4), 124 (13), 107 (82), 165 (6), 95 (64), 85 (53), 79 (43), 69 (77), 55 (63), 43 (100).

1-Bromo-4,8-dimethyldodeca-3,7-diene (13) In a 48 % portion, hydrobromic acid (3 ml) was added to stirred and ice-cooled **12** (6.0 mmol, 1.26 g), and the mixture was stirred for 20 min at 0 °C. Distilled water was added, the organic layer was separated, and the aqueous layer was extracted with hexane. The combined organic solutions were washed with saturated $NaHCO_3$ solution and brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The crude product was purified by column chromatography on silica gel (hexane) yielding 1.0 g (80 %). IR υ_{max}, cm^{-1}: 2963, 2932, 2855, 1671, 1445, 1375, 1267, 1207, 1122, 852. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.88 (t, *J* 6.96Hz, 3H), 1.31 (m, 4H), 1.61 (d, *J* 8.80Hz, 3H), 1.69 (dq, *J* 10.16Hz *J* 1.18Hz, 3H), 2.03 (m, 6H), 2.56 (q, *J* 7.32Hz, 2H), 3.34 (t, *J* 7.32Hz, 2H), 5.11 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 14.02, 15.88, 16.26, 22.39, 23.40, 26.36, 30.24, 31.77, 32.82, 39.33, 39.71, 39.96, 120.86, 121.57, 123.63, 124.35, 135.54, 138.65. MS: *m/z* (%): 271 (1), 259 (1), 215 (2), 187 (7), 165 (3), 151 (1), 137 (1), 123 (2), 111 (16), 95 (7), 81 (9), 69 (100), 55 (43), 41 (16).

5,9-Dimethyltrideca-4,8-dienal (14) The Grignard reagent was prepared from bromide 13 (1.0 g, 3.65 mmol) and magnesium (0.088 g, 3.65 mmol) in dry diethyl ether (2 ml). To this, was added slowly a solution of *N,N*-dimethylformamide (DMF) (0.3 ml, 4 mmol) in diethyl ether (5 ml) at 0 °C over a period of 5 min. An instant exothermic reaction takes place with the formation of a sticky white precipitate. The mixture then was brought to room temperature and stirred for 1 h. Subsequently, the reaction mixture was carefully quenched with hydrochloric acid solution (1 mol/L) until the solution became acidic. The product was extracted with diethyl ether. The extract was washed with saturated $NaHCO_3$ solution and brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane–ethyl acetate 8:2) to give **14** in 52 % yield. IR υ_{max}, cm^{-1}: 2932, 2845, 2707, 1729, 1654, 1441, 1390. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.91 (m, 3H), 1.31 (m, 4H), 1.60 (d, *J* 8.80Hz, 3H), 1.70 (dq, *J* 10.16Hz *J* 1.18Hz, 3H), 2.03 (m, 6H), 2.36 (q, *J* 7.32Hz, 2H), 2.45 (t, *J* 7.32Hz, 2H), 5.11 (m, 2H), 9.81 (t, *J* 1.64, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 13.32, 15.80, 21.64, 30.05, 39.35, 39.35, 44.00, 122.08, 122.74, 123.93, 124.30, 135.40, 135.72, 136.99, 137.26, 202.63. MS: *m/z* (%): 222 (2), 204 (1), 189 (1),

178 (3), 165 (3), 147 (3), 137 (9), 119 (3), 111 (11), 93 (12), 81 (7), 69 (100), 55 (52), 53 (5), 41 (18).

5,13,17-Trimethylhenicosa-5,12,16-trien-9-ol (15) As described above, compound **3** (3.8 mmol, 0.78 g) was converted into **15** (0.37 g, 56 % yield) using magnesium turnings (3.8 mmol, 0.092 g). To this, a solution of **14** (1.9 mmol, 0.422 g) in dry diethyl ether (3 mL) was gradually added. IR υ_{max}, cm^{-1}: 3264, 2963, 2932, 2855, 1671, 1457, 1380, 1079, 923. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.88 (m, 6H), 1.32 (m, 8H), 1.50 (m, 4H), 1.60 (m, 6H), 1.67 (m, 3H), 2.02 (m, 12H), 3.6 (m, 1H), 5.12 (m, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 14.00, 15.87, 15.97, 22.35, 22.68, 23.40, 24.30, 26.56, 30.16, 31.57, 37.40, 37.63, 39.38, 39.76, 40.07, 71.64, 123.79, 124.05, 124.53, 124.82, 135.32, 135.63, 135.78, 135.99, 136.22. MS: *m/z* (%): 300 (1), 287 (1), 263 (2), 245 (4), 219 (3), 203 (2), 178 (4), 163 (9), 149 (10), 135 (13), 121 (16), 109 (20), 95 (43), 81 (43), 69 (100), 55 (65), 41 (25).

5,13,17-Trimethylhenicosan-9-ol (16) In the same manner as described above for **6**, compound **15** (0.37 g, 1.07 mmol) was converted into **16** (0.37 g, 1.04 mmol, 97 % yield). IR υ_{max}, cm^{-1}: 3276, 2963, 2926, 2861, 1460, 1386. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.88 (m, 16H), 1.12 (m, 6H), 1.30 (m, 18H), 1.43 (m, 10H), 3.62 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 14.22, 19.70, 23.10, 24.54, 29.28, 32.78, 36.73, 36.84, 37.08, 37.34, 37.84, 72.06. MS: *m/z* (%): 336 (4), 308 (1), 294 (1), 278 (2), 252 (1), 238 (1), 227 (18), 208 (6), 168 (6), 157 (26), 139 (15), 125 (23), 111 (44), 97 (69), 83 (100), 69 (76), 57 (76), 43 (63).

5,9,17−Trimethylhenicosane (17) As described above for **7**, compound **16** (0.37 g, 1.04 mmol) was converted into **17** (0.14 g, 0.44 mmol, 42 % yield). IR υ_{max}, cm^{-1}: 2961, 2928, 2857, 1469, 1378. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.89 (m, 16H), 1.10 (m, 4H), 1.30 (m, 30H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 14.16, 19.75, 23.08, 24.46, 27.03, 29.30, 29.78, 30.10, 32.71, 36.80, 37.16, 37.47. MS: *m/z* (%): 338 (1), 323 (1), 309 (1), 295 (1), 281 (6), 253 (3), 239 (1), 225 (2), 211 (7), 183 (6), 169 (7), 155 (17), 141 (11), 126 (4), 113 (21), 99 (34), 85 (81), 71 (73), 57 (100), 43 (71).

Chemical Standards 1-Hexenal and 1-hexanol were purchased from Acros Organics (Geel, Turnhout, Belgium). Hexanoic acid, hexyl acetate, octyl acetate, and tridecane were purchased from Aldrich Chemical Company (Milwaukee, WI, USA).

Results and Discussion

The chromatographic profile of extracts from males and females *P. picta* showed one male-specific compound, with retention time (Rt) of 21.94 min, and Kovats Index (KI) of 2232 on an RTX-5 column (2203 on an RTX-Wax column and 2238 on a DB-1 column) (Fig. 1) (Kovats, 1965). Gas chromatography-electroantennogram detection analysis of the male extract showed that only the antennae of female *P. picta* responded strongly to this male-specific compound (Fig. 2).

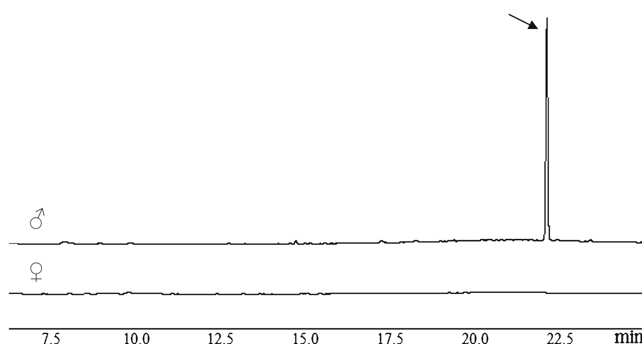


Fig. 1 Gas chromatographic analysis of volatiles obtained from male and female *Phthia picta* adults (the arrow marks the male-specific compound)

The FTIR spectra of the male-specific compound exhibited bands in the region of asymmetrical axial deformation of methyl groups at 2960 cm^{-1}, a band of symmetrical axial deformation of methyl groups overlapping the CH_2 band of asymmetrical axial deformation at 2928 cm^{-1}, and symmetrical axial deformation at 2858 cm^{-1}. Symmetrical bending vibration of methyl groups also was observed at 1379 cm^{-1} and asymmetrical bending vibration at 1468 cm^{-1}. These data suggested that the male-specific compound could be a saturated methyl-branched hydrocarbon (Fig. 3a) (Silverstein et al., 2005).

The mass spectrum of the male-specific compound showed a molecular ion at *m/z* 338 (Fig. 3b), suggesting a $C_{24}H_{50}$ molecular formula for the hydrocarbon. The absence of linearity for the C_{14} units confirmed the existence of methyl branches in the molecule, in agreement with FTIR spectral data (Silverstein et al., 2005). The high relative

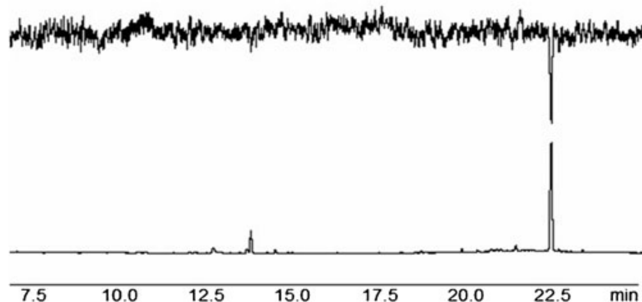


Fig. 2 Coupled gas chromatograph-electroantennogram detection analysis of male *Phthia picta* antenna of a female to the aeration extract from a *P. picta* male; the flame ionization detector response is below

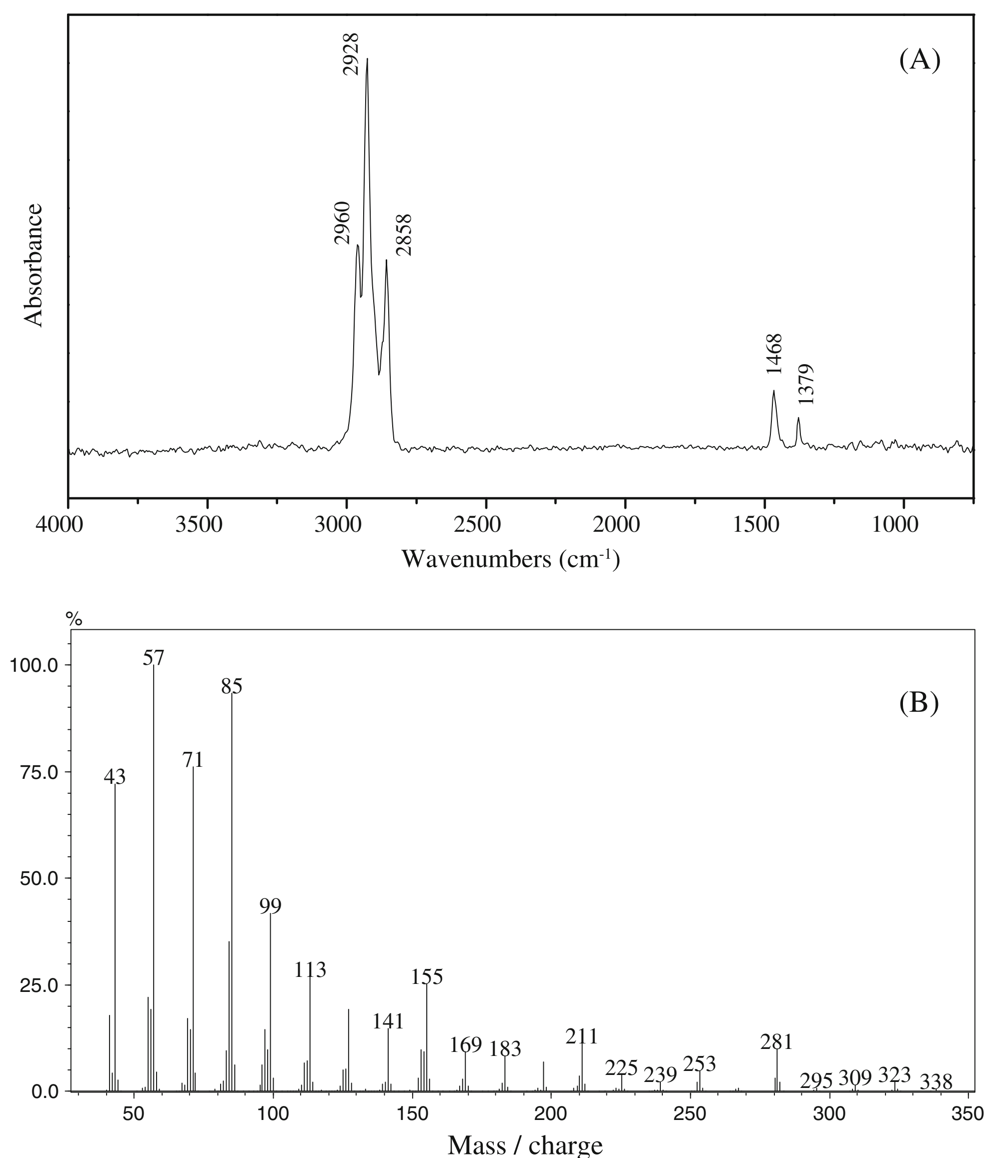
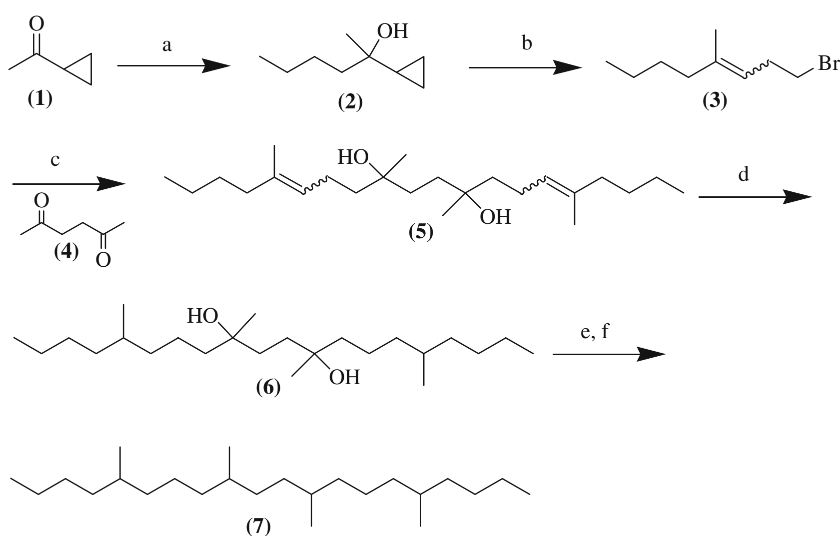


Fig. 3 **a** Coupled gas chromatography-infrared spectroscopy analysis of the male-specific *Phthia picta* compound. **b** Electron impact ionization mass spectrum of the male-specific compound of *P. picta*



Scheme 1 Synthesis of 5,9,12,16-tetramethyleicosane. (**a**) *n*-BuBr, Mg^0, Et_2O, 95 %, (**b**) HBr 48 %, 0 °C, 83 %, (**c**) Mg^0, Et_2O, hexan-2,5-dione **(4)**, 80 %, (**d**) H_2/PdC, hexane, 90 %, (**e**) *p*-TSA, benzene, reflux, (**f**) H_2/PdC, hexane, 25 % for both steps

abundance of *m/z* 85 ion, associated with the *m/z* 57 ion, suggested the presence of a methyl group at C_5; the high relative abundance of *m/z* 155 ion suggested a second methyl group at C_9; the *m/z* 211 ion suggested the presence of a third methyl group at C_{12}; and the *m/z* 281 ion suggested the presence of a fourth methyl branch at C_{16}. Based on these data, 5,9,12,16-tetramethyleicosane was our initial candidate structure for the natural product.

5,9,12,16-Tetramethyleicosane was synthesized as a mixture of all stereoisomers as showed in Scheme 1. The Grignard reaction of buthylmagnesium bromide with cyclopropylmethylketone **(1)** yield the alcohol **2** in 95 % yield (Biernacki

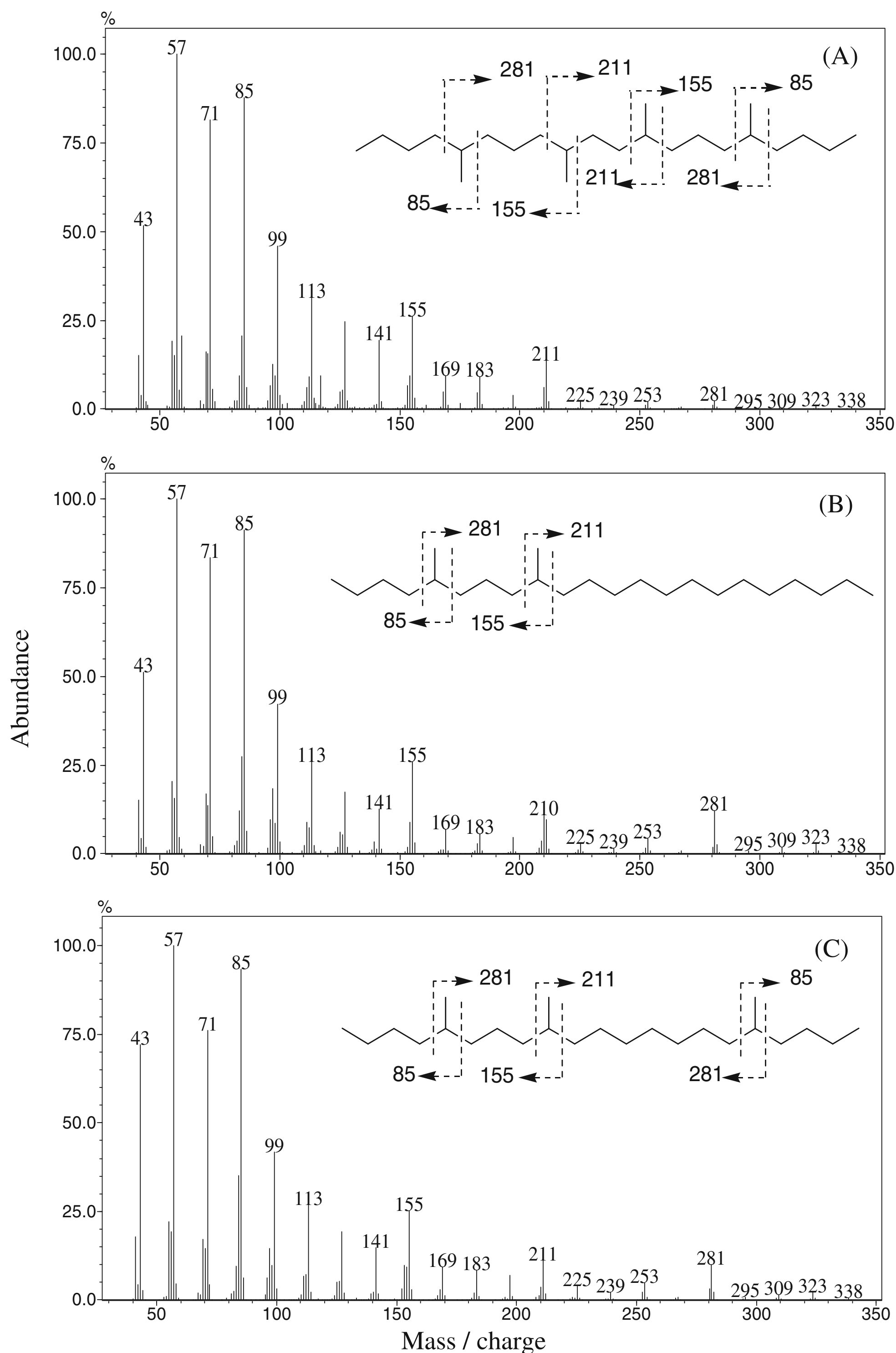
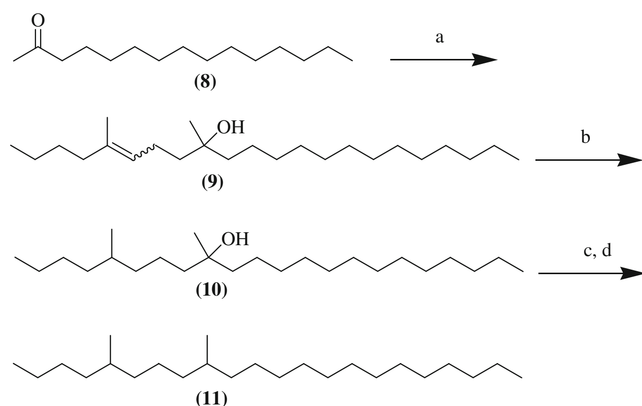


Fig. 4 Mass spectra and the fragmentations of synthetic compounds: (**a**) 5,9,12,16-tetramethyleicosane; (**b**) 5,9-dimethyldocosane; (**c**) 5,9,17-trimethylhenicosane



Scheme 2 Synthesis of 5,9-dimethyldocosane. (**a**) **3**/Mg^0, Et_2O, 66 %, (**b**) H_2/PdC, hexane, 97 % (**c**) *p*-TSA, benzene, reflux, (**d**) H_2/PdC, hexane, 32 % for both steps

and Gdula, 1979; Mori and Murata, 1994; Zarbin et al., 1998), which was treated with 48 % bromic acid that, through Julia's cylcopropane cleavage, led to bromide **3** in 83 % yield. The Grignard reagent prepared from **3** was allowed to react with hexan-2,5-dione **(4)**, previously prepared by Jones oxidation of the commercially available hexan-2,5-diol in 98 % yield (Ferreira and Zarbin, 1996; Kim et al., 2011), providing the key intermediate **5**, which was submitted to catalytic hydrogenation with Pd/C in hexane affording the saturated alcohol **6** (Zarbin et al., 2000). Finally, diol **6** was dehydrated with *p*-TSA in benzene (Utermoehlen et al., 1987), obtaining the corresponding alkene that was directly hydrogenated over Pd/C leading to 5,9,12,16-tetramethyleicosane **7** in 14 % overall yield after six steps (Scheme 1).

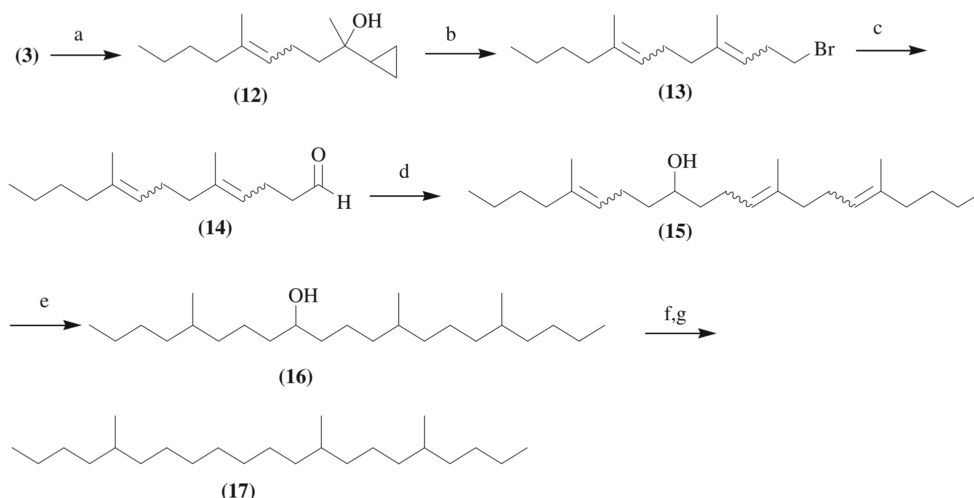
Synthetic **7** produced a broad GC peak, and a mass spectrum similar to that of the natural product (Fig. 4a). However, its Rt was almost one minute earlier on the RTX-5 column, with different Kovats indexes (2157 on the RTX-5 column, 2103 on the RTX-WAX column, and 2161 on the DB-1 column).

Due to the fact that the synthetic compound with four methyl branches exhibited lower Kovats indexes than those of the natural product, we suspected that 5,9-dimethyldocosane could be the true structure. The methyl branches at C_5 could be associated to the fragments *m/z* 85 and *m/z* 281, while the methyl branch at C_9 would be in accordance with *m/z* 155 and *m/z* 211 fragments.

5,9-Dimethyldocosane was synthesized as a mixture of all four stereoisomers using a similar methodology as deployed for Scheme 1 (Scheme 2). The Grignard reagent prepared from bromide **3** obtained in the first synthesis was added to commercial pentadecan-2-one (**8**), leading to the alcohol **9** in 66 % yield, which was catalytically hydrogenated with Pd/C to give saturated alcohol **10** in 97 % yield. Dehydration (Zarbin et al., 2003b) followed by hydrogenation afforded the final compound, 5,9-dimethyldocosane (**11**), in 21 % overall yield in four steps.

The mass spectrum of **11** (Fig. 4b) also was similar to the natural product, however, its Rt was approximately one minute longer than that of the natural product on the RTX-5 column (KI 2279 on the RTX-5 column, 2353 on the RTX-WAX column, and 2283 on the DB-1 column).

The Kovats indexes obtained from the previous synthesized molecules suggested that the natural product should contain three methyl branches in a C_{21} carbon chain. The accumulated data collected strongly supported methyl branches at positions C_5 and C_9. So, the remaining candidate structure with strong diagnostic ions at *m/z* 85, 155, 211, and 281 seemed to be a molecule with the third methyl branch at position C_{17}. A third methyl branch at C_{12} also could produce ions at *m/z* 155 that would be compatible with mass spectrum. However, in this case, it would be expected to see an even mass ion from this fragment with single methyl group (*m/z* 154/155), similar to the one found for the 5,9-dimethyldocosane **11** (*m/z* 210/211, see Fig. 4b). Therefore, we synthesized 5,9,17-trimethylhenicosane as showed in Scheme 3.



Scheme 3 Synthesis of 5,9,17-trimethylhenicosane. (**a**) Mg^0, Et_2O, **1,** 85 %, (**b**) HBr 48 %, 80 % (**c**) Mg^0, Et_2O, DMF, 52 %, (**d**) **3**/Mg^0, Et_2O, 56 %, (**e**) H_2/PdC, hexane, 97 % (**f**) *p*-TSA, benzene, reflux, (**g**) H_2/PdC, hexane, 42 % for both steps

Again, a sequence of Julia's rearrangements was used as the key reaction (Mori and Murata, 1994). The Grignard reagent prepared from previously synthesized bromide **3** was added to cyclopropylmethylketone (**1**) leading to the alcohol **12** in 85 % yield. This alcohol was treated with 48 % bromic acid, obtaining the new bromide **13** in 80 % yield. The Grignard reagent prepared from **13** was added to N,N-dimethylformamide (DMF) in ether to give, upon acidic work-up, the corresponding aldehyde **14** in 52 % yield (Olah et al., 1984; Xiao et al., 2010). A Grignard reagent prepared from bromide **3** was added to aldehyde **14**, leading to the alcohol **15** in 56 % yield, which was hydrogenated, and then suffered dehydration and hydrogenation (Utermoehlen et al., 1987) affording the desired 5,9,17-trimethylhenicosane (**17**) in 12 % overall yield after seven steps.

5,9,17-Trimethylhenicosane did, indeed, produce and an identical mass spectrum (Fig. 4c), retention times, and Kovats indexes on the three GC columns employed, as did the natural product. These data confirm that the male-produced compound from *Phthia picta* is 5,9,17-trimethylhenicosane.

The behavioral responses of *P. picta* males and females to synthetic 5,9,17-trimethylhenicosane were evaluated in a Y-tube olfactometer. In the bioassay, approximately 80 % of the females were attracted to the synthetic compound ($P<0.001$). In contrast, when males were tested, attraction was not different from the control ($P=0.206$) (Fig. 5). The fact that only females were attracted to the treatment suggests that the 5,9,17-trimethylhenicosane is a sex pheromone produced by *P. picta* males. This is the first time that a hydrocarbon has been identified as a pheromone in Coreidae bugs.

Besides sex pheromone, true bugs produce large quantities of strong-smelling and irritating defensive chemicals that are released when these insects are disturbed (Aldrich et al., 1982; Aldrich, 1988; Aldrich et al., 1993; Durak and Kalender, 2007). Odorous compounds are produced by both adults and immatures that defend against predation (Staddon, 1979). These chemicals also may have a role as alarm pheromones (Gunawardena and Bandumathie, 1993; Leal et al., 1994). The defensive/alarm compounds of adult bugs are produced in metathoracic scent glands (MSG) usually composed of a reservoir and a pair of lateral glands that each connect to the reservoir through a duct (Staddon, 1979, 1986; Aldrich, 1988; Durak and Kalender, 2007; Durak, 2008).

The metathoracic scent gland of true bugs in the infraorder Pentatomomorpha (Tian et al., 2011; Weirauch and Schuh, 2011) is a compartmentalized gland that includes a pair of lateral accessory glands and a median reservoir (Aldrich et al., 1978; Aldrich, 1988). This gland produces defensive secretions characteristic of heteropterans. However, in certain Lygaeidae (Aldrich et al. 1997, 1999) and Alydidae (e.g., Aldrich et al., 2000), attractant pheromones are released from the lateral accessory glands of males or females, thus bypassing the reservoir. Therefore, our analyses of *P. picta* included sampling by dissecting the lateral accessory glands and reservoirs of adult males and females separately, as well as aerations of the sexes.

Fig. 5 Responses of *Phthia picta* males and females to synthetic 5,9,17-trimethylhenicosane in Y-olfactometer test (** statically significant; $P<0.001$, binomial test)

Dissections of the MSG reservoir and lateral glands separately for *P. picta* males and females, followed by GC-MS analysis of the extracts, showed different results. The MSG reservoirs of males and females each contained the typical coreid defensive compounds; i.e., 1-hexanal, 1-hexanol, hexanoic acid, hexyl acetate, octyl acetate, and tridecane (Fig. 6C) (Aldrich et al., 1993; Leal et al., 1994). These secretory components exhibited mass spectra that matched the spectra of the aforementioned components in the NIST library, and full identification was confirmed by GC coinjection with authentic standards. However, the contents of lateral gland showed a pronounced difference between the sexes. While almost no components were detected in the lateral glands of females, the corresponding extract from males contained one compound in high concentration, whose Kovats indexes and mass spectra matched perfectly

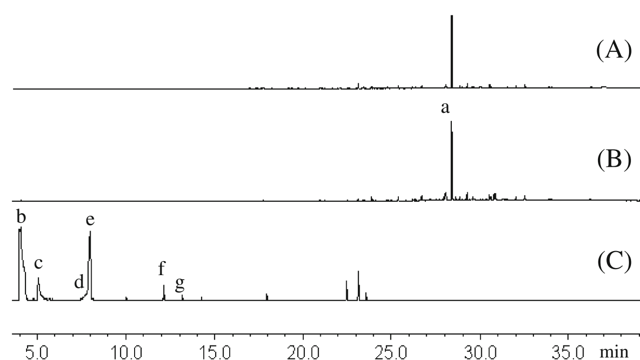


Fig. 6 Gas chromatographic analysis of compounds obtained from *Phthia picta* adult males: (**A**) volatiles obtained by aeration; (**B**) extract of lateral gland of the MTG; (**C**) extract of MSG reservoir compounds: (a) 5,9,17-trimethylhenicosane, (b) 1-hexanal; (c) 1-hexanol; (d) hexanoic acid; (e) hexyl acetate; (f) octyl acetate; and (g) tridecane. Remaining peaks are unidentified

with the male-specific compound detected by aeration (Fig. 6A and B).

In summary, we identified the male-produced sex pheromone of *P. picta* as 5,9,17-trimethylhenicosane, and we also described the contents of the reservoir of metathoracic gland from males and females. In addition, we determined that the sex pheromone is produced in the lateral glands of the MSG of males in this species. The results herein described could become useful for managing this elusive and unpredictable pest.

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Male-Produced Sex Pheromone of the Stink Bug *Edessa meditabunda*

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Abstract *Edessa meditabunda* is a secondary pest within the piercing-sucking stink bug complex that attacks soybean crops in Brazil. The behavioral responses of males and females to aeration extracts from conspecifics suggested the presence of a male-produced sex pheromone. Gas chromatographic (GC) analysis of male and female aeration extracts revealed the presence of two male-specific compounds in a ratio of 92:8. Gas chromatographic - electroantennographic detection (GC-EAD) assays indicated that the major component is bioactive for females, supporting the behavioral data. Analysis of the mass and infrared spectra of the male-specific compounds suggested that they were both methyl-branched long-chain methyl esters. On the basis of the mass spectra of the respective hydrocarbons obtained by micro derivatizations, the structures of these methyl esters were proposed to be methyl 4,8,12-trimethylpentadecanoate (major) and methyl 4,8,12-trimethyltetradecanoate (minor). An 11 step synthetic route that was based on a sequence of Grignard reactions, starting from cyclopropyl methyl ketone, was developed to obtain synthetic standards with a 7.9 % overall yield for the major compound and a 9.9 % yield for the minor. The synthetic standards co-eluted with the natural pheromones on three different GC stationary phases. Y-tube olfactometer assays showed that the synthetic standards, including the major compound alone and a mixture of the major and minor compounds in the proportion found in natural extracts, were strongly attractive to females.

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Introduction

Soybeans (*Glycine max*) one of the most profitable and widespread crops in the Brazil (Conab, 2011), are strongly attacked by a complex of Pentatomidae (Panizzi and Slansky, 1985; Zarbin et al., 2009). The main pentatomid soybean pests are *Dichelops furcatus, D. melacanthus, Euschistus heros, Nezara viridula,* and *Piezodorus guildinii* (subfamily Pentatominae), and *Edessa meditabunda* (subfamily Edessinae) (Corrêa-Ferreira and Panizzi, 1999; Lourenção et al., 1999; Souza, 2007; Zarbin et al., 2009). In the central western region of Brazil, the Neotropical pentatomid, *E. meditabunda*, is one of the most important and abundant species detected in soybean fields (Souza, 2007).

Every year, millions of liters of insecticides are used to control stink bugs in Brazilian soybean crops (Corrêa-Ferreira and Moscardi, 1996). New methods for controlling these insects need to be developed as insecticide treatments are not efficient, and insecticide residues are health hazards and serious obstacles to exportation. The use of semiochemical methods, especially aggregation and sex pheromones, is a more environmentally benign management approach (Borges et al., 2007).

Out of all of the pentatomid species that attack soybeans in Brazil, attractant pheromones have been identified for three species: *E. heros* (Borges et al., 1998, 1999), *N. viridula* (Aldrich et al., 1987, 1989; Borges et al., 1987; Tillman et al., 2010), and *P. guildinii* (Borges et al., 2007). The genus *Edessa* is the largest in the Pentatomidae, containing approximately 260 described species (Silva et al., 2006); however,

information about the communication system in these species is sparse. Recently, Silva et al. (2012) described the mating behavior and vibratory communication of *E. meditabunda*, but information on the chemical communication of these species is lacking.

The goal of this research was to identify and synthesize the attractant pheromone of *E. meditabunda*, and to test the attractiveness of the synthesized pheromone candidates in laboratory experiments. We are describing herein the first identification of a sex pheromone for an *Edessa* species.

Methods and Materials

Insects A colony of *E. meditabunda* was started with insects collected at the EMBRAPA soybean fields, Londrina, Paraná, Brazil (23° 11′S, 51° 11′W). Adults were sexed and separated from nymphs, and maintained in plastic cages (35×20×20 cm) at 25±2°C, 70±5 % humidity and L14:D10 photoperiod. The colony was reared on soybean seeds (*Glycine max*), green beans (*Phaseolus vulgaris*), peanuts (*Arachis hypogaea*), and glossy privet fruits (*Ligustrum lucidum*). The food was replaced every 3 days.

Collection of Volatiles Volatiles were collected via the aeration method (Zarbin et al., 1999). Five males and 5 females were placed separately in glass chambers (33×3.5 cm ID) containing privet fruits. The collecting apparatus was maintained at the same temperature and photoperiod as the colony. A continuous 1 L/min flow of humidified and charcoal-filtered air was pulled through each chamber, carrying the volatiles to glass traps (11×1 cm ID) containing 60 mg of the adsorbent polymer Super-Q (Alltech Associates Inc., Deerfield, IL, USA). The adsorbed volatiles were eluted from the polymer once a day with double-distilled hexane (2 ml) and concentrated with argon to 50 µl (10 µl per insect). Extracts were stored at -20°C for chemical analyses and bioassays. To determine the day cycle, collections were made every 12 h. Additionally, collections were made every 2 h, over two consecutive days, during a photoperiod of higher pheromone release.

Chemical Analyses Initial analyses were carried out using a Shimadzu GC2010 gas chromatograph (GC) equipped with an FID detector and an RTX-5 (Restek, 30 m×0.25 m×0.25 µm film thickness) capillary column with helium as the carrier gas. The GC was operated in splitless mode (250°C). The temperature program was 50°C for 1 min, increasing at 7°C per min to 250°C with a 10-min hold. For the analyses determining the Kovats indices (Lubeck and Sutton, 1983) and coinjection of the natural product with the synthetic standard, two other GC columns were employed: RTX-WAX (Restek, 30 m×0.25 m×0.25 µm film thickness) and HP-1 (Agilent, 30 m×0.25 m×0.25 µm film thickness) capillary columns.

GC-mass spectrometry (MS) data were acquired using a Shimadzu QP2010-Plus electron ionization mass detector. The GC was equipped with an RTX-5 (30 m×0.25 m×0.25 µm) capillary column. The injector mode and temperature program were the same as those described above.

Extracts also were analyzed by GC-Fourier transform infrared spectroscopy (FTIR) with a Shimadzu GC2010 coupled to a DiscovIR-GC (Spectra Analysis, Marlborough, MA, USA) infrared detector (4000–750 cm^{-1}, resolution 8 cm^{-1}). The capillary column, injector mode and temperature program were the same as those used for the analyses described above.

^{1}H- and ^{13}C-NMR spectra of the synthetic compounds were recorded on a Bruker ARX-200 spectrometer (200 and 50 MHz, respectively) as $CDCl_3$ solutions. Chemical shifts are expressed in ppm relative to TMS.

GC-electroantennographic detection (GC-EAD) analysis data were acquired using a Shimadzu GC2010 coupled to a Syntech® electroantennographic detector (Hilversum, Netherlands). The GC was equipped with an RTX-5 capillary column (30 m×0.25 mm×0.25 µm), and operated in splitless mode (250°C) with a temperature program starting at 100°C for 1 min and increasing 7°C per min to 250°C. The column effluent was split in a 3:1, with one part going to the FID (270°C) and three parts going through the heated transfer line into humidified airstream (200 ml/min) directed to the electrodes. The antennae was detached from the scape base along with some muscular tissue and fixed between the two stainless electrodes (Syntech probe) using conductive gel (Signa gel, Parker Labs, USA). The electroantennograms were recorded using the Syntech GC-EAD32 program (version 4.6) (Ambrogi et al., 2012).

Micro-derivatizations

Lithium Aluminum Hydride ($LiAlH_4$) Reduction Aeration extract (50 µl) from males was treated with 5 µl of a dry ethereal solution of $LiAlH_4$ (~5 mg/ml) for approximately 1 min. A few µl (~10) of water were added, followed by extraction with ether and drying with anhydrous Na_2SO_4 (Attygalle, 1998).

Reduction to the Carbon Skeleton The resulting $LiAlH_4$ reduction product (50 µl) was converted to the respective mesylate by treatment with 400 µl of a 1 % solution of methanesulfonyl chloride in pyridine. After 24 h, the excess was treated with methanol and the solvent was evaporated under argon. The residue was dissolved in ethyl ether and reduced with a dry ether solution of $LiAlH_4$ (~5 mg/ml) for 3 h at room temperature. A few µl (~10) of water were

added, followed by extraction with ether and drying with anhydrous Na_2SO_4 (Attygalle, 1998).

Syntheses

^1H, ^{13}C-NMR, MS, and FTIR data of all the synthetic compounds are presented as supplementary material, available online.

2-cyclopropylpentan-2-ol (4) The Grignard reagent was prepared by the slow addition of bromopropane (2.45 g, 20 mmol) to a suspension of Mg^0 (266 mg, 22 mmol) in anhydrous ethyl ether (10 ml) under an inert atmosphere at room temperature. After observing the formation of the Grignard reagent, a solution of cyclopropylmethylketone **(3)** (2.02 g, 24 mmol) in diethyl ether (5 ml) was added dropwise. The reaction mixture was stirred overnight, and quenched by adding ice (2 g) followed by the addition of a saturated solution of NH_4Cl (5 ml). The aqueous layer was extracted with ether (3×15 ml), the organic layer was washed with brine, and dried over Na_2SO_4. The pure product was obtained by flash chromatography (hexane/ethyl acetate: 9/1), giving compound **4** at a 96 % yield (2.46 g, 19.2 mmol).

2-cyclopropylbutan-2-ol (5) This compound was made with a method analogous to that used to prepare compound **4,** at a 92 % yield (2.59 g, 22.7 mmol), from bromoethane (2.69 g, 24.7 mmol).

1-bromo-4-methylhept-3-ene (6) HBr 48 % (9.7 ml) was added to a strongly stirred solution of the alcohol **4** (2.4 g, 18.8 mmol) at 0°C. The mixture was stirred for 15 min between 0 and 5°C. The reaction was extracted with hexane, washed with a saturated solution of $NaHCO_3$, and dried with anhydrous Na_2SO_4. The crude product was purified by flash column chromatography (hexane), resulting in compound **6** at a 99 % yield (3.61 g, 18.6 mmol

1-bromo-4-methylhex-3-ene (7) This compound was made with a method analogous to that used to prepare compound **6,** at a 98 % yield (3.87 g, 21.5 mmol) starting from compound **5** (2.5 g, 21.9 mmol).

2-cyclopropyl-6-methylnon-5-en-2-ol (8) This compound was made with a method analogous to that used to prepare compound **4,** at a 60 % yield (2.00 g, 10.2 mmol) starting from compound **6** (3.30 g, 17 mmol).

2-cyclopropyl-6-methyloct-5-en-2-ol (9) This compound was made with a method analogous to that used to prepare compound **4,** at a 62 % yield (2.23 g, 12.4 mmol) starting from compound **7** (3.6 g, 20.0 mmol).

1-bromo-4,8-dimethylundeca-3,7-diene (10) HBr 48 % (3.7 ml) was added to a strongly stirred solution of the alcohol **8** (1.80 g, 9.17 mmol) at 0°C. The mixture was stirred for 30 min and held between 0 and 5°C. The reaction was extracted with hexane, washed with saturated solutions of NaCl and $NaHCO_3$, and dried with anhydrous Na_2SO_4. The crude product was purified by flash column chromatography (hexane), resulting in compound **10** with an 84 % yield (1.99 g, 7.70 mmol).

1-bromo-4,8-dimethyldeca-3,7-diene (11) This compound was made with a method analogous to that used to prepare compound **10,** at a 91 % yield (2.45 g, 10 mmol) starting from compound **9** (2.0 g, 10 mmol).

2-cyclopropyl-6,10-dimethyltrideca-5,9-dien-2-ol (12) This compound was made with a method analogous to that used to prepare compound **4,** with a 60 % yield (1.08 g, 4.09 mmol) starting from compound **10** (1.80 g, 6.81 mmol).

2-cyclopropyl-6,10-dimethyldodeca-5,9-dien-2-ol (13) This compound was made with a method analogous to that used to prepare compound **4,** at a 62 % yield (1.39 g, 5.57 mmol) starting from compound **11** (2.20 g, 8.98 mmol).

1-bromo-4,8,12-trimethylpentadeca-3,7,11-triene (14) HBr 48 % (610 μL) was added to a strongly stirred solution of alcohol **12** (0.90 g, 3.41 mmol) at 0°C. The reaction was extracted with hexane, washed with saturated solutions of NaCl and $NaHCO_3$, dried with anhydrous Na_2SO_4, and the solvent was evaporated. The crude product was purified by flash chromatography (hexane), resulting in compound **14** with a 63 % yield (0.70 g, 2.15 mmol).

1-bromo-4,8,12-trimethyltetradeca-3,7,11-triene (15) This compound was made with a method analogous to that of compound **14,** at a 70 % yield (0.84 g, 2.68 mmol) starting from compound **13** (1.2 g, 3.83 mmol).

4,8,12-trimethylpentadeca-3,7,11-trien-1-yl acetate (16) Anhydrous HMPA (2 ml) was added to a round-bottom flask containing 175 mg (2.3 mmol) of sodium acetate while stirring. The mixture was cooled to 0°C and 0.60 g (1.83 mmol) of the bromide **14** was added dropwise. After 12 h, the reaction was extracted with hexane (3×15 ml), washed with brine, rinsed several times with distilled water, and dried with Na_2SO_4. Compound **16** was purified by flash chromatography (hexane/EtOAc: 9/1) with a 77 % yield (0.43 g, 1.41 mmol).

4,8,12-trimethyltetradeca-3,7,11-trien-1-yl acetate (17) This compound was made with a method analogous to that

used to prepare compound **16,** at a 78 % yield (0.49 g, 1.67 mmol) starting from compound **15**.

4,8,12-trimethylpentadecyl acetate (18) Compound **16** (0.4 g, 1.31 mmol) in 5 ml of hexane was hydrogenated over Pd/C (10 %, 5.0 mg) at room temperature under a hydrogen atmosphere (25 psi) in a Parr® apparatus for 2 h. The mixture was filtered, and the filtrate was evaporated at reduced pressure to obtain the acetate **18** at a 89 % yield (0.37 g, 1.17 mmol).

4,8,12-trimethyltetradecenyl acetate (19) This compound was made with a method analogous to that used to prepare compound **18,** at an 83 % yield starting from compound **17**.

4,8,12-trimethylpentadecan-1-ol (20) A dry THF solution (1 ml) of the acetate **18** (0.3 g, 0.96 mmol) was slowly added to a suspension of $LiAlH_4$ (77 mg, 2 mmol) in THF (5 ml) cooled to 0°C. The mixture was stirred for 5 h at room temperature, then cooled to 0°C, hydrolyzed with an NaOH aqueous solution (15 %, 0.5 ml), and extracted several times with ethyl ether. The combined ether extracts were washed with water, dried over Na_2SO_4 and concentrated. The crude product was purified by flash chromatography (hexane/ethyl acetate: 8/2), resulting in the alcohol **20**, with an 80 % yield (0.21 g, 0.77 mmol).

4,8,12-trimethyltetradecan-1-ol (21) This compound was made with a method analogous to that used to prepare compound **20,** at an 82 % yield (0.21 g, 0.82 mmol) starting from compound **19** (0.3 g, 1.00 mmol).

Methyl 4,8,12-trimethylpentadecanoate (1) Jones reagent (~1 ml) was added to a solution of compound **20** (0.18 g, 0.67 mmol) in acetone at room temperature with magnetic stirring until the orange color remained. After 12 h, the excess CrO_3 was consumed by adding methanol. The mixture was concentrated under vacuum, the residue was diluted with water, and extracted with ethyl ether. The ethereal layer was washed with brine, dried with anhydrous $NaSO_4$, and evaporated under vacuum. The residue obtained was diluted in hexane (5 ml), and an ethereal solution of diazomethane was added at 0°C until the resulting solution shared the yellow color of the ethereal solution. The mixture was stirred at room temperature for 30 min, and concentrated under reduced pressure. The pure product was obtained by column chromatography (hexane/ethyl acetate : 9/1), resulting in compound **1** at an 80 % yield in two steps (0.16 g, 0.54 mmol).

Methyl 4,8,12-trimethyltetradecanoate (2) This compound was made with a method analogous to that used to prepare compound **1,** at an 85 % yield (0.17 g, 0.60 mmol) starting from compound **21** (0.18 g, 0.70 mmol).

Olfactometer Bioassays The behavioral responses of *E. meditabunda* to natural and synthetic compounds were tested in a Y-tube olfactometer using humidified, charcoal-filtered air at 2.5 L/min (Zarbin et al. 2007). The olfactometer consisted of a Y-shaped glass tube (4×40 cm) with two 20 cm arms. The odor sources, which consisted of pieces of filter paper (2×2 cm) impregnated with the synthetic compounds, natural extracts or hexane (control), were placed at the ends of the arms. An insect was introduced into the base of the olfactometer, and the behavior of the insect was observed over 20 min. An insect walking upwind toward the odor source for more than 5 cm in any of the arms, and remaining in the arm for more than 2 min, was considered a positive response. An insect remaining in the main tube was considered a negative response. Each insect was tested only once, representing one replicate experiment. The experiments were performed at the time of day of highest pheromone release, and the odor source was replaced after each test. Insects that did not choose either of the arms were excluded from statistical analysis. The data were analyzed using a *Chi-square* test with the BioEstat program (version 5.0) (Ayres et al., 2003).

Initially, four bioassay experiments were performed; the responses of males to odors from males, males to odors from females, females to odors from males, and females to odors from females. Because only females were attracted to the odors from males, males were not used in subsequent experiments.

Two experiments were conducted to determine the biological activity of the synthetic major and minor male-specific compounds. Females were tested with the synthetic major male-specific compound (methyl 4,8,12-trimethylpentadecanoate **1**, 50 μg), and with a blend of the two male-specific compounds (methyl 4,8,12-trimethylpentadecanoate **1**, 50 μg plus methyl 4,8,12-trimethyltetradecanoate **2**, 4.4 μg) at a ratio of 92:8.

Results

Evidence of a Sex Pheromone Comparison of the chromatograms of aeration extracts collected from male and female *E. meditabunda* adults showed the presence of two male-specific compounds (compounds **1** and **2**) along with other compounds that were common to both sexes (Fig. 1). The ratio between compounds **1** and **2** was calculated to be 92:8, respectively, based on the areas of the GC peaks detected by FID. The respective Kovats Indices were calculated on three different columns and are shown in Fig. 1.

Y-tube olfactometer bioassays employing natural extracts showed that aeration extracts of males are significantly more attractive to females than the solvent control [treatment 15 (68 %), control 8 (32 %), $N=22$, $P<0.05$]. The same extract,

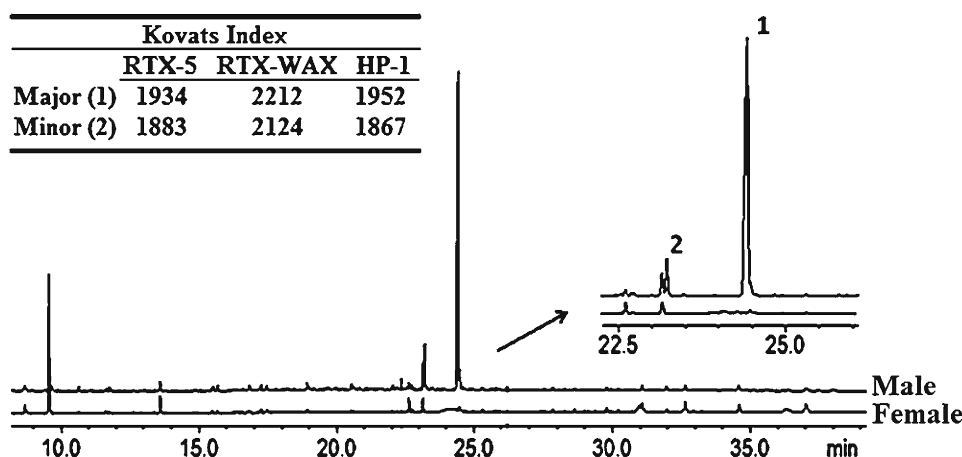


Fig. 1 Gas chromatograms of volatiles produced by *Edessa meditabunda* adults and Kovats Indices of the two male-specific compounds analyzed on RTX-5, RTX-WAX and HP-1 capillary columns. Chromatograms obtained by using a RTX-5 capillary column

however, was not attractive to conspecific males. On the other hand, the experiments carried out with volatiles from females did not exhibit attractiveness to either sex, and most of the insects in these experiments made no choice, remaining in the main olfactometer arm. These results are consistent with the GC profile of the extracts, and suggest that males produce a sex pheromone, as previously reported for other stink bugs such as *Thyanta pallidovirens* (Millar, 1997) and *Acrosternum hilare* (McBrien et al., 2001), and not an aggregation pheromone that attracts both sexes, as is the case for *N. viridula* (Harris and Todd, 1980b) and *P. hybneri* (Leal et al., 1998).

GC-EAD assays employing aeration extracts from males and the antennae from both sexes were conducted, and only antennae of females exhibited activity for the major male-specific volatile **1** (Fig. 2), thus confirming the evidence of a male-produced sex pheromone mediating the behavior of *E. meditabunda*. However, these electrophysiological responses were not detected for the minor component **2**, even after several analyses.

Dynamics of Production of the Male-Specific Components The production of the major male-specific compound was dependent on light. Although the compounds were detected throughout all monitoring periods, approximately 71 % of the daily release occurred during the 12 h of photophase (Fig. 3a). There were practically no differences between release during the six 2-h periods evaluated during photophase. Only production during the first period of photophase was significantly different from production during the other photophase periods (Fig. 3b).

Structural Identification The GC-FTIR spectrum of compound **1**, and GC-MS spectra obtained from compounds **1** and **2** are shown in Fig. 4. The infrared spectra of compound **1**, which is similar to that of compound **2** (not shown), shows two bands at 1175 cm^{-1} and 1196 cm^{-1}, indicating a C-O single bond; a C = O stretching band characteristic of esters at 1744 cm^{-1} (Silverstein et al., 2005) is also present. The mass spectra of compounds **1** and **2** are similar, exhibiting the same base peak at *m/z* 87, and molecular ion at *m/z* 298 for **1** (Fig. 4a) and *m/z* 284 for **2** (Fig. 4b). The base peak at *m/z* 87 suggests a branch close to the ester function. The base peak and the high relative intensities of the fragments at *m/z* 157 and 241 for compound **1**, and at *m/z* 157 and 227 for compound **2**, indicates a methyl branched structure. The difference of 14 mass units between the molecular ions and the fragments at *m/z* 227 and 241, suggest that compound **2** lacks a CH_2 in the main carbon chain relative to compound **1**.

To determine the structures of these compounds, several micro-derivatizations were carried out with the natural products. First, to determine what kind of ester was present, a reduction with $LiAlH_4$ was employed. A fragment at *m/z*

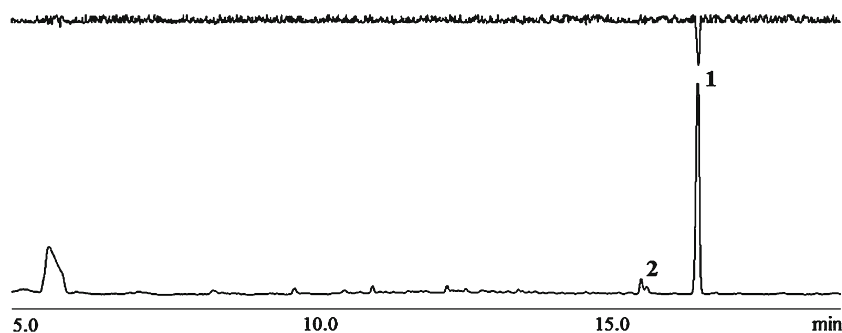


Fig. 2 Electroantennogram response in females (top) to an aeration extract of *Edessa meditabunda* males

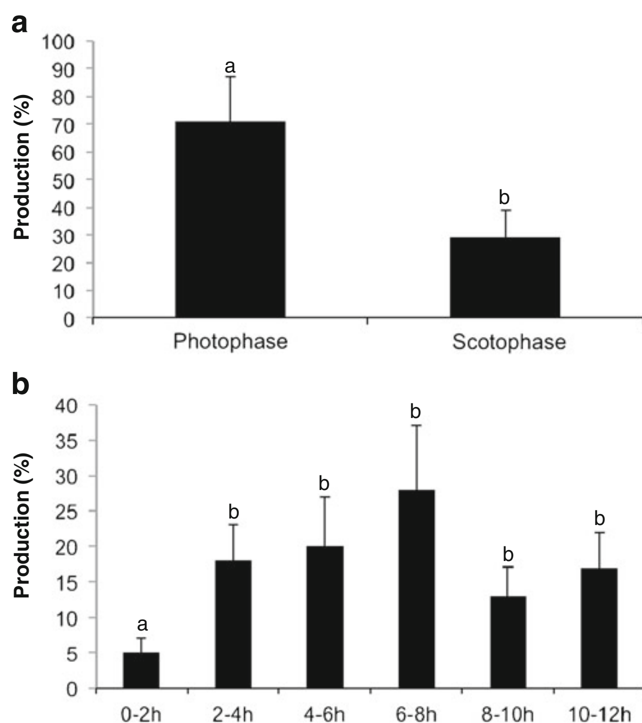


Fig. 3 Comparison of the amounts of the major component **1** collected from the aeration of *Edessa meditabunda* males: **a** between the photophase and scotophase (ANOVA + Tukey test, $P<0.01$, $P=0.005$); **b** during different times of the 12 h photophase (ANOVA + Tukey test, $P<0.05$). Mean values followed by the same letter are not significantly different ($N=3$)

252 related to the loss of water (M^+ - 18) was observed in the mass spectrum of the resulting alcohol, indicating a molecular ion at *m/z* 270, for a difference of 28 units from the parent compound (M^+ at *m/z* 298). This difference indicates a reduction of a methyl ester present on the natural compound **1**. The same pattern was observed in the minor derivative, also suggesting a methyl ester structure. The alcohol structure of the derivative was confirmed by the presence of a broad O–H stretching band at 3252 cm^{-1} in the FTIR spectrum. On the basis of these data, compounds **1** and **2** may have the molecular formulae $C_{19}H_{38}O_2$ and $C_{18}H_{36}O_2$, respectively.

To determine the number and positions of the possible methyl branches on the main carbon chain, the alcohol derivative was converted to a mesylate and reduced with $LiAlH_4$ to the corresponding hydrocarbon (Scheme 1A). The same procedure was performed using $LiAlD_4$ instead the $LiAlH_4$ to produce the hydrocarbon with 3 deuterons in the first carbon of the main chain (Scheme 1B).

The mass spectrum of the hydrocarbon derivative of the major compound exhibited high relative intensities of the fragments at *m/z* 71, 141 and 211, diagnostic fragments for the methyl branches at positions 4, 8, and 12 of the carbon chain (Fig. 5a). Slight increases in the relative intensities of these fragments were observed due to the symmetry of the resulting hydrocarbon. These methyl branch positions were corroborated by analyzing the mass spectrum of the $LiAlD_4$ hydrocarbon product; in the deuterated molecule, the symmetry was broken, and the fragments at *m/z* 74, 144, and 214 also had slightly elevated intensities (Fig. 5b). Considering that the two male-specific compounds are known to be methyl esters, and that they contain three methyl branches, methyl 4,8,12-trimethylpentadecanoate was proposed as the identity of the major compound **(1)**, and methyl 4,8,12-trimethyltetradecanoate was proposed as the identity of the minor compound **(2)** (Fig. 6).

Synthesis of Methyl 4,8,12-trimethylpentadecanoate (1) and Methyl 4,8,12-trimethyltetradecanoate (2) A synthetic methodology to obtain **1** and **2** was developed to confirm the proposed structures of the pheromones, with Julia olefination as a key step (Scheme 2), in a route similar to that proposed by Mori and Murata (1994) to synthesize the sex pheromones of the stink bugs *E. heros* and *E. obscurus*.

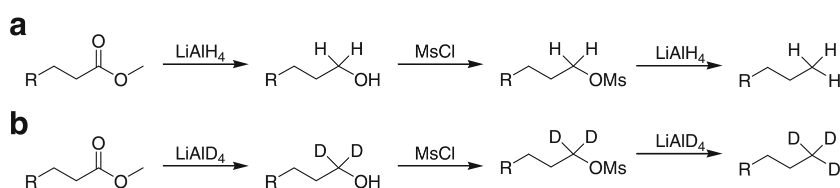
According to Scheme 2, 2-cyclopropylpentan-2-ol **(4)** was prepared at a 96 % yield by adding cyclopropylmethylketone **(3)** to a solution containing propylmagnesium bromide. The alcohol **4** formed was subjected to a Julia olefination with HBr 48 %, causing the opening of the cyclopropyl ring, yielding bromide **6** at 99 % (Mori and Murata, 1994). The stereoisomeric mixture had an *E*- to *Z*-**6** ratio of 3:1 observed by GC, as described previously (Biernacki and Gdula, 1979).

Preparing the Grignard reagent derived from compound **6** (Zarbin et al. 1998), adding it to cyclopropylmethylketone **(3),** and performing Julia olefination on the resulting alcohol **8**, gave bromide **10** with a 50 % yield over the two steps. The triene **14**, a direct precursor of the pheromone, was synthesized as a mixture of the eight stereoisomers by performing the same Grignard reaction and Julia olefination sequence using bromide **10**, with a 38 % yield over the two steps. The direct acetylation of the compound **14** using sodium acetate in HMPA (Larock, 1974) gave the ester **16** at a 77 % yield. Ester **16** was submitted to a catalytic hydrogenation under Pd/C, resulting in the hydrogenated acetate **18** with an 89 % yield (Zarbin et al., 2000). The reduction of the carboxyl group of the acetate **18** with $LiAlH_4$ resulted in the alcohol **20** with an 80 % yield (Vidal et al., 2010). The Jones oxidation (Zarbin et al., 2000) of the alcohol **20** resulted in the corresponding carboxylic acid, which was directly treated with an ethereal solution of diazomethane (Furniss et al., 1989), affording the desired ester **1** with a 57 % yield over the two steps. The overall yield of methyl 4,8,12-trimethylpentadecanoate **(1)** was 7.9 % from compound **3** after 11 steps.

The minor component, methyl 4,8,12-trimethyltetradecanoate **(2)**, was synthesized based on the same route starting from bromoethane, with an overall yield of 9.9 % from compound **3** after 11 steps.

Fig. 4 Electron impact mass spectra of **1** (**a**) and **2** (**b**), and the infrared spectrum of **1** (**c**)

The two synthetic compounds were co-injected with the natural extracts of males, and they co-eluted on all three different GC columns tested (RTX-5, HP-1 and RTX-WAX). The mass and infrared spectra of the synthetic pheromones did not differ from the spectra of the natural compounds.



Scheme 1 Derivatization sequences with male volatile extracts to obtain the carbon skeletons of the major male-specific compounds of *Edessa meditabunda* (**A**: $LiAlH_4$ derivative; **B**: $LiAlD_4$ derivative

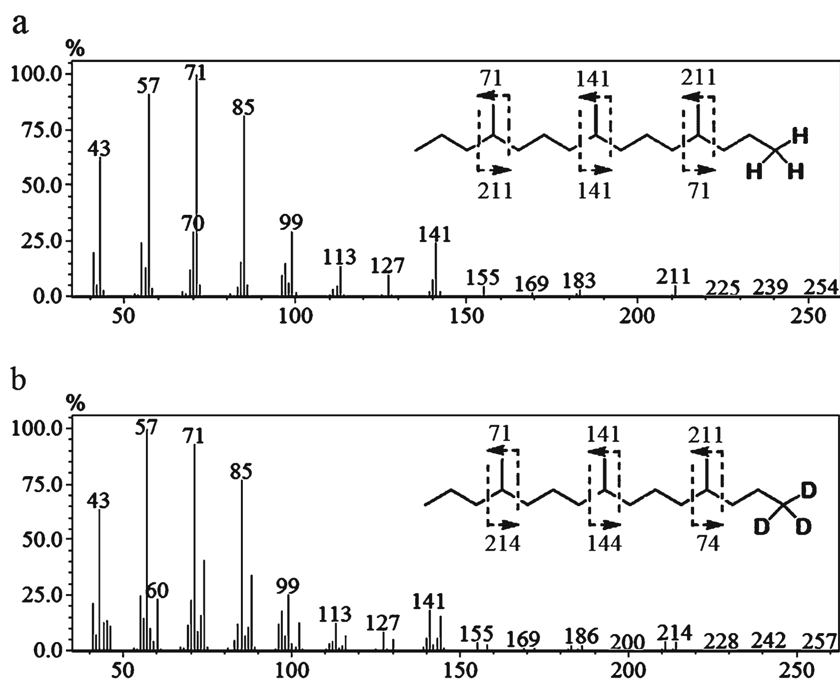


Fig. 5 Mass spectra of $LiAlH_4$ (**a**) and $LiAlD_4$ (**b**) hydrocarbon derivatives from the natural male-specific compound **1**

Y-tube Bioassays Employing the Synthetic Pheromones A Y-tube olfactometer was used to test the biological activities of the synthetic male-specific compounds (Fig. 7). The first experiment consisted of testing only the major male-specific compound (**1**) against the control (only solvent); females were significantly attracted to this compound [treatment 15 (75 %), control 5 (25 %), N=20, P=0.025]. The second experiment was performed with a mixture of the two synthetic compounds at the same ratio as found in volatile extracts of males against the control. Female attraction to the mixture was highly significant [treatment 16 (80 %), control 4 (20 %), N=20, P=0.007].

Discussion

Pheromone Production In the Pentatomidae, single bugs or small groups sometimes release more male-specific compounds during aeration than do larger groups (Ho et al., 2005; Zahn et al., 2008). For example, when the stink bug, *Murgantia histrionica*, was aerated individually or in groups of five insects, the males released higher amounts of sex-specific compounds than when they were aerated in groups of ten or more. It has been suggested that males can detect the presence of other males, which influences rates of pheromone release (Zahn et al., 2008). The use of five insects per aeration chamber for our *Edessa* aerations seemed appropriate, as the analyses produced clean chromatograms with small amounts of defensive compounds and large amounts of male-specific compounds.

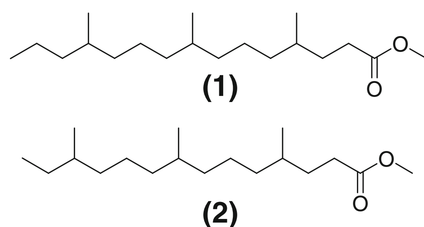
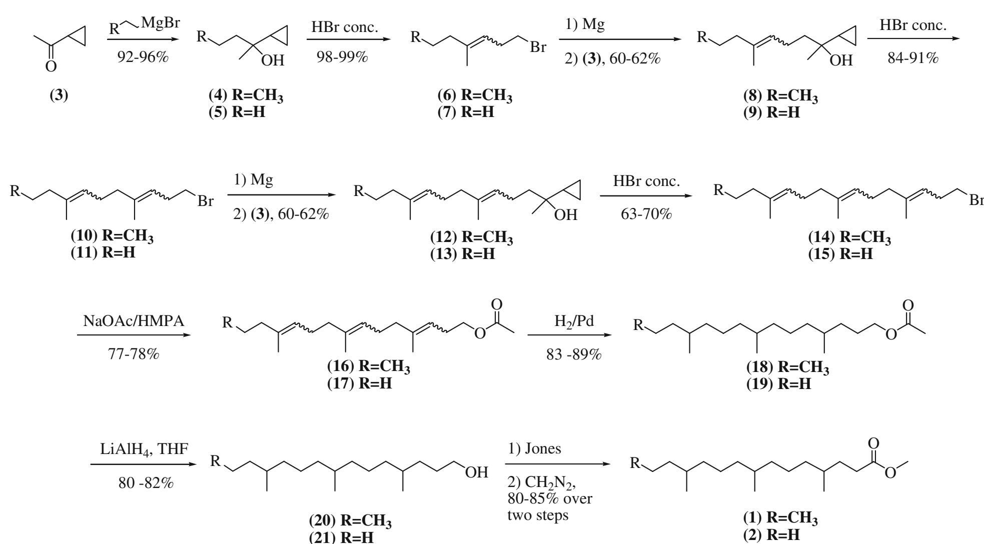


Fig. 6 Proposed structures for the major (**a**) and minor (**b**) male-specific compounds released by *Edessa meditabunda*

In addition, *M. histrionica* males release pheromone primarily during photophase (Zahn et al., 2008), as does *E. meditabunda*. However, most other phytophagous pentatomids studied engage in reproductive activity occurring during the late afternoon to early evening [e.g., the *Chlorochroa* genus (Fish and Alcock, 1973; Ho and Millar, 2001a, c), *N. viridula* (Harris and Todd, 1980a), *E. heros* (Borges et al., 1998) and *T. pallidovirens* (Wang and Millar, 1997)].

Thus far, all studies with aggregation or sex pheromones produced by Pentatomidae have shown that the males are responsible for attractant pheromone production (Moraes et al., 2008). Once on the same plant, the insects of both sexes emit substrate-borne vibratory signals that are used as a directional short-distance cues to locate the mate (Čokl, 2008). Some explanations for why males are responsible for aggregation and sex pheromone release have been proposed but, until now, none has been proven. One of these hypotheses is that



Scheme 2 Synthetic route for the male-specific compounds released by *Edessa meditabunda*

the male bugs are responsible for the recolonization process characteristic of many heteropteran species, such as the stink bugs *Podisus maculiventris,* in which males often search for food first and then call females to the new habitat using pheromones (Aldrich et al., 1984), and *E. servus*, in which sexually mature males migrate to corn before females (Herbert and Toews, 2011). Another suggestion is that because stink bug pheromones are used by parasitoids and predators to locate their prey (Aldrich, 1995; Powell, 1999), males have evolved to be smaller by developing more quickly, assuming the risk of discovery by natural enemies as the pheromone producers for the potential pay-off of mating with more females (Ho and Millar, 2001a).

Chemical Identification Methyl 4,8,12-trimethylpentadecanoate **(1)** and methyl 4,8,12-trimethyltetradecanoate **(2)** are novel molecules in pheromone chemistry, but the structures of these compounds are analogous to the sex pheromones produced by *E. heros* (Aldrich et al., 1991) and *E. obscurus* (Borges and Aldrich, 1994), methyl 2,6,10-trimethyltridecanoate and methyl 2,6,10-trimethyldodecanoate.

The Pentatomidae is a group that demonstrates structural chemical patterns of attractive pheromone components for some genera where several species have been studied (Moraes et al., 2008). In particular, the genus *Euchistus*, for which the pheromones of seven species have been identified, the pheromones of all include methyl (*E*,*Z*)-2,4-decadienoate as an aggregation pheromone component (Aldrich et al., 1991, 1994; Borges et al., 1998; Krupke et al., 2001). Although the genus *Edessa* is the largest of the Pentatomidae genera (Silva et al., 2006), heretofore only defensive compounds have been identified (Howard and Wiemer, 1983; Borges and Aldrich, 1992).

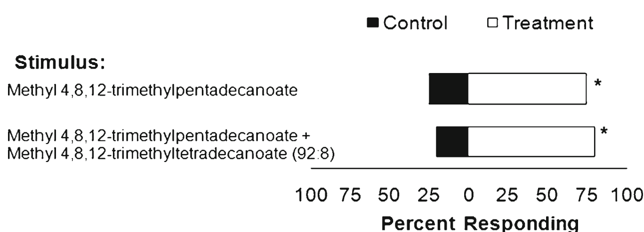


Fig. 7 Results of Y-tube bioassays testing the attraction of *Edessa meditabunda* females to the major synthetic compound, methyl 4,8,12-trimethylpentadecanoate **(1)**, or to a blend of the two synthetic compounds found only in male volatiles, methyl 4,8,12-trimethylpentadecanoate **(1)** and methyl 4,8,12-trimethyltetradecanoate **(2)** (92:8). *Chi-square* test, $N=20$, * $P<0.05$

Y-tube Bioassays The major male-specific compound, methyl 4,8,12-trimethylpentadecanoate **(1)**, and extracts of conspecific males showed biological activity on *E. meditabunda* females, inducing a behavioral response. The presence of the minor male-specific compound, methyl 4,8,12-trimethyltetradecanoate **(2)**, which was not EAD-active**,** did not improve attraction or act as a repellent; therefore, the significance of this compound in the pheromone blend remains unclear.

These data corroborate previous research on stink bug pheromones (Leal et al., 1998; Ho and Millar, 2001c; McBrien et al., 2001), where laboratory tests showed the function of the major compound as a pheromone but did not identify the roles of minor components (Ho and Millar, 2001b). However, field bioassays are still needed to determine whether the main pheromone compound alone may be sufficient for use as an attractant, and whether the minor compound has a function, such as attractiveness improvement or blend specificity.

In summary, the existence of a male-produced sex pheromone released by the stink bug *E. meditabunda* was determined. Two male-specific compounds were identified, methyl 4,8,12-trimethylpentadecanoate (**1**) and methyl 4,8,12-trimethyltetradecanoate (**2**); however, addition of **2** to the main component **1** did not significantly improve attraction of females in laboratory bioassays These novel compounds were synthesized as a mixture of all stereoisomers, and their biological activities were confirmed in laboratory tests. We are now working in a stereoselective synthesis of all the eight possible stereoisomers of compounds **1** and **2**, in order to determine the influence of chirality on behavior, as well as to determine the absolute configuration of these natural molecules. Field experiments employing the synthetic pheromones are underway, and the results will appear in due course.

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Sex Pheromone Communication in Two Sympatric Neotropical Stink Bug Species *Chinavia ubica* and *Chinavia impicticornis*

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Abstract *Chinavia* and *Nezara* spp. stink bugs (Heteroptera: Pentatomidae) include over100 species, with highest diversity in Afrotropical and Neotropical regions. Species thus far studied in these genera utilize *trans*-(*Z*)-(4*S*)-bisabolene epoxide (BE) and *cis*-(*Z*)-(4*S*)-BE as major sex pheromone components, with species specificity ensured by different ratios of the two compounds. Gas chromatography (GC) and coupled GC-mass spectrometry (GC-MS) analyses of a volatiles from *C. ubica* males revealed the presence of two BE isomers in approximately a 90:10 ratio, which were shown by microprobe ^{1}H NMR to be *cis*-(*Z*)-BE and *trans*-(*Z*)-BE isomers, respectively. Analyses of volatiles from *C. impicticornis* males suggested the presence of a single isomer, *trans*-(*Z*)-BE, in high purity (>90 %). The absolute configurations of the isomers produced by *C. ubica* and *C. impicticornis* were determined using chiral GC analysis (β-DEX column). Oxidative microchemistry of synthetic standards of *cis*-(*Z*)-(4*S*)-BE and *trans*-(*Z*)-(4*R*)-BE, and volatiles from male of *C. ubica*, revealed the absolute stereochemistry of the *cis*-(*Z*)-BE to be (1*R*,2*S*,4*S*) [*cis*-(Z)-(4*S*) for short]. Similarly, analyses of *trans*-(*Z*)-(4*S*)-BE and *cis*-(*Z*)-(4*R*)-BE standards, and volatiles from males of *C. ubica* and *C. impicticornis*, revealed the absolute stereochemistry of the *trans*-(*Z*)-BE to be (1*S*,2*R*,4*S*) [*trans*-(*Z*)-(4 S) for short]. Olfactometer bioassays with synthetic BEs confirmed attraction of female *C. ubica* and *C. impicticornis* to conspecific synthetic pheromone, but not to heterospecific synthetic pheromone. *Chinavia impicticornis* appeared not to discriminate behaviorally between the conspecific pheromone and its enantiomer. Coupled GC-electroantennography with antennae from females suggested that *C. ubica* and *C. impicticornis* possess olfactory receptors for both *cis*-(*Z*)-(4*S*)-BE and *trans*-(*Z*)-(4*S*)-BE. The results in this study confirm that *C. ubica* and *C. impicticornis*, as for other *Chinavia* and *Nezara* spp., utilize *cis*-(*Z*)-(4*S*)-BE and *trans*-(*Z*)-(4*S*)-BE as sex pheromone components, with different ratios guaranteeing species specificity. Furthermore, the results suggest that the absolute stereochemistry of BEs may be less important for conspecific recognition than the relative stereochemistry between the epoxide group and the alkyl substituent on the bisabolene ring.

Keywords Soybean pest · Sex pheromone · Bisabolene epoxide · Olfactometer bioassay · Heteroptera Pentatomidae

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Introduction

Chinavia and *Nezara* spp. stink bugs (Heteroptera: Pentatomidae) include over 100 species, with highest diversity in Afrotropical and Neotropical regions. The genus *Chinavia* Orian, which includes Afrotropical, Nearctic, and Neotropical species, was until recently considered to be a subgenus of *Acrosternum* Fieber (Schwertner and Gracia, 2006). In Brazil, *C. ubica* (Rolston, 1983) and *C. impicticornis* (Stål, 1872) are sympatric, both causing severe damage to soybean and other crops such as cotton and sunflower (Panizzi et al., 2007). In addition to herbivorous damage, these insects also facilitate infection by plant pathogens (Daugherty, 1967; Clarke and Wilde, 1970).

Several studies have focused on chemical communication in green stink bugs, including the identification of sex pheromones (Borges et al., 1987; Baker et al., 1987; Aldrich et al., 1987, 1993; Borges, 1995). Borges et al. (1987) showed that the attractant pheromone of the southern green stink bug, *N. viridula*, influences long-range orientation of females, and suggested that the pheromone is produced from the abdominal sternites of males, which was confirmed in later studies (Cribb et al., 2006). The major attractant components produced by males of *Chinavia* and *Nezara* spp., comprise *trans*-(*Z*)-(4*S*)-bisabolene epoxide (BE) and *cis*-(*Z*)-(4*S*)-BE, with pheromone specificity being guaranteed by the ratio of isomers (Borges et al., 1987; Aldrich et al., 1987; Brezot et al., 1994; McBrien et al., 2001). Brazilian populations of *N. viridula* produce *trans*-(*Z*)-(4*S*)-BE and *cis*-(*Z*)-(4*S*)-BE in a 2:1 ratio (Borges et al., 1987; Baker et al., 1987), whereas populations of *N. viridula* and *Chinavia* spp. from other geographical regions produce *cis*-(*Z*)-(4*S*)-BE in relatively higher amounts (Baker et al., 1987; Aldrich et al., 1987, 1993; McBrien et al., 2001). In addition to variation in isomer ratios, the release rate of pheromone components varies considerably among species, which is apparently an important factor in suitable mate location (Aldrich, 1995).

Previous laboratory bioassays suggested that the ratio between the *cis*-(*Z*)-BE and *trans*-(*Z*)-BE isomers appears to be crucial for intraspecific recognition between individuals of *Nezara* and *Chinavia* spp. (Borges, 1995; McBrien et al., 2001). As both *C. ubica* and *C. impicticornis* have similar geographical distribution and host plants, we investigated how specificity in intra-specific communication is achieved. Therefore, the sex pheromone composition was investigated, and electroantennography and bioassay studies were undertaken with different BE isomers.

Methods and Materials

Insects Chinavia ubica and C. impicticornis were obtained from laboratory colonies started from adults collected in soybean fields during 2004 – 2011, near the EMBRAPA Genetic Resources and Biotechnology Laboratory, Brasília, DF, Brazil (15° 47′S and 47° 55′ W). Bugs were reared on raw peanuts (*Arachis hypogaea*), soybeans (*Glycine max*), fresh green beans (*Phaseolus vulgaris*), and water, at 26 ± 1.0°C and 65 % r.h. under a 14 L:10D photoperiod (light, 06:00 to 20:00 h). Insects were kept in 8-L plastic containers (100-150/container), with the food supply being renewed three times per week. A 15 cm^2 plastic mesh (~ 40 mesh) was placed against the inner wall of each container as an oviposition substrate and shelter for the bugs. Egg masses were collected daily, and incubated in 9 cm I.D. plastic Petri dishes until hatch. When nymphs molted to second instars, they were transferred to plastic containers and reared as above. To prevent interactions between the sexes, males were separated from females after their imaginal molt and cuticular hardening (ca. 24 h after molting). Sexually mature adults, ≥ 8 d after the final molt, were used for all bioassays.

Volatile Collection Volatiles were collected from groups of 50 males or females of *Chinavia ubica* and *C. impicticornis* by aeration (N=30 volatile collections of each gender). To minimize emission of defensive compounds, insects were introduced carefully into 1 L glass containers (Zhang et al., 2003). Air (1 l / min) was drawn sequentially through a bed of 4-12 mesh activated charcoal (Fisher Scientific, Pittsburgh, PA, USA), the glass container, and two adsorbent traps (10 cm x 0.5 cm I.D.) containing Super Q (100 mg each; Alltech Associates, Inc., Deerfield, IL, USA) using a negative pressure air-flow. Collection from males and females started 9 d after the final molt to the adult stage. Insects were fed fresh green beans (replaced three times per wk), and aerated continuously for 20 d, changing the adsorbent traps daily. Traps were eluted with hexane (0.5 ml), and the combined eluates were stored at -20°C until needed for further use. Volatile collections were concentrated under a gentle stream of nitrogen to yield a solution of approximately 0.1 bug-equivalent/µl of solution (~500 µl) prior to bioassays.

Column Chromatography Volatile extracts collected from male *C. ubica* and *C. impicticornis* were subjected to silica gel column chromatography (500 mg; 6.0 cm x 0.5 cm I.D.; (80/100 mesh, Merck, Darmstadt, Germany). Fifteen 24 h volatile collections from 50 virgin males of each species in hexane, combined and concentrated to ca. 1 ml under a gentle stream of nitrogen, were loaded onto the column, which was eluted sequentially with pentane (3 x 1 ml), 5 % diethyl ether in pentane (3 x 1 ml), 10 % diethyl ether in pentane (3 x 1 ml), and diethyl ether (3 x 1 ml). The first two fractions collected using 10 % diethyl ether in pentane were shown by coupled gas chromatography-mass spectrometry (GC-MS) analysis (see details below) to contain bisabolene epoxide (BE) isomers (90 % pure by GC). The fractions containing BE isomoers were concentrated under a gentle stream of nitrogen to yield a solution of approximately 1 bug-equivalent/µl of solution (~750 µl).

Chemical Analysis of Volatile Extracts and Chromatography Fractions Aeration extracts collected from female and male *C. ubica* and *C. impicticornis*, and column chromatography fractions (prepared as described above), were analyzed using a Shimadzu 17A GC equipped with a DB-5 column (30 m x 0.25 mm ID, 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA), and a flame ionization detector (FID). The oven temperature programme was 50°C/2 min,

then 15°C/min to 250°C. Data were collected with Autosystem Software in ASCII format, and processed using Origin 5.0 software (Microcal). Injections were made in splitless mode. For coupled GC-MS analysis, selected extracts were analyzed using an Agilent GC7890A coupled to a 5975MSD instrument equipped with a quadrupole analyzer, a non-polar DB-5 column (30 m×0.25 mm ID, 0.25 μm film, J&W Scientific, Folsom, CA, USA) and a splitless injector, with helium as the carrier gas. Ionization was by electron impact (70-eV, source temperature 200°C). Data were collected and analyzed with GCMS MSD ChemSation Software (Agilent, USA). Identifications were made by comparison of spectra with library databases (NIST 2005), and confirmed by using authentic standards. For ^{1}H NMR analysis, collected column chromatography fractions (prepared as described above) enriched in BEs were concentrated to dryness under a gentle flow of nitrogen, then redissolved in ca. 50 μl of deuterated acetone (C_3D_6O), and transferred into microprobe NMR tubes. ^{1}H NMR spectroscopy was performed using a Bruker Avance 500 MHz NMR spectrometer equipped with a 2.5 mm microprobe. Chemical shifts (in parts per million) from the 1D ^{1}H NMR data were compared with literature values (Chen et al., 2000). For quantification of male-specific compounds in aeration samples, heneicosane in hexane (2 μg/ml) was added as an internal standard to estimate the quantity of compound produced per insect per 24 h of volatile collection.

Absolute Configuration of C. ubica cis-(Z)-BE To confirm the absolute configuration of *cis*-(*Z*)-BE produced by *C. ubica*, the procedure of McBrien et al. (2001) was employed. Commercially available (-) and (+)-limonene oxides (0.5 mg/ml, 2 ml) were added separately to KIO_4 (100 mg) and $KMnO_4$ (5 mg), and the mixtures were stirred for 10 h at ambient temperature. An extract of *C. ubica* (equivalent to 250 bug-hours) containing approximately 0.2 mg/ml of *cis*-(*Z*)-BE was submitted to the same procedure. The synthetic (4*S*) and (4*R*)-acetyl-1,2-epoxy-1-methylcyclohexane samples obtained via oxidation of (-) and (+)-limonene oxide, as well as BE present in the *C. ubica* extract, were analysed using a chiral GC column (β-DEX™ 225, 30 m×0.25 mm ID, d_f 0.25 μm, matrix non-bonded; 25 % 2,3-di-O-acetyl-6-O-TBDMS-β-cyclodextrin in SPB-20 poly(20 % phenyl/80 % dimethylsiloxane) phase; Supelco, Inc.). The oven temperature was programmed 90°C/2 min) at 5°C/min to 210°C (20 min). Injections were made in splitless mode with helium as the carrier gas, injector temperature at 250°C, and detector temperature at 270°C.

Absolute Configuration of C. ubica and C. impicticornis trans-(Z)-BE To confirm the absolute configuration of *trans*-(*Z*)-BE produced by *C. ubica* and *C. impicticornis*, the procedure of McBrien et al., (2001) was employed. Synthetic *trans*-(*Z*)-(4*S*)-BE and *cis*-(Z)-(4*R*)-BE (prepared as described below) were converted to (4*S*) and (4*R*)-α-bisabolene respectively by dilution in methylene chloride (0.65 mg in 2 ml), and addition to a mixture of zinc powder (200 mg), NaI (44 mg), and NaOAc (15 mg) in AcOH (1 ml). The mixture was stirred for 5 h under nitrogen atmosphere, then filtered through glass wool, diluted in water (1 ml), and extracted with CH_2Cl_2 (2 x 2 ml). The organic phase was washed with water (2 ml), followed by dilute NaOH (1 M, 2 x 2 ml), brine (2 x 2 ml), and dried over anhydrous $MgSO_4$. The same procedure was used with pooled extracts of *C. ubica* and *C. impicticornis*, each extract containing 250 bug-hour equivalents. The synthetic (4*R*) and (4*S*)-α-bisabolenes were analyzed by chiral GC (β-DEX™ 225) run isothermally at 90°C.

Chemicals (*Z*)-(1*R*,2*S*,4*S*)-BE and (*Z*)-(1*S*,2*R*,4*S*)-BE were synthesized from (-)-limonene oxide, and (*Z*)-(1*S*,2*R*,4*R*)-BE and (*Z*)-(1*R*,2*S*,4*R*)-BE from (+)-limonene oxide, following the method of Chen et al. (2000). Identifications were confirmed by comparison of ^{1}H and ^{13}C NMR data (Bruker Avance 500 MHz), and MS data (EI, 70 eV, 5795 MSD Agilent coupled to a GC 7890 using a HP-5 column, 30 m x 0.25 mm ID, 0.25 μm film thickness) to data reported in Chen et al. (2000).

Electrophysiology Electroantennogram (EAG) recordings were made using Ag-AgCl glass electrodes filled with saline solution (composition as in Maddrell, 1969, but without glucose). An antenna from a female bug was excised and suspended between the two electrodes. The signals were passed through a high impedance amplifier (UN-06, Syntech, The Netherlands), and analysed using a customized software package (Syntech).

Coupled GC-electroantennogram (GC-EAG) The GC-EAG system, in which the effluent from the GC column is simultaneously directed to the antennal preparation and the GC detector has been described previously (Wadhams, 1990). The effluent from the transfer line to the antenna was delivered into a purified airstream (1 l/min) flowing continuously over the preparation. Chromatography of extracts was achieved using an Agilent 6890 GC equipped with a cold on-column injector and FID. The GC column was 50 m× 0.32 mm I.D. HP-1 programmed from 30°C/2 min at 15°C/min to 250°C. The carrier gas was helium. The outputs from the EAG amplifier and the FID were monitored simultaneously, and analyzed using Syntech software. The antennae of female *C. ubica* or *C. impicticornis* were tested using extracts of *C. ubica* or *C. impicticornis* males. At least 5 replicates were performed for each sample and species.

Olfactometer Bioassays A two-choice olfactometer, modified from Borges and Aldrich (1994a), was used to test the biological activity of volatiles from live insects, aeration samples from males, column chromatography fractions, and synthetic compounds. The olfactometer was positioned horizontally on a bench in a room with bright fluorescent overhead lights (4 x 40 W). The temperature in the bioassay room was maintained at 26.0 ± 1.0°C. The olfactometer was rotated between replicates to avoid positional bias. The apparatus was cleaned after each five replicates with fragrance-free liquid soap, rinsed thoroughly with water, and dried in a convection oven at 180°C. To begin an assay, a single *C. ubica or C. impicticornis* individual was introduced gently into the release chamber with an artist's paint brush (Camel Hair, number 1), and allowed to acclimatise for 3 min while the remainder of the apparatus was assembled. After attaching the treatment and control chambers and starting the air flow, the test bug's behavior was recorded by an observer for 15 min/replicate. The first choice of the insect was determined as the first arm of the olfactometer that an insect chose and subsequently remained in for at least 100 s. Insects that did not leave the release chamber of the olfactometer during the first 10 min were considered "non-responders", and were not included in the data analyses.

Bioassays of Live Insects The intraspecific attraction of insects of both sexes to volatiles from conspecific males (*C. ubica, N*=72 for females and *N*=36 for males and *C. impicticornis, N*=51 for females and *N*=36 for males), and to volatiles from conspecific females (*C. ubica*, *N*=50 for females and *N*=35 for males and *C. impicticornis, N*=36 for females and *N*=34 for males) was investigated. Test individuals were offered a choice between volatiles from 5 live, sexually mature virgin insects or a clean air control. The 5 live insects used as sources of volatiles were changed after 10 replicates.

Bioassays of Aeration Extracts The volatile collections obtained from *C. ubica* and *C. impicticornis* from both genders were concentrated to obtain a solution containing 0.1 bug-equivalent/μl of hexane. For all bioassays, 5 μl of the aeration sample were applied to a piece of filter paper (1.5 x 0.5 cm); filter paper with solvent alone (hexane, 5 μl) served as the control. Bioassays of volatiles from *C. ubica* males were performed with C. *ubica* females (*N*=85) and males (*N*=26), and bioassays of volatiles from *C. ubica* females were performed with *C. ubica* males (*N*=42) and females (*N*=27). Volatiles from *C. impicticornis* males were bioassayed with *C. impicticornis* females (*N*=46) and males (*N*=35), and volatiles from *C. impicticornis* females were tested with *C. impicticornis* females (*N*=50) and males (*N*=35). Because males did not respond to live males, or to volatiles from males and females for either species, bioassays with aeration fractions from males were carried out only with females.

Bioassays of Column Chromatography Fractions Column chromatography fractions obtained from male of *C. ubica* and *C. impicticornis* were concentrated to obtain a solution containing 1 bug-equivalent/μl of solvent. For all bioassays 5 μl of each fraction were applied to pieces of filter paper (1.5 x 0.5 cm); filter paper treated with only the solvent (hexane, 5 μl) served as a control. To evaluate whether other compounds present in volatile extracts had synergistic or antagonist effects on attraction, column chromatography fractions from *C. ubica and C. impicticornis* containing only the male-specific compounds were bioassayed with conspecific females (*C. ubica N*=39 and *C. impicticornis N*=52). To determine whether *C. ubica* females could recognize males of *C. impicticornis,* and whether *C. impicticornis* females could recognize males of *C. ubica* using the volatile compounds of the other species, bioassays were carried out using cross-specific extracts and fractions from males. Females of *C. ubica* were tested against the crude extract (*N*=30), and the BE-containing fraction (*N*=54) from *C. impicticornis* males, and females of *C. impicticornis* were tested against the crude extract (*N*=30) and BE-containing fraction (*N*=33) of *C. ubica* males.

Bioassays of Synthetic Compounds To evaluate if the insects can discriminate between the different isomers and ratios of *cis*-(*Z*)-BE and *trans*-(*Z*)-BE, the four synthetic isomers (*cis*-(*Z*)-(4*S*)-BE, *trans*-(*Z*)-(4*S*)-BE, *cis*-(*Z*)-(4*R*)-BE, and *trans*-(*Z*)-(4*R*)-BE) were tested in bioassays. *Chinavia ubica* females were exposed to the following treatments: *cis*-(*Z*)-(4*S*)-BE (*N*=53), *cis*-(*Z*)-(4*R*)-BE (*N*=42), *trans*-(*Z*)-(4*S*)-BE (*N*=42), and *trans*-(*Z*)-(4*R*)-BE (*N*=34) vs. 5 μl of hexane control; *cis*-(*Z*)-(4*S*)-BE vs. *trans*-(*Z*)-(4*R*)-BE (*N*=60); *trans*-(*Z*)-(4*S*)-BE versus *trans*-(*Z*)-(4*R*)-BE (*N*=78) (5 μl of a 1 mg/ml solution in hexane of each compound). *Chinavia ubica* females also were exposed to 5 μl of a solution containing 1 mg/ml of 90:10 mixture of *cis*-(*Z*)-(4*S*)-BE+*trans*-(*Z*)-(4*S*)-BE vs. 5 μl of hexane (*N*=40); vs. 5 μl of *trans*-(*Z*)-(4*S*)-BE (1 mg/ml) (*N*=40); and vs. 5 μl of *cis*-(*Z*)-(4*S*)-BE (1 mg/ml) (*N*=40). *C. impicticornis* females were exposed to the following treatments: 5 μl of *cis*-(*Z*)-(4*S*)-BE (1 mg/ml) (*N*=49), 5 μl of *cis*-(*Z*)-(4*R*)-BE (1 mg/ml) (*N*=45), 5 μl of *trans*-(*Z*)-(4*S*)-BE (1 mg/ml) (*N*=42), and 5 μl of *trans*-(*Z*)-(4*R*)-BE (1 mg/ml) (*N*=47) vs. 5 μl of hexane, as control; 5 μl of *trans*-(*Z*)-(4*S*)-BE (1 mg/ml) versus 5 μl of *trans*-(*Z*)-(4*R*)-BE (1 mg/ml) (*N*=41); and 5 μl 90:10 mixture of *cis*-(*Z*)-(4*S*)-BE+*trans*-(*Z*)-(4*S*)-BE (1 mg/ml) vs. 5 μl of hexane control (*N*=40).

General Procedure of Bioassays Solutions of test stimuli, comprising 5 μl of the volatile collections from males and

females of *C ubica* or *C. impicticornis*, column chromatography fractions from males of each species or the synthetic compounds, were spotted onto a strip of filter paper (1.5 x 0.5 cm). The paper was placed in one of the arms of the olfactometer. The volatile collections comprised volatiles collected from 50 males over 24 h. Controls, i.e., filter papers (1.5 x 0.5 cm) treated with hexane, were placed in the other arm.

Statistical Analysis Data on the responses of males and females to different treatments (live insects, aeration extracts and fractions thereof, and synthetic standards, vs. controls) were analysed using *Chi square* to test the hypotheses of non-preference (probability of choice each arm of the olfactometer=0.5). When the number of insects responding to each treatment in each set of bioassays was lower than 5 (< 20 % of insect choosing an arm of the olfactometer), the statistical analysis was not applied and the insects were considered as not responding to the treatment tested.

Results

Olfactometer Bioassays of Aeration Extracts Virgin female *C. ubica* and *C. impicticornis* were significantly attracted to the volatiles from conspecific virgin males (χ^2 test, $P<0.001$; Fig. 1), but not to volatiles from conspecific virgin females (Fig. 1). Males were not significantly attracted to volatiles from conspecific virgin males or virgin females (Fig. 1). Virgin female *C. ubica* and *C. impicticornis* were attracted to aeration extracts from conspecific virgin males (χ^2 test, $P<0.001$; Fig. 2), but were not attracted to extracts from conspecific virgin females (Fig. 2), and virgin male *C. ubica* and *C. impicticornis* were not attracted to extracts collected from either conspecific males or females (Fig. 2). Virgin female *C. ubica* and *C. impicticornis* were attracted to combined column chromatography fractions prepared from aeration extracts of conspecific virgin males (χ^2 test, $P<0.001$; Fig. 3). There was no significant attraction of virgin female *C. ubica* and *C. impicticornis* to aeration extracts, or combined column chromatography fractions thereof from heterospecific virgin males (Fig. 4).

Chemical Analysis of Aeration Extracts GC analysis of volatile extracts from groups of 50 sexually mature male and female *C. ubica* and *C. impicticornis* revealed the presence of a series of compounds common to both genders; i.e., undecane (**1**), dodecane (**2**), (*E*)-2-decenal (**3**), tridecane (**4**), and (*E*)-2-decenyl acetate (**5**) (Fig. 5). GC analysis also revealed the presence of three male specific compounds released by *C. ubica* (compounds **6, 7,** and **8**; Fig. 5A), and two male specific compounds released by *C. impicticornis* (compounds **6** and **7**; Fig. 5B). Coupled GC-MS analysis,

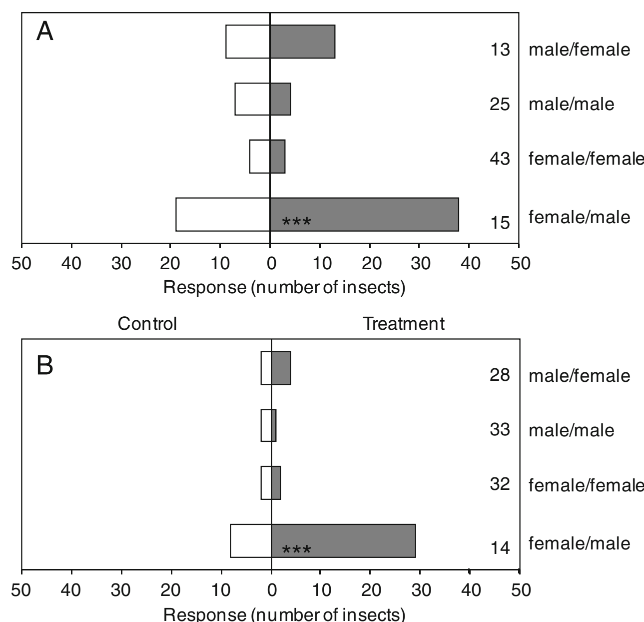

Fig. 1 Response (number of insects) of *Chinavia ubica* **a** and *Chinavia impicticornis* **b** virgin males and females to volatiles from conspecific virgin males and females in a two-choice olfactometer assay. Male/female: response of males to live females, male/male: response of males to live males, female/female: response of females to live females, females/males: response of females to live males. Numbers on right hand side indicate number of insects that did not make a choice. *** indicates statistically significant difference in response to treatment vs. control (χ^2 test $P<0.001$)

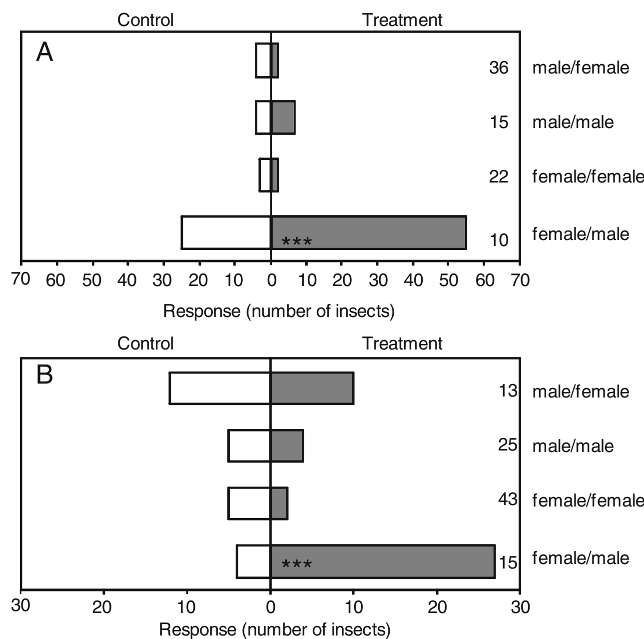

Fig. 2 Response (number of insects) of *Chinavia ubica* **a** and *Chinavia impicticornis* **b** virgin males and females to conspecific aeration extracts in a two-choice olfactometer assay. Male/female: response of males to female extracts, male/male: response of males to male extracts, female/female: response of females to females extracts, females/males: response of females to male extracts. Numbers on right hand side indicate number of insects that did not make a choice. *** indicates statistically significant difference in response to treatment vs. control (χ^2 test $P<0.001$)

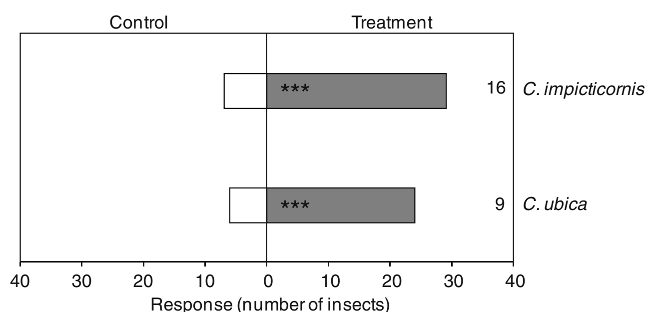

Fig. 3 Response (number of insects) of *Chinavia ubica* and *Chinavia impicticornis* virgin females to conspecific aeration extract from males for column chromatography fractions containing bisabolene epoxides (BEs). *C. ubica*: response of *C. ubica* females to male extracts of *C. ubica*, *C. impicticornis*: response of *C. impicticornis* females to male extracts of *C. impicticornis*. Numbers on right hand side indicate number of insects that did not make a choice. *** indicates statistically significant difference in response to treatment vs. control ($\chi2$ test $P<0.001$)

along with comparison of mass spectral data with an MS database (NIST, 2005) tentatively identified **6** as α-bisabolene, and **7** and **8** as bisabolene epoxide isomers (Fig. 5). For *C. ubica*, microprobe ^{1}H NMR analysis of combined column chromatography fractions enriched in the bisabolene epoxides **7** and **8**, and comparison of the data with literature values (Chen et al., 2000), suggested that the minor compound **7** possessed *trans*-(*Z*) stereochemistry, whereas the predominant compound **8** possessed *cis*-(*Z*) stereochemistry. For *C. impicticornis*, microprobe ^{1}H NMR analysis of combined liquid chromatography fractions, and comparison of the data with literature values (Chen et al., 2000), suggested that compound **7** possessed *trans*-(*Z*) stereochemistry. From quantitative GC analysis, virgin male *C. ubica* were shown to

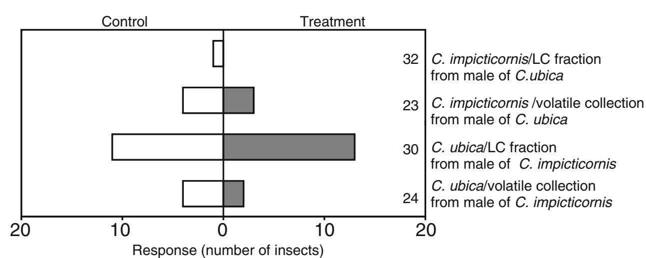

Fig. 4 Response (number of insects) of *Chinavia ubica* and *Chinavia impicticornis* virgin females to volatiles from heterospecific males and column chromatography (LC) fractions containing bisabolene epoxides (BEs). *C. impicticornis*/LC fraction from male of *C. ubica*: response of *C. impicticornis* females to LC fraction from male of *C. ubica*. C. *impicticornis*/volatile collection of male of *C. ubica*: response of *C. impicticornis* females to volatile collections from male of *C. ubica*, *C. ubica*/LC fraction from male of *C. impicticornis*: response of *C. ubica* females to LC fraction from male of *C impicticornis*. *C. ubica*/ volatile collection from male of *C. impicticornis*: response of *C. ubica* females to volatile collections from male of *C. impicticornis*. Numbers on right hand side indicate number of insects that did not make a choice. There was no significant response to any treatment (χ^2 test $P>0.05$)

release 0.23±0.24 μg/24 h of **7, and** 3.03±4.14 μg/24 h of **8** (mean±standard error, $N=40$); the ratio between the two bisabolene epoxides was consistent (0.07; 0.069-0076 95 % confidence interval). Virgin male *C. impicticornis* were shown to release 0.12±0.19 μg/24 h ($N=78$) of **7**.

Absolute Configuration of cis-(Z)-BE from C. ubica To determine the absolute configuration of *cis-(Z)*-BE, an extract from males was submitted to oxidation using KIO_4 and $KMnO_4$, producing *cis*-4-acetyl-1,2-epoxy-1-methylcyclohexane. The configuration of the product was confirmed as (4*S*) by comparison of the retention time on a chiral column (β-DEX™) with standards obtained from the oxidation of (-) and (+)-limonene oxide products; .i.e., (4*S*)- and (4*R*)-acetyl-1-methylcyclohexane, respectively. The product from the *C. ubica* micro-chemical reaction co-eluted with second peak; i.e., the (4*S*)-isomer. As the microprobe NMR data had demonstrated the relative stereochemistry to be the *cis*-configuraion, the absolute stereochemistry of the *cis*-(Z)-BE isomer was determined as (1*R*,2*S*,4*S*) or *cis*-(*Z*)-(4*S*).

Absolute Configuration of trans-(Z)-BE from C. ubica and C. impicticornis To confirm the absolute configuration of *trans*-(*Z*)-BE produced by *C. ubica and C. impicticornis,* synthetic *trans*-(*Z*)-(4*S*)-BE and *cis*-(*Z*)-(4*R*)-BE were converted to (4*S*)- and (4*R*)-α-bisabolene, respectively. The same procedure was used with pooled extracts of *C. ubica* and *C. impicticornis*. The (4*R*)- and (4*S*)-α-bisabolene micro-chemical products eluted at 100.84 and 101.17 min, respectively, while the product from the pooled extract of *C. ubica* and *C. impicticornis* co-eluted with the second peak, confirming that the *trans*-(*Z*)-BE isomer possessed (4*S*) stereochemistry. As the microprobe NMR data had demonstrated the relative stereochemistry to be the *trans*-configuration, the absolute stereochemistry of the *trans*-(*Z*)-BE isomer was determined as (1*S*,2*R*,4*S*) or *trans*-(*Z*)-(4 S).

Olfactometer Assays of Synthetic Compounds Virgin *C. ubica* females were attracted to *cis*-(*Z*)-(4*S*)-BE (χ^2 test, $P<0.01$; Fig. 6A), *trans*-(*Z*)-(4*S*)-BE (χ^2 test, $P<0.001$; Fig. 6A), *cis*-(*Z*)-(4*R*)-BE (χ^2 test, $P<0.01$; Fig. 6A) and *trans*-(*Z*)-(4*R*)-BE (χ^2 test, $P<0.01$; Fig. 6A) compared to the hexane control. When females of *C. ubica* were tested with a mixture of *cis*-(*Z*)-(4*S*) and *trans*-(*Z*)-(4*S*)-BE in a 90:10 ratio (i.e., the same ratio produced by males of *C. ubica*) they preferred this mixture compared to a hexane control (χ^2 test, $P<0.001$; Fig. 6A), and when compared to *trans*-(*Z*)-(4*S*)-BE (i.e., the major component produced by *C. impicticornis*) (χ^2 test, $P<0.01$; Fig. 6A). However, female *C. ubica* did not discriminate between the 90:10 mixture, and *cis*-(*Z*)-(4*S*)-BE. In addition, *C. ubica* females preferred *cis*-(*Z*)-(4*S*)-BE when compared to *cis*-(*Z*)-(4*R*)-

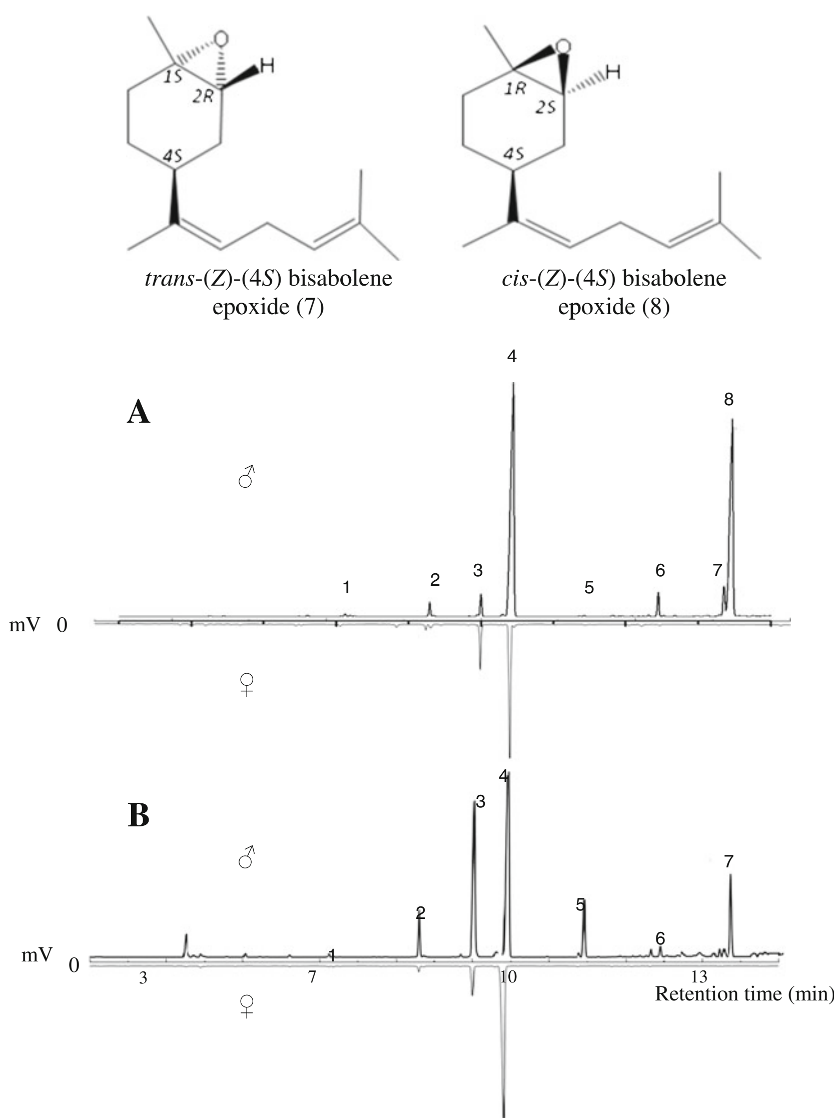

Fig. 5 GC analysis of aeration extracts collected from **a** virgin male and female *Chinavia ubica* and **b** virgin male and female *Chinavia impicticornis.* 1) Undecane 2) dodecane 3) (*E*)-2-decenal 4) tridecane 5) (*E*)-2-decenyl acetate 6) α-bisabolene, 7) *trans*-(*Z*)-(4*S*) bisabolene epoxide and, 8) *cis*-(*Z*)-(4*S*) bisabolene epoxide

BE (χ^2 test, $P<0.05$), but did not distinguish between *trans*-(*Z*)-(4*S*)-BE and *trans*-(Z)-(4*R*)-BE.

Virgin female *C. impicticornis* preferred *trans*-(*Z*)-(4*S*)-BE (i.e., the major component produced by male *C. impicticornis*) (χ^2 test, $P<0.01$; Fig. 6B), and its *trans*-(*Z*)-(4*R*)-BE enantiomer (χ^2 test, $P<0.001$; Fig. 6B), compared to the hexane control. However, females were not attracted to either *cis*-(*Z*)-(4*S*)-BE or *cis*-(*Z*)-(4*R*)-BE, and did not discriminate between *trans*-(*Z*)-(4*S*)-BE and *trans*-(*Z*)-(4*R*)-BE (Fig. 6B). Furthermore, *C. impicticornis* females were not significantly attracted to the 90:10 mixture of *cis*-(*Z*)-(4 S)-BE and *trans*-(*Z*)-(4*S*)-BE (i.e., the major components produced by male *C. ubica*), compared to the hexane control (Fig. 6B).

Electroantennogram (EAG) Responses GC-EAG using antennae from female *C. ubica* revealed responses to both *cis*-(*Z*)-(4*S*)-BE and *trans*-(*Z*)-(4*S*)-BE present in the aeration extract collected from virgin male *C. ubica* (Fig. 7A), and the *trans*-(*Z*)-(4*S*)-BE present in the extract from virgin male *C. impicticornis* (Fig. 7B). For antennae from *C. impicticornis* females, responses were recorded to the *trans*-(*Z*)-(4*S*)-BE present in the extract from virgin male *C. impicticornis,* and both *cis*-(*Z*)-(4*S*)-BE and *trans*-(*Z*)-(4*S*)-BE present in the aeration extract collected from virgin male *C. ubica* (Fig. 8A&B).

Discussion

Olfactometer bioassays with males and females of *C. ubica* and *C. impicticornis*, using live insects, showed that males produce and emit a volatile sex pheromone similar to attractant pheromones identified earlier for *Nezara viridula* (Borges, 1995; Tillman et al., 2010), *Euschistus heros* (Borges and Aldrich, 1994a), *Thyanta perditor* (Moraes et al., 2005a), *T. pallidovirens* (McBrien et al., 2002), *C. hilaris* (McBrien et al., 2001), *Tibraca limbativentris*

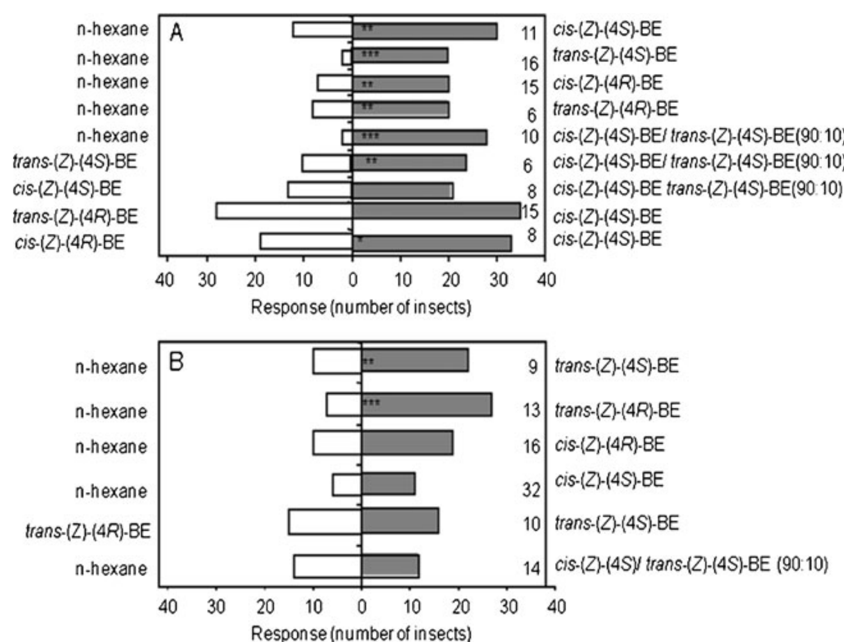

Fig. 6 Response (number of insects) of *Chinavia ubica* **a** and *Chinavia impicticornis* **b** females to synthetic *cis*-(*Z*)-(4*S*)-BE, *cis*-(*Z*)-(4*R*)-BE, *trans*-(*Z*)-(4*S*)-BE, *trans*-(*Z*)-(4*R*)-BE and to a mixture of *cis*-(*Z*)-(4*S*)-BE and *trans*-(*Z*)-(4*S*)-BE in the proportion found in *C. ubica* (90:10). Numbers on right hand side indicate number of insects that did not make a choice. Asterisks indicate a significant difference in the choices of insects. * $P<0.05$, ** $0.05<P<0.001$ and *** $P<0.001$ χ^2 test

(Borges et al., 2006), and *Piezodorus guildinii* (Borges et al., 2007). Chemical analysis of aeration extracts from male and female *C. impicticornis* and *C. ubica* showed the presence of three male specific compounds, i.e., (*Z*)-α-bisabolene, *cis*-(*Z*)-(4*S*)-BE, and *trans*-(*Z*)-(4*S*)-BE. These isomers have been identified in different populations of stink bugs; i.e., *Nezara* and *Chinavia* spp., around the world (Baker et al., 1987; Borges et al., 1987; Aldrich et al., 1989, 1993; McBrien et al., 2001). In addition to the male-specific compounds, a series of compounds common to both genders was identified: undecane, dodecane, (*E*)-2-decenal, tridecane, and (*E*)-2-decenyl acetate. (*E*)-2-Decenal and (*E*)-2-decenyl acetate are major metathoracic scent gland components in *Chinavia* spp. and *Nezara viridula,* whereas other stink bug species, such as *Euschistus* spp. and *Dichelops melacanthus,* produce greater amounts of (*E*)-2-hexenal, (*E*)-2-octenal, and (*E*)-2-octenyl acetate in their scent glands (Borges et al., 2007; Moraes et al., 2008). While the role of these compounds as kairomones for stink bug natural enemies has been established (Borges and Aldrich, 1994b; Laumann et al., 2009), their role in other aspects of stink bug chemical ecology still needs to be fully elucidated.

The specificity of the male-produced sex pheromone in stink bugs may be ensured by different relative abundances of *cis*- and *trans*-BEs (Brezot et al., 1994; Miklas et al., 2000; McBrien et al., 2001; Moraes et al., 2008). All populations of *Nezara viridula* studied to date from the U.S., Italy, and Brazil emit a ratio of *trans*-:*cis*-(Z)-BE between 2-4:1, whereas a Japanese population of *N. viridula* emits a 1:1 ratio of isomers (Aldrich et al., 1993). Males of *Chinavia* spp. typically produce the same compounds, but in an inverse proportion, with higher amounts

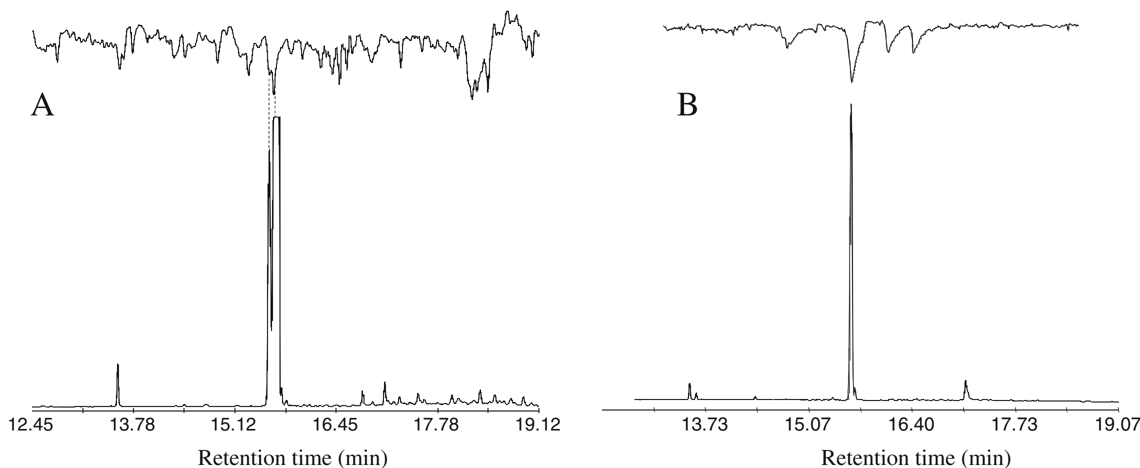

Fig. 7 Coupled GC-electroantenogram responses of **a** antennae from *Chinavia ubica* females to aeration extract from virgin *C. ubica* males; **b** antennae from *C. ubica* to aeration extract from virgin *C. impicticornis* males. Lower trace = flame ionization detector (FID) response. Upper trace = EAG response

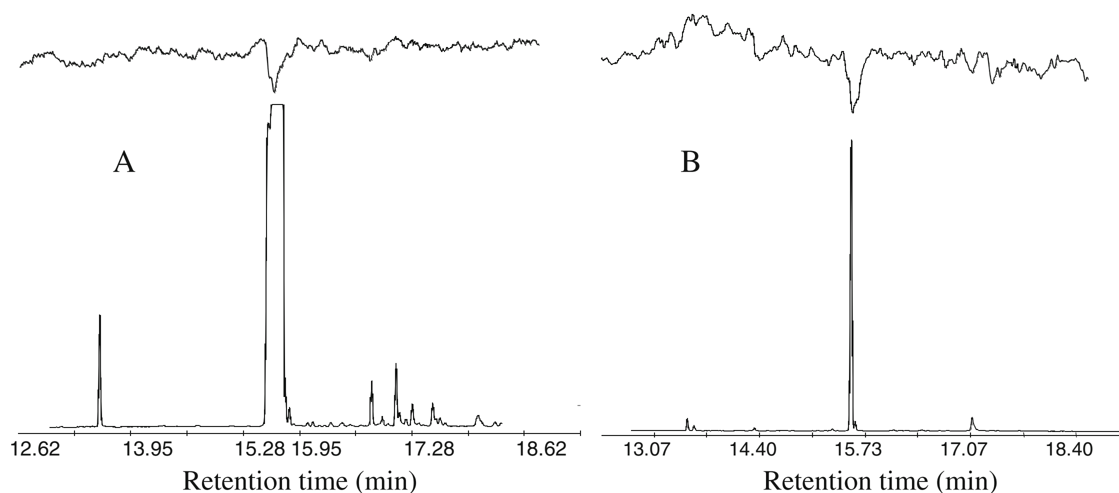

Fig. 8 Coupled GC-electroantenogram responses of **a** antennae from *Chinavia impicticornis* females to aeration extract from virgin *C. ubica* males; **b** antennae from *C. impicticornis* females to aeration extract from virgin *C. impicticornis* males. Lower trace = flame ionization detector (FID) response. Upper trace = EAG response

of *cis*-(*Z*)-BE or in the same proportions; for example, the U.S. species, *C. pennsylvanica,* emits a 1:1 ratio of *cis*- and *trans*-(Z)-BE (Aldrich et al., 1989, 1993). Therefore, as expected in this study, *C. ubica* emitted *cis*-(Z)-BE in higher amounts than *trans*-(Z)-BE. Unexpectedly, however, *C. impicticornis* emitted only a *trans*-(Z)-BE isomer, which chemotaxonomically suggests that this species may be closer to *Nezara* than to *Chinavia* (Aldrich, 1995; Borges, 1995; Moraes et al., 2008).

Electroantennography recordings with antennae from females suggest that *C. ubica* and *C. impicticornis* possess olfactory receptor neurons for both *trans*-(*Z)*-(4*S*)-BE and *cis*-(*Z)*-(4*S*)-BE. While this can be expected for *C. ubica*, where both compounds are utilised as sex pheromone components, the antennal response of *C. impicticornis* for *cis*-(*Z)*-(4*S*)-BE, despite the lack of a pheromonal role, suggests that *Chinavia* spp. stink bugs are able to locate and discriminate conspecifics through antennal perception of specific blends of *trans*-(*Z)*-(4*S*)-BE and *cis*-(*Z)*-(4*S*)-BE.

Other stink bug species appear to use pheromone stereochemistry to ensure that they will find the correct partner (Borges, 1995; Costa et al., 2000; McBrien, et al., 2001, 2002; Moraes et al, 2005a; Mori, 2007). Male *E. heros* produce only one isomer, methyl (2*S*,6*R*,10*S*)-trimethyltridecanoate, as a volatile sex pheromone, with the other possible isomers being either less active or inactive (Borges and Aldrich, 1994a; Costa et al., 2000). However, in field tests, a racemic mixture of all eight possible isomers attracts *E. heros* females, similar to live males or the synthetic methyl-(2*S*,6*R*,10*S*)-trimethyltridecanoate (Borges et al., 1998, 2011).

The results in this study show that, for *Chinavia* spp., as for other stink bug species, the specific attractant pheromone blends make a major contribution to reproductive isolation in sympatric species. An important additional discovery was that female *C. impicticornis* did not distinguish between *trans*-(*Z)*-(4*S*)-BE and *trans*-(*Z)*-(4*R*)-BE isomers. This suggests that the absolute stereochemistry of BEs may be less important for conspecific recognition than the relative stereochemistry between the epoxide group and the alkyl substituent on the bisabolene ring. The amount of pheromone produced also is probably important for conspecific recognition and reproductive success of these species (Aldrich, 1995). For stink bugs, in addition to specific pheromone composition and emission, the use of signals of other modalities such as vision, touch, and substrate-bone vibratory signals appear to be important for reproductive success (Borges et al., 1987, McBrien et al., 2002; Miklas et al., 2003; Moraes et al., 2005b; Polajnar and Colk, 2008). Knowledge of reproductive behavior of stink bugs, together with the other signals involved in the different reproductive steps, could help to develop optimized strategies for controlling these notorious global agricultural pests.

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Efficient Sex Pheromone Trapping: Catching The Sweetpotato Weevil, *Cylas formicarius*

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Abstract The sweetpotato weevil, *Cylas formicarius* (Fabricius) (Coleoptera: Brentidae), is the most serious pest of sweetpotato around the world, damaging sweetpotatoes in the field and in storage, as well as being a quarantine pest. Because the larval period is spent within vines or tubers, and the adults are nocturnal, chemical control frequently is not effective. In addition, there are few natural enemies, and pheromone-based trapping does not appear to reduce the damage level. In the present study, we evaluated a number of parameters that affect pheromone-based trap catch, including trap design, trap size, trap color, and height at which the traps are placed. Pherocon unitraps caught higher numbers than ground, funnel water, or delta traps. Medium-sized traps (13×17.5 cm) were more effective than larger or smaller traps. In a color-choice test, *C. formicarius* preferred red over gray, brown, blue, white, yellow, black, or red traps; light red was more attractive than other shades of red. Maximum catches were obtained when the traps were set 50 cm above the crop canopy. Light-red unitraps with pheromone lures caught more adults than identical traps without lures, suggesting that *C. formicarius* is influenced by both visual and olfactory cues. Pheromone-baited light-red unitraps, 13×17.5 cm, installed 50 cm above the crop canopy, were the most effective at catching *C. formicarius* adults, and they appear to have the greatest potential for use in trap-and-kill strategies and eradication programs.

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Introduction

The sweetpotato, *Ipomoea batatas* (L.) Lam. (Convolvulaceae), is one of the most important food crops in the world, particularly in emerging countries, where it is a food staple (Woolfe, 1992). Its production is severely affected by several insect pests (Chalfant et al., 1990), of which the sweetpotato weevil, *Cylas formicarius* (Fabricius) (Coleoptera: Brentidae), is the most serious worldwide (Austin et al., 1991). Feeding by this weevil elicits terpenoid production in sweet-potato storage tubers that results in damaged, unpalatable tubers (Uritani et al., 1975). Sutherland (1986) reported that yield losses due to *C. formicarius* damage range from 5 to 80 %. The weevil completes its life cycle within either the tubers or vines of the sweetpotato plant, but prefers tubers (Strong, 1983).

Because of the high incidence of *C. formicarius* on sweetpotatoes, some growers in the Mariana Islands of the South Pacific have become frustrated and given up cultivation entirely. Even small weevil populations can cause severe damage to tubers, especially because the pest is present throughout the year in warm conditions (Sutherland, 1986), with weevil incidence extremely high during the hot and humid season (Chalfant et al., 1990). In Micronesia, *C. formicarius* infestations are particularly severe. Our preliminary trapping studies conducted over a year indicate that populations are high throughout Guam. Guam and other Micronesian Islands are in the midst of a decline in sweetpotato production because of the impact of this weevil (Hwang, 2001). Anecdotally, according to local growers and authorities, thousands of sweetpotato plants in this

region have been damaged by *C. formicarius*. Although some control methods exist, chemical application is both undesirable and expensive. Without effective control, weevil populations are likely to cause very high or complete loss of sweetpotato production in Guam and other Micronesian Islands.

Chemical control in the field reduces *C. formicarius* numbers, but with varying degrees of success (Talekar, 1983). The protected position of the larvae, developing within the vines and tubers, limits the effectiveness of chemical treatments (Sutherland, 1986). Chalfant et al. (1990) reported a range of hormones and insect growth regulators that have varying effects on *C. formicarius,* but further research on these techniques is required. Cultural controls, such as the use of *C. formicarius*–resistant cultivars of *I. batatas*, non-infested planting material, and crop rotation, along with various management regimes, also reduce pest infestations (Jansson et al., 1987).

The cryptic feeding habits of the larvae, and the nocturnal activity of the adults make detection and control of infestations difficult. Resistant varieties of sweetpotato have not yet been used by growers (Downham et al., 2001). Sex-pheromone lures are effective in detecting infestations at low population levels, and for use as a component of control and management (Reddy and Guerrero, 2004, 2010). Coffelt et al. (1978) isolated and bioassayed the female-produced sex pheromone of *C. formicarius*. Heath et al. (1986) purified, identified, and synthesized the active component as (*Z*)-3-dodecen-1-ol (*E*)-2-butenoate*,* and field studies on the development of traps and lures for this species were carried out (Jansson et al., 1992, 1993). The electrophysiological and behavioral responses of *C. formicarius* showed the synthetic compound to be highly attractive in the field, highlighting its potential for use in pest control (Sureda et al., 2006). Mass trapping has suppressed populations of *C. formicarius* males in several countries (e.g.*,* Yasuda, 1995), although there has not always been concomitant reductions in the infestation rates or increases in sweetpotato yields (see, e.g., Braun and Van De Fliert, 1999). However, use of the pheromone in a pest-management strategy in India has resulted in a considerable reduction of damage, leading to a 53 % increase in production of marketable tubers (Pillai et al., 1993). Trapping also has reduced the number of roots damaged by *C. formicarius* in the Caribbean (Alcázar et al., 1997, Jackson and Bohac, 2006). It has been estimated that the use of pheromone-baited traps as part of an integrated pest-management program for *C. formicarius* could reduce one to three insecticide applications per season (Hwang, 2000).

Characteristics such as trap design, size, color, and height are known to influence the efficacy of pheromone-baited traps for other species of tropical weevils (Reddy et al., 2011). Therefore, we undertook field studies to evaluate the effects of trap characteristics that may increase the effectiveness of trapping programs for *C. formicarius*.

Methods and Materials

Experimental Field Sites Experiments were carried out on the island of Guam (USA) at 4 locations: Latte Heights (13°26′ N, 144°48′ E, 79.9 m above sea level), Dededo (13°30′ N, 144°51′ E, 96.9 m), Mangilao (13.43°N, 144.80°E, 54.3 m), and the University of Guam's Agricultural Experiment Station (AES) in Yigo (13.31°N, 144.52°E′, 138 m). The prevailing temperature, relative humidity, and wind velocity were recorded during the experimental period. Nearby sweetpotato fields were covered densely with various invasive plant species such as *Chromolaena odorata* (L.) R.M. King & H. Rob. (Asteraceae), *Panicum maximum* Jacq (Poaceae)*, Lantana camara* (L.) (Verbenaceae), and *Bidens alba* (L.) (Asteraceae). In addition, the morning glory, *Ipomoea triloba* (L.), known to be a major alternative host for C. *formicarius* (Austin et al., 1991), was present at most of the study locations.

Trap Design In the first experiment, four different types of traps, ground, funnel water, Pherocon unitrap, and Pherocon delta were evaluated. The ground trap (Fig. 1a) was constructed in our laboratory from a 120×60×0.5 cm piece of white corrugated plastic board, with a 50×8 cm slit baffle fitted at the top to prevent weevils from escaping (Reddy et al., 2005). Traps were sealed at all corners and along edges

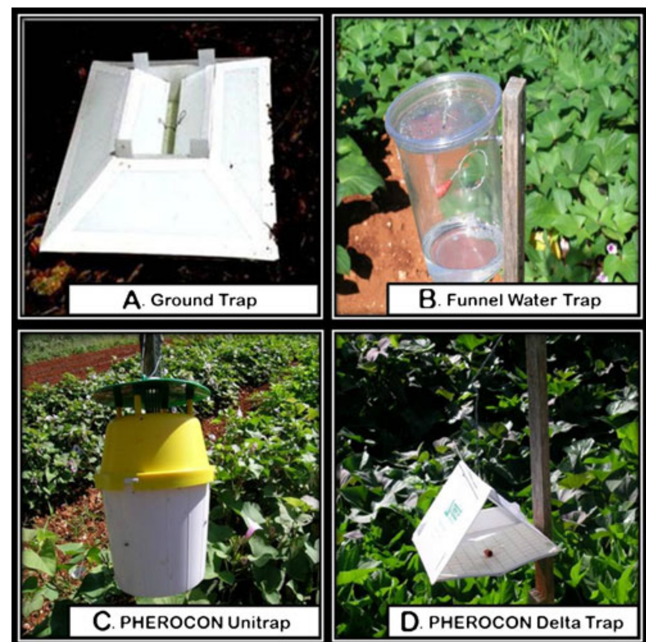

Fig. 1 The four trap designs used

with marine adhesive sealant, and water mixed with a liquid dishwashing detergent (1–3 %) was placed in the bottom container to retain adults. The lower outer edges of the ground traps were covered with earth to prevent weevils from crawling under them.

Funnel water traps (Fig. 1b) were commercially available from Trécé Incorporated (Adair, OK, USA). Each trap consisted of a transparent, covered plastic cup (1 L capacity) with two 3 cm circular holes in the sides, near the top; 250 ml of water mixed with detergent (1–3 %) were placed in the bottom of the cup. The trap was fitted with a wire linkage allowing it to be hung from a wooden post in the field.

The Pherocon unitraps (also called bucket traps), 20.5 cm high × 13 cm diam. also were obtained from Trécé Incorporated (Fig. 1c). Each unitrap consisted of a funnel-shaped white plastic receptacle, with a yellow plastic lid and holder for lures, mounted over a bucket for retaining captured insects. Because the unitrap incorporated a funnel ending in a pot from which insects could not escape, no detergent solution was used.

The Pherocon VI delta traps (Fig. 1d) also were from Trécé Incorporated. Each trap consisted of a sheet of white cardboard folded into a triangular tube with partially open ends. The sticky trapping surface was provided by an exchangeable card (18.5 cm^2) that slid in and out swiftly and easily. The partial end closures could be opened flat for counting of the catch and for exchange of trapping cards. The trap was fitted with a wire linkage allowing it to be hung from a wooden post in the field.

Pheromone Lures Pheromone lures of rubber septa loaded with *Z*3-dodecenyl-*E*2-butenoate, sealed in an impermeable bag for shipping and storage, were obtained from Chem Tica Internacional S.A. (San José, Costa Rica). The lure packs, each containing 10 mg of pheromone and emitting the active ingredient at <0.01 mg/day (Material Safety Data Sheet, ChemTica Internacional, S.A.), were stored at 4 °C until use. Lures were suspended on wires inside the ground traps, Pherocon unitraps, and funnel water traps. In the Pherocon delta traps, the lures were placed at the centers of the exchangeable sticky cards. Although *C. formicarius* pheromone can remain active in the field for 30–64 d (Jansson et al., 1992), our lures were changed at 30-d intervals, as recommended by Hwang (2000).

Effect of Trap Design The four trap types, with their pheromone lures, were placed at randomly chosen locations about 10 m apart in sweetpotato fields at the 4 test locations. Tests were replicated three times at each site to yield 12 replications. Traps without pheromone lures were used as controls. Overall, 96 traps were used: 8 treatments (4 trap designs, each with and without lures) × 3 replications × 4 sites. Each week, the trapped adult weevils were removed and counted and their numbers recorded. Traps were washed and rinsed, and new detergent solution added. We rotated trap positions weekly at each location to diminish positional effects on trap catch. The study was conducted from Feb–June 2010.

Effect of Trap Size In the second experiment, the effectiveness of four sizes of Pherocon unitraps (20.5 cm height × 18 cm diam., 20.5 × 13 cm, 17.5 × 7.5 cm, 11 × 9 cm) was compared. At each site, three traps of each size were set up and their positions rotated weekly. Tests were replicated three times at each site to yield 12 replications. The study used 48 traps (4 trap sizes × 3 replications × 4 sites). The experiment was conducted from July–Oct. 2010.

Effect of Trap Color In the third experiment, 13-cm diam. Pherocon unitraps were covered entirely with brown, black, gray, yellow, red, white, green, or blue vinyl tape and tested independently (8 traps colors × 3 replications × 4 sites) at the same sites. The experiment was carried out from Nov. 2010–Feb. 2011. Color characteristics of the tape were determined using a Konica Minolta CR-410 Chromometer (Minolta Instrument Systems, Ramsey, NJ, USA) and are given in Table 1.

Effect of Shade of Color In the fourth experiment, different shades of red (light red, tomato red, dark red, and candy-apple red) were evaluated. The trap used was again the 13-cm diam. Pherocon unitrap, and the shades were tested independently (4 shades of red × 3 replications × 4 sites) at the same sites. The experiment was conducted from Mar–June 2011. Different red trap-color measurement values are given in Table 2.

Effect of Trap Height In the fifth experiment, 60 (traps with 5 different heights × 3 replications × 4 sites) light red, 13-cm-diam. Pherocon unitraps were placed at ground level and at 50, 100, 150, and 200 cm above the crop canopy, 10 m apart. Trap height was adjusted according to crop growth in the field. Observations on trap catches were made at weekly intervals. The experiment was carried out from July–Sept. 2011.

Relative Effects of Visual and Olfactory Cues In the sixth experiment, light red, 13-cm-diam. Pherocon unitraps, placed 50 cm above the crop canopy, were baited with pheromone lures or left unbaited (1 trap with, and 1 without, lure × 3 replications × 4 sites = 24 traps). The experiment was carried out at the same sites from Oct.–Dec. 2011.

Statistical Analysis Trap catch data were transformed by log $(x+1)$ to fit the assumption of homogeneity of variance for

Table 1 Specifications of the colors of traps used

Trap color	L*	a*	b*	Chroma (C)	Hue angle (h°)
Black	30.44±0.06	0.42±0.03	−1.08±0.04	1.16±0.05	–
Brown	35.26±0.18	3.98±0.03	3.94±0.02	5.60±0.03	44.66±0.11
Gray	39.83±0.11	−0.17±0.02	−2.23±0.01	2.24±0.01	85.64±0.47
Yellow	82.57±0.02	−2.92±0.03	84.02±0.27	84.07±0.27	91.99±0.02
Red	42.84±0.11	49.88±0.28	19.44±0.20	53.54±0.34	21.29±0.09
White	92.29±0.03	1.34±0.01	−2.59±0.04	2.91±0.03	–
Green	43.50±0.08	−27.32±0.03	1.72±0.09	27.37±0.03	176.39±0.19
Blue	36.02±0.10	15.19±0.10	−35.82±0.12	38.91±0.14	292.98±0.08

Means (± SD) were generated from three observations

L* indicates a measure of "lightness" that runs through the center of the color chart; 100 at the top represents white, and zero at the bottom represents black

The a* axis, which runs left to right on the color chart, indicates a red shade when greater than zero (positive) and a green shade when lower than zero (negative). Similarly, the b* axis, which runs vertically through the color chart, indicates a yellow shade when positive and a blue shade when negative (Wrolstad et al., 2005)

Chroma is related to the saturation of a color, with lower chroma values being less saturated

The hue angle is expressed on a 360° grid on which 0°=red, 90°=yellow, 180°=green, and 270°=blue

ANOVA. Because all responses used were count variables, a one-way Poisson ANOVA model was fitted, by means of the GLIMMIX Procedure SAS Version 9.3 (SAS Institute, 2009). The least square means test was used to make multiple comparisons for differences among treatments.

Results

Effect of Trap Design Traps of all the designs baited with pheromone lures captured *C. formicarius* but differed in numbers of adults caught: mean catches were in the order Pherocon unitrap > funnel water trap > ground trap > Pherocon delta trap. Pherocon unitraps captured more adults than funnel water traps (F=22.4; df=7, 21; P<0.05; Fig. 2), and ground traps more than Pherocon delta traps. Traps without lures (control) caught no adults. During the experimental period, the average temperature was 28.5 °C, the average relative humidity 65–80 %, and the average wind velocity 5.8 m.sec^{-1}. The Pherocon unitrap, which caught more adults than all the other traps, was selected for all further experiments.

Effect of Trap Size The smaller and larger traps were less effective than medium-sized traps. Pherocon unitraps of 13 cm diam. caught more (F=8.6; df=3,23; P<0.05; Fig. 3) *C. formicarius* adults (59.7±1.2 adults/trap) than did the next larger size (16.9±0.8 adults/trap) and the two smallest size traps (29.1±1.3 and 32.0±1.6 adults/trap, respectively); catches in the two smallest traps did not differ

Table 2 Specifications of the shades of red used on traps

Trap color	L*	a*	b*	Chroma (C)	Hue angle (h°)
Candy-apple red	43.13±0.06	49.34±0.23	19.53±0.28	53.06±0.21	21.59±0.31
Tomato red	47.92±0.02	43.82±0.06	26.86±0.01	51.40±0.06	31.51±0.03
Light red	50.03±0.06	47.62±0.06	28.79±0.08	55.65±0.09	31.16±0.04
Dark red	42.76±0.06	43.06±1.20	19.67±0.02	47.34±1.09	24.56±0.61

Means (± SD) were generated from three observations

L* indicates a measure of "lightness" that runs through the center of the color chart; 100 at the top represents white, and zero at the bottom represents black

The a* axis, which runs left to right on the color chart, indicates a red shade when greater than zero (positive) and a green shade when lower than zero (negative). Similarly, the b* axis, which runs vertically through the color chart, indicates a yellow shade when positive and a blue shade when negative (Wrolstad et al., 2005)

Chroma is related to the saturation of a color, with lower chroma values being less saturated.

The hue angle is expressed on a 360° grid on which 0°=red, 90°=yellow, 180°=green, and 270°=blue

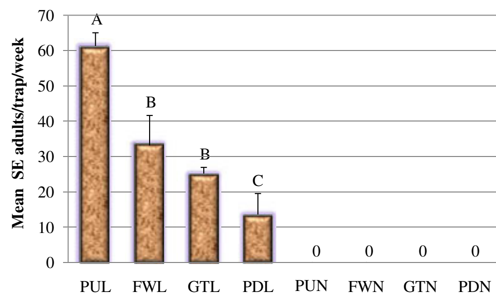

Fig. 2 Mean (± SE) numbers of adult *Cylas formicarius* caught by Pherocon unitrap with pheromone lure (PUL), funnel water trap with lure (FWL), ground trap with lure (GTL), Pherocon delta trap with lure (PDL), Pherocon unitrap without lure (PUN), funnel water trap without lure (FWN), ground trap without lure (GTN), and Pherocon delta trap without lure (PDN). Different capital letters indicate differences among treatments (two-way ANOVA with Poisson model, least square means, $P<0.005$). Means were generated from 12 replicates (3 replicates per location×4 sites)

from each other. Therefore, the 13 cm traps were used for all further experiments. During the experimental period, the average temperature was 30.2 °C, the average relative humidity 65–80 %, and the average wind velocity 5.2 m.sec^{-1}.

Effect of Trap Color Red unitraps caught more adult *C. formicarius* than those of any other color tested (F=11.31, df=7, $P<0.05$; Fig. 4), followed, in descending order, by grey, brown, blue, white, yellow, black and green traps; green traps caught fewer adults than any other. Therefore, red traps were used for all subsequent experiments. During the experimental periods, the average temperature was 27.8 °C, the average relative humidity 65–80 %, and average wind velocity was 4.4 m.sec^{-1}.

Effect of Shade of Red Light-red unitraps caught more adults (F=10.22, df=3, $P<0.05$; Fig. 5) than traps of other shades. Candy-apple red caught the fewest, whereas tomato

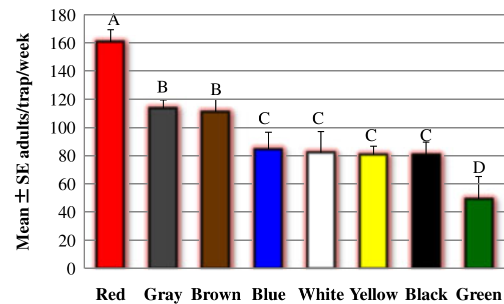

Fig. 4 Mean (± SE) numbers of adult *Cylas formicarius* caught in pheromone-baited Pherocon unitraps of different color. Different capital letters indicate differences among treatments (one-way ANOVA with Poisson model, least square means, $P<0.01$). Bars represent means of 12 replicates (3 replicates per location×4 sites)

red and dark red traps did not differ. Therefore, light-red traps were used for all subsequent experiments. During the experimental period, the average temperature was 29.4 °C, the average relative humidity 65–80 %, and the average wind velocity 6.2 m.s^{-1}.

Effect of Trap Height Traps installed 50 cm above the crop canopy caught more adults (F=8.14, df=4, $P<0.01$; Fig. 6) than traps positioned on the ground or at other heights. Therefore, traps were positioned at 50 cm above the ground height for the remaining experiment. During the experimental periods, the average temperature was 27.3 °C, the average relative humidity 65–80 %, and the average wind velocity 4.4 m.sec^{-1}.

Relative Effects of Visual and Olfactory Cues Light-red Pherocon Unitraps baited with pheromone lures caught more adults (mean of 1028.8±18.5) than those without lures (mean of 22.0±8.8; F=13.22, df=1, $P<0.001$). During the experimental period, the average temperature was 30.5 °C, the average relative humidity 65–80 %, and the average wind velocity 7.8 m.sec^{-1}.

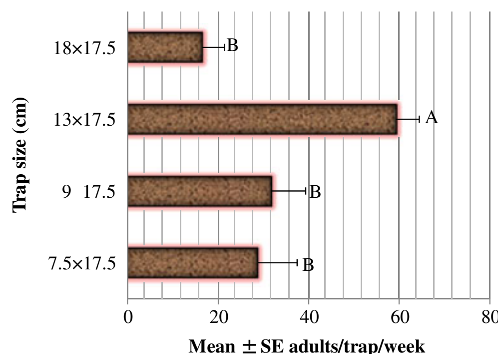

Fig. 3 Mean (± SE) numbers of adult *Cylas formicarius* caught in pheromone-baited Pherocon unitraps of different size. Different capital letters indicate differences among treatments (one-way ANOVA with Poisson model, least square means, $P<0.05$). Bars represent means of 12 replicates (3 replicates per location×4 sites)

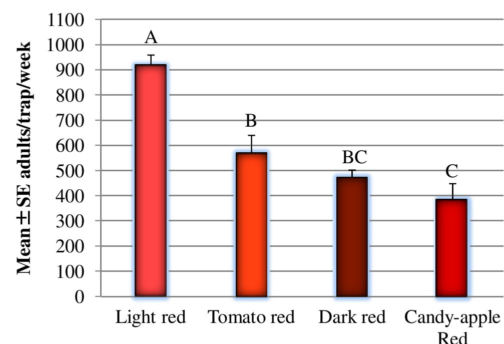

Fig. 5 Mean (± SE) numbers of adult *Cylas formicarius* caught in pheromone-baited Pherocon unitraps of different shades of red. Different capital letters indicate differences among treatments (one-way ANOVA with Poisson model, least square means, $P<0.01$). Bars represent means of 12 replicates (3 replicates per location×4 sites)

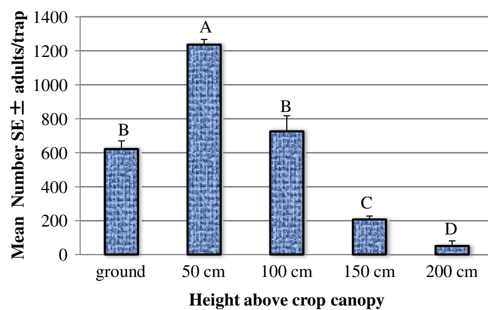

Fig. 6 Mean (± SE) numbers of adult *Cylas formicarius* caught in light red unitraps mounted at different heights in a sweet-potato field. Different capital letters indicate differences among treatments (one-way ANOVA with Poisson model, least square means, $P<0.05$). Bars represent means of 12 replicates (3 replicates per location × 4 sites)

Discussion

Nearly 10 years ago, Japanese scientists eradicated West Indian sweetpotato weevil, *Euscepes postfasciatus* (Fairmore) (Coleoptera: Curculionidae), from the Kume islands of Okinawa (Kuba et al., 2000) by means of the search and kill method. For *C. formicarius*, a similar approach should be considered. Because *C. formicarius* is more widespread in most sweetpotato growing areas than was *E. postfasciatus*, it will not be easy to eradicate. Pheromone-baited traps are useful for detecting the spread of *C. formicarius* and in alerting both growers and scientists to its presence, both in sweet-potato plants and in wild *Ipomoea* plants that harbor the weevil. Widespread indiscriminate destruction of *Ipomoea* spp. is not advisable, because these plants could be important to the local ecosystem, but infested plants around current or past sweetpotato plantings could be identified by means of pheromone-baited traps, and then uprooted, and burned.

Another approach, that of release of sterile males, is in use in the Okinawa islands for the control of *C. formicarius* and *E. postfasciatus* (Moriya, 1997), but this technique may have limited value because, unlike melon fly, *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae) (Koyama et al., 2004), *C. formicarius* adults are not especially active fliers. Like other members of the family Brentidae, *C. formicarius* adults crawl from place to place (Chalfant et al., 1990), limiting the extent to which sterile males might mate with normal females. While *C. formicarius* is not a strong flier, it readily mates when on foliage, and this is what is targeted with the male sterilization approach. In addition to *C. formicarius*, other Coleoptera also have been treated in sterile insect technique (SIT) programs, such as the cockchafer, *Melolontha vulgaris* L. (Scarabaeidae), and the boll weevil, *Anthonomus grandis* Boheman (Curculionidae), with remarkable results (Dyck et al., 2005). Nevertheless, an efficient pheromone-trapping technique is required for *C. formicarius*, either to use in conjunction with SIT programs, or for management or eradication.

According to Jackson and Bohac (2006), although current trap designs are effective for monitoring *C. formicarius*, they are cumbersome, difficult to sustain, or costly. Those authors suggested that more work is needed to develop simple, inexpensive, and effective traps for monitoring *C. formicarius*. Our results showing that Pherocon unitraps were more effective than other types for *C. formicarius* apparently contrast with the study of Smit et al. (1997), who reported that Unitraps performed relatively poorly for the congeneric *C. puncticollis* (Boheman) and *C. brunneus* F. (Coleoptera: Curculionidae). They also reported that Unitraps were commercially available, but were too expensive for routine use in Uganda. Although, the trap design we used was identical to that in their study, the different relative trap efficiencies may be due to differences in behavior between the African sweetpotato weevils they studied and *C. formicarius*.

Of the 10 trap designs evaluated by Jackson and Bohac (2006), a funnel trap (a modification of a water-pan trap) and the Pherocon sticky trap were the most effective for capturing adults. The better performance of the unitraps, compared to the generic water funnel trap in our study, may have been due to the continuous rainfall received by our study areas. Simple water-pan traps often are used in developing countries (Pillai et al., 1993), but we found them to be the least effective.

In our study, the medium-sized (13-cm diam.) trap outperformed both larger and smaller traps. The importance of trap size for other weevil species has been highlighted in a previous study (Reddy et al., 2011). Trap-catch differences for *Arhopalus rusticus nubilus* (Le-Conte) (Coleoptera: Cerambycidae) and *Xyleborus* spp. between 8- and 16-unit traps may be related to differences in trap surface area for interception of beetles, or to preference for taller vertical silhouettes (Hoover et al., 2000). In contrast, catches of the reproduction weevil, *Hylobius pales* Herbst (Curculionidae), in 16-unit traps were 54 % lower than those in 8-unit traps (Miller and Crowe, 2009).

The study reported here is the first to demonstrate a color preference by *C. formicarius,* although the importance of color preference by nocturnal insects and other curculionid weevils has been demonstrated previously (Reddy et al., 2011). Abdallah and Al-Khatri (2005) reported that more adult *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae) were attracted to red and orange, than to blue, traps. Our findings differ from those of Smit et al. (1997), who observed that trap color was not critical for *C. puncticollis* and *C. brunneus*, although red traps had lower catches than yellow and white traps, which were the predominant colors available in Uganda.

In our study, traps installed 50 cm above the crop canopy had higher catches than those at other heights. These results differ from those of Yasuda et al. (1992), who reported that sticky traps, at the same height as the crop canopy, caught more *C. formicarius* than traps at heights up to 300 cm. Because nocturnal weevils habitually crawl to the topmost leaves of sweetpotato plants (Proshold et al., 1986), pheromone traps surrounded by sweetpotato foliage should capture more insects than traps that are not surrounded (Jansson et al., 1992). Trap height is known to influence capture of other weevil pests. For example, Faleiro (2006) reported the greatest *R. ferrugineus* catches at 1.0 m above the ground. Smit et al. (1997) noted that raising the trap until the entrance holes were 15 or 30 cm above the canopy improved catches of *C. puncticollis*, whereas catches of *C. brunneus* were unaffected by trap height. Our results on trap height effectiveness contrast with those on *C. formicarius* by Proshold et al. (1986), who reported that raising the trap above crop height critically reduced adult catches.

Visual cues alone influence the behavior or catches of weevil populations (Reddy and Raman, 2011). In the present study, we found that light red traps caught more *C. formicarius* than other traps. We suggest that trap color could be important because the flight activity of *C. formicarius* may occur during crepuscular times. Björklund et al. (2005) reported that traps baited solely with odor or solely with visual stimuli catch more pine weevils, *Hylobius abietis* (L.) (Coleoptera: Curculionidae), than stimulus-free traps. Odor and visual stimuli also have been shown to have additive effects on trap catch (Reddy, 2012), and traps with a combination of odor and visual stimuli catch more weevils than traps with odor or visual stimuli alone (Björklund et al., 2005). In the current study, traps baited with pheromone captured more *C. formicarius* than did identical traps without pheromone. This result is in agreement with previous observations on several other weevils that use olfactory (pheromones), rather than visual, cues (see Reddy et al., 2011, and references therein), although Tansey et al. (2010) reported that the cabbage seedpod weevil, *Ceutorhynchus obstrictus* (Marsham) (Coleoptera: Curculionidae) also is influenced by visual cues. Reeves (2011) described the importance and use of vision for locating host plants, with some recent examples showing that vision can be even more important than olfaction.

We conclude that trap design, size, color, and height affect the response of *C. formicarius* to pheromone-baited traps. In particular, the 17.5×13-cm, light-red Pherocon unitraps baited with pheromone lures and installed 50 cm above the crop canopy gave the highest catches of *C. formicarius*. These results are useful and should be taken into consideration when trap-and-kill strategies are developed.

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Influence of Diet on Fecundity, Immune Defense and Content of 2-Isopropyl-3-Methoxypyrazine in *Harmonia axyridis* Pallas

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Abstract Food type can affect all functional aspects of an insect's life. We investigated the effects of different diet regimes on life history parameters of the ladybird beetle *Harmonia axyridis.* Furthermore, we tested the importance of elytral color, sex, and diet on chemical and immune defense in this species. We also compared hemolymph from cohorts of *H. axyridis* and *Coccinella septempunctata* (Coleoptera: Coccinellidae) fed different diets to examine effects on the 2-isopropyl-3-methoxypyrazine (IPMP) content in these beetles. No effects of diet on the duration of larval development and on adult weight were found. We detected, however, significantly higher fecundity and oviposition rates when female *H. axyridis* were reared on pea aphids than when reared on eggs of *Ephestia kuehniella*. Males and females did not differ in their immune response. Elytral color affected both immune defense and chemical defense. The antimicrobial activity of the hemolymph differed only when morphotypes were tested against *E. coli*. Moreover, we observed an effect of elytral pigmentation on IPMP content. The *succinea* 2 type (orange without dots) had the lowest IPMP content in two out of three feeding regimes compared to the *succinea* 1 (orange with dots) type. Depending on diet, IPMP contents differed in both species leading to higher contents either in *H. axyridis* or *C. septempunctata.* Furthermore, aphid species ingested during larval development significantly affected IPMP content in adult beetles. These results implicate new aspects for risk assessment of *H. axyridis* in viticulture.

Keywords IPMP · Elytral color · *Acyrthosiphon pisum* · Aphids · *Coccinella septempunctata* · *Ephestia kuehniella* · Immune system · MIC tests · Coleoptera · Viticulture · Insect pathogen · Wine · Antimicrobial

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Introduction

Ladybird beetles (Coleoptera: Coccinellidae) have a broad prey range that includes aphids, psyllids, scale insects, lepidopteran eggs, and chrysomelids (Hodek and Honek, 1996). At high densities, when prey is scarce or of poor quality, cannibalism is common for some species (Osawa, 1993; Snyder et al., 2000). Diet has, nevertheless, a significant impact on the performance of coccinellids (Specty et al., 2003; Lanzoni et al., 2004, Soares et al., 2005; Berkvens et al., 2008; Jalali et al., 2009). Despite their polyphagy, coccinellids often are specific with regard to their essential food (Hodek, 1993). The aphid species preyed upon and the plant species on which these prey aphids feed can affect larval developmental time, adult longevity, body weight, and fecundity (Hukusima and Kamei, 1970; Fukunaga and Akimoto, 2007).

The Multicolored Asian ladybird beetle, *Harmonia axyridis,* is native to northeast Asia. It has been introduced to different regions of the world (i.e., France or USA) as a biocontrol agent (Tedders and Schaefer, 1994; Ferran et al., 1996) and became widely established in Europe and America (Koch, 2003). In the fall and prior to hibernation, it feeds on sugar-containing fruits, especially grapes (Galvan et al., 2008). In contrast, the seven-spot ladybird beetle *Coccinella*

septempunctata, a coccinellid beetle native to Europe and invasive to the USA, is a more aphid-specific predator (Hodek and Honek, 1996).

Harmonia axyridis is a polymorphic species, and some phenotypes differ in their fitness. The variable pigmentation of the elytra in *H. axyridis* results in more than 100 recognizable color morphs (Soares et al., 2003). Phenotypes can be divided into five groups: non-melanic *succinea* (red with 0–19 black dots), melanic *conspicua* (black with two red dots), *spectabilis* (black with four red dots), *axyridis* (more than four red dots), and entirely black *nigra* morphs (Soares et al., 2001). This striking feature has led to a number of studies that have examined differences in mating and predatory behavior (Osawa and Nishida, 1992; Seo et al., 2008; Su et al., 2009; Sloggett, 2010) and physiology (Soares et al., 2001, 2005; Bezzerides et al., 2007; Berkvens et al., 2008) for the different phenotypes of this coccinellid species. Moreover, a positive relationship of elytral redness with the content of an alkaloid defense compound (harmonine) in the beetles was reported by Bezzerides et al. (2007). Contrary to chemical defense against predators and parasitoids, immune defense in Coleoptera has received less attention (Bulet et al., 1991; Gross et al., 2008; Haine et al., 2008). Armitage et al. (2003) found no evidence for cuticular melanin content and costs of immune defense in meal worm beetles (Tenebrionidae). For other insects, namely Lepidoptera, some studies have shown a correlation between the amount of cuticle melanization and immune function, while others have reported a lack of such correlation (Wilson et al., 2001; Lee et al., 2008; Karl et al., 2010). To date, no study has related elytral color to immune defense in *H. axyridis*.

The hemolymph of coccinellid beetles contains defensive substances against natural enemies (Klausnitzer and Klausnitzer, 1997). Additionally, some of these substances may act as aggregation pheromones (Al Abassi et al., 1998) or belong to the beetle's immune system. For *H. axyridis*, Gross et al. (2010) observed stronger antimicrobial activity in their hemolymph than in *C. septempunctata*. The main defensive chemicals identified in the hemolymph of *H. axyridis* and *C. septempunctata* are methoxypyrazines (and therein mainly 2-isopropyl-3-methoxypyrazine (IPMP)) (Al Abassi et al., 1998; Pickering et al., 2004; Cai et al., 2007; Kögel et al., 2012b). During grape harvesting and processing, beetles often get crushed or reflex bleed. This causes a specific off-flavor in wine called "ladybird taint" (Pickering et al., 2004, 2005; Galvan et al., 2007; Ross et al., 2010; Kögel et al., 2012a). The influence of food on chemical defense compounds in coccinellid beetles' hemolymph is currently unknown. Thus, this study examined the effect of diet on the content of 2-isopropyl-3-methoxypyrazine and antimicrobial compounds in the hemolymph of *H. axyridis* and *C. septempunctata*.

The performance and IPMP content of *H. axyridis* and *C. septempunctata* fed on eggs of *Ephestia kuehniella* were compared to beetles reared on pea aphids (*Acyrthosiphon pisum*). For this purpose, we evaluated different fitness parameters between the beetles fed on either of the two diets in the laboratory according to Gross et al. (2004a). Additionally, we investigated the influence of several other aphid species for *H. axyridis* on IPMP content in the wild. Finally, we controlled for correlation of elytral pigmentation and antimicrobial activity in the hemolymph of *H. axyridis* by comparing the phenotypes prevailing in our laboratory colony against three different model microorganisms and an insect pathogen.

Methods and Materials

Insects Permanent laboratory colonies of *H. axyridis* and *C. septempunctata* were maintained under controlled conditions (alternating temperature regime during one week (168 h): 100 h at 25°C, 68 h at 20°C ; 16:8 hL/D, 60 % rh) in a climate chamber at the Julius Kühn-Institute (JKI), Dossenheim, Germany. Groups of about 100 larvae were kept either (1) on a diet of pea aphids (*Acyrthosiphon pisum*) that were reared on beans (*Vicia faba,* tannin reduced cultivar "Tattoo"), (2) on *A. pisum* and grape juice, or (3) on frozen eggs of meal moths (*Ephestia kuehniella,* provided by Koppert Biological Systems, The Netherlands) until pupation. All cohorts received water by soaked cotton pads *ad libitum*. In rearing dishes (50× 40×12 cm), the larvae were kept from egg hatching until pupation. Mortality, developmental time, and numbers of pupae were recorded daily. After emergence, the newly hatched adults were sexed and weighed on a balance (MC 210S, Sartorius, Germany). The beetles were kept in rearing cages (40×30×30 cm) and fed the same diet as during the larval stage until they were used for the analysis of their hemolymph (antimicrobial activity, IPMP content). Preimaginal survival was monitored as the number of adults obtained from the pupae of each cohort. Fecundity and other oviposition parameters were monitored for a total period of 49 d. Fifteen pairs of beetles for each food treatment (aphids or *E. kuehniella* eggs) were kept individually in Petri dishes (9 cm diam). The number of eggs and egg clutches were recorded daily. Males were replaced when they had died.

For some experiments, adult beetles were divided into three different morphological groups based on elytral coloration: 1) melanic *spectabilis* morphs: black elytra with two red dots on either elytron, 2) *succinea 1* morph with many intensively black dots on orange colored elytra, and 3) *succinea 2* morphs with none or very few, faint black markings on orange elytra. These three morphotypes occurred in our laboratory colony and in the field populations found in the vegetation surrounding the facilities of the Julius Kühn-Institut, Dossenheim.

Field-collected Beetles For IPMP analysis, pupae of *H. axyridis* were collected in April 2011 in South Germany (Dossenheim, Siebeldingen and surroundings) from leaves of *Prunus persica, P. cerasus, P. spinosa*, *P. domestica, P. mahaleb, Malus domestica, Acer platanoides, Hedera helix, Sambucus nigra*, and *Corylus avellana.* After eclosion, adults were kept without food (experiment 1) or fed the particular aphid species found on this specific host plant that had also served as prey during larval development (experiment 2). Aphids were identified as *Myzus varians* on *P. persica, Brachycaudus helichrysi* on *P. domestica, Dysaphis plantaginea* on *M. domestica*, *Drepanosiphon platanoides* on *A. platanoides*, *Aphis hederae* on *H. helix,* and *A. sambuci* on *S. nigra*. The aphid species on the remaining plants could not be identified.

In experiment 1, the IPMP contents of the hemolymph of the adult ladybird beetles were analyzed 3 d after emerging without providing any food for the hatched adults. In experiment 2, newly hatched adults were fed for 5 d with aphids obtained from the respective plants where pupae were collected. After this feeding period, the IPMP content of these beetles was measured by GC-MS analyses. Additionally, 30 adults each of *H. axyridis* and *C. septempunctata* were collected on grape berries of several *Vitis* hybrids from a vineyard around Geilweilerhof, Siebeldingen (September 2011) and their IPMP contents were also analyzed.

Microorganisms Gram positive *Bacillus subtilis* and *B. thuringiensis* ssp. *tenebrionis* (strain 10 BI 256–82), gram negative *Escherichia coli* (K12/D31), and the yeast *Saccharomyces cerevisiae* (DSM 70499) were used as model test organisms. All strains were obtained from the collection at the Julius Kühn-Institut, Dossenheim. Prior to the assays, liquid Mueller Hinton Broth was inoculated with test bacteria; yeast was cultivated in Sabouraud Dextrose Broth. Population growth of the overnight cultures (28°C) was monitored through its optical density as absorbance at 600 nm. Prior to the assays, each culture was diluted to a final concentration of 10^6 CFU.

Determination of Antimicrobial Activity in the Hemolymph of Harmonia axyridis The antimicrobial activity in the hemolymph of adult *H. axyridis* was evaluated with broth dilution assays in 96-well microtiter plates (Sarstedt). The assays to determine the minimal inhibitory concentration (MIC) of each hemolymph pool were conducted as described in Gross et al. (2010). Hemolymph was collected from live, unchallenged adults. Insects were used for the assays after they had reached sexual maturity, i.e., after the onset of oviposition in each cohort. Beetles were separated by sex, elytral color, and diet. We prepared 40 μl hemolymph solutions at a concentration of 5 μl hemolymph /ml sterile water. This stock solution was applied to the first well of a serial dilution plate, and then diluted 1:2 with each of the following pipetting steps until the 12th well in each row of the plate. The amount removed from the last well was discarded. Aliquots of 100 μl of bacterial or fungal cultures were added to each of the 96 wells. Depending on the amount of hemolymph obtained from the beetles for each hemolymph pool, two or three rows were filled per test factor and microorganism. All tests were done in four independent replications. In each assay, the growth inhibition of all test organisms was compared to an antibiotic as a positive reference. Starting concentrations of 50 μg gentamycin/ml sterile water and of 50 μg nystatin/ml dimethylsulfoxid were used, respectively, as controls for bacterial and fungal growth inhibition. Test plates were incubated in a shaker (160 rpm) at 28°C. After 20 h, growth inhibition was evaluated visually as clear wells. This visual control was confirmed with a spectrophotometer through changes in absorption at 600 nm (Microplate Reader Fluostar Omega, BMG Labtech, USA). The minimal inhibitory concentration (MIC) is defined as the minimal concentration of hemolymph or antibiotic that causes complete growth inhibition of the microorganism tested (DIN norm 58940). For each sample, the most abundant minimal inhibitory concentration for the rows from each hemolymph pool and the four replications was taken as MIC for the tested factor and microorganism.

Test of Antimicrobial Properties of Methoxypyrazine Compounds In order to test for potential antimicrobial properties of methoxypyrazine compounds present in the hemolymph of *H. axyridis*, we used commercially available 2-isobuthyl-3-methoxypyrazine (IBMP), IPMP, and 2-secbuthyl-3-methoxypyrazine (SBMP) (SIGMA Aldrich Nr. 297666, Nr. 243132 and Nr. 243116) at a concentration of 1 μg/ml, as these compounds were previously identified in the hemolymph of *H. axyridis* (Cai et al., 2007; Cudjoe et al., 2005; Kögel et al., 2012b). The solutions were used in MIC assays in concentrations of 1 μg/ml and 0.01 μg/ml (diluted 1:100 in a water/ethanol (1:1) mixture) against the four microorganisms mentioned above. Four replications were carried out for each substance and concentration.

Comparison of Diet Effects on the Chemical Defense (IPMP content) of H. axyridis and C. septempunctata Beetles were weighed and sexed prior to the analyses. A single beetle was subsequently crushed in 10 ml of Milli-Q-water (Millipore Corporation, USA) in a mortar. The sample then was transferred to a 20 ml round bottom glass headspace vial. Three g of sodium chloride and 10 ng of an internal standard (2-isopropyl-3-ethoxypyrazine (IPEP)) were added. For each feeding group and beetle species or morphotype, 10 replications were done.

Headspace-sampling and Chemical Analysis Volatile compounds present in the hemolymph of both ladybird species

were extracted using headspace-solid phase microextraction (HS-SPME). Beetle headspace was sampled using a divinylbenzene/carboxen/polydimethylsiloxane fiber (Sigma Aldrich Nr. 57328-U DVB/Carboxen/PDMS) and a manual fiber assembly. The sample vial was heated to 40°C for 20 min in a water bath before fiber exposure to request a subsequent headspace equilibration. After exposure in the headspace for 30 min at a constant temperature of 40°C under continuous stirring, the fiber was thermally desorbed in a GC injection port in splitless mode (Agilent 6890) at 250°C for 2 min and further thermally cleaned for 3 min with 10 ml/min split flow. Coupled gas chromatography–mass spectrometry (GC-MS) was used for analyses. Pyrazines were separated by the following procedure: the GC program was started at 40°C and held for 6 min; then temperature was raised to 100°C at 15°C/min; at 3°C/min to 160°C; at 25°C/min to 200°C; and finally temperature was held at 200°C for 5 min. The column was a DB-Wax (30 m length, 0,250 mm I.D., 0.50 µm film thickness; J&W Scientific). Helium was used as carrier gas. For the first 12 min, an FID was used and afterwards the system was switched to a MS (Agilent 5975B) in SIM Mode. The selected mass channels were *m/z* 137 and 152 for IPMP and 137 and 151 for IS.

GC-Data Analysis Data peaks were analyzed using the ChemStation D.02.00.611 software (Agilent Technologies). Identification of IPMP in beetles' hemolymph was done by comparing mass spectra and retention time with synthetic IPMP as reference substance. Concentrations of the reference samples were similar to those found in target samples. Calibration curves were prepared for five concentrations ranging from 0.5, 1.0, 1.5, 2.0 and 2.5 ng of IPMP and IPEP as internal standard per liter in water solution. Quantification was done by comparing the peak areas of IPMP and internal standard in counts. The standard addition method was used to identify possible matrices effects. Due to equal extraction methods, absolute and relative quantities of IPMP could be calculated. The contents are represented as ng IPMP/g fresh weight of beetles.

Statistical Analysis The numbers of eggs and egg clutches, adult body weight, and larval development times were statistically compared between different treatments (food) with Mann–Whitney *U*-tests (Sachs, 1992). The sex ratio and percentage preimaginal survival of beetles reared on different diets were compared by chi^2-tests. Due to normality and homogeneity of data, the comparisons of IPMP contents between different treatments were analyzed by ANOVA followed by *post-hoc*-tests of Least Significant Difference between means (LSD). Statistical analyses were done with SPSS statistics software 19 (IBM 2011).

Results

Diet Effects on Life History Parameters and Fecundity of Harmonia axyridis The total duration of the larval developmental time (i.e. four larval stages) was not different for the two diet regimes (Table 1, Mann–Whitney *U* test, diet 1 $N=104$ pupae obtained, diet 2 $N=166$ pupae obtained, $P>0.05$). Preimaginal survival ($\chi^2=0.09$, $df=1$, $P>0.05$), adult weight (Mann–Whitney *U* test, $N>30$ beetles per sex and diet, $P>0.05$), and sex ratio ($\chi^2=0.42$, $df=1$, $P>0.05$) were also similar (Table 1). By contrast, all fecundity parameters differed significantly between aphid and *E. kuehniella* eggs diets (Table 1). Aphid fed female *H. axyridis* laid on average more than twice the number of egg clutches (Mann–Whitney *U* test, $N=15$, $P<0.001$), and the total number of eggs doubled the eggs laid by *E. kuehniella* egg fed females (Mann–Whitney *U* test, $N=15$, $P<0.001$). Thus, fecundity (eggs/female) and oviposition rate (eggs/female/day) was more than twice as high for aphid fed beetles than for egg fed beetles (Table 1 and Fig. 1, Mann–Whitney *U* test, $P<0.001$). Survival of female beetles in the oviposition trials did not differ between diets.

Determination of the Antimicrobial Activity in the Hemolymph No difference in the antimicrobial activity of the hemolymph between female and male beetles was observed. Thus, data were combined for both sexes in Fig. 2. Independent from the diet ingested, antimicrobial activity in the hemolymph of *H. axyridis* was most effective against *S. cerevisiae*, followed by gram negative *E. coli*, intermediate against gram positive *B. subtilis*, and least effective against *B. t. thuringiensis* (Fig. 2). Since differences in MIC values of one dilution step up or down lay within the normal variation of this serial dilution test, only differences in MIC values against the growth of *E. coli*, spanning various dilution steps, which were observed for *succinea 2* morphs, can be regarded as a true effect of diet. No other differences in antimicrobial activity between the two diets or between color morphs were observed. Furthermore, no interactions of elytral color and feeding regime on antimicrobial activity in the hemolymph was found. According to DIN norm sheet 58940, a statistical analysis of MIC values is neither necessary nor possible (Deutsches Institut für Normung, 2010).

H. axyridis succinea 2 fed with aphids had lower MIC values against *E. coli* than *succinea 1*. In general, we observed more variability between morphs in the measurable growth inhibition for aphid reared beetles than for the beetles reared on *E. kuehniella* eggs (Fig. 2). Neither of the

Table 1 Life history parameters for *Harmonia axyridis* under two different feeding regimes. Larvae and adults were fed either live individuals of *Acyrthosiphon pisum* reared on *Vicia faba* or frozen eggs of *Ephestia kuehniella*. Two generations were compared for each diet

	A. pisum	*E. kuehniella*
Larval developmental time (d)	12.47±0.41[a]	11.94±0.74[a]
Preimaginal survival (%)	94.00[a]	89.45[a]
Adult weight (g) male	0.030±0.005[a]	0.030±0.004[a]
Adult weight (g) female	0.032±0.005[a]	0.033±0.004[a]
Sex ratio (% female)	48.4[a]	40.2[a]
Total number of egg clutches	218[a]	101.5[b]
Total number of eggs (N=15)	10,885[a]	5,193.5[b]
Mean number eggs/d	222.2±133.5[a]	106±73.2[b]
Fecundity (N=15) (total eggs/female during 49 d)	788.8[a]	384.5[b]
Oviposition rate (N=15) (eggs/female/d)	16.1±9.3[a]	7.9±5.5[b]

Data presented are mean values +/- standard errors. [a], [b]indicate significant differences (P<0.05).

pyrazine solutions caused a detectable growth inhibition in any of the four microorganisms tested.

Differences in IPMP Contents between C. septempunctata and Morphotypes of H. axyridis within One Feeding Group The contents of IPMP differed significantly between the different morphotypes of *H. axyridis*: *H. axyridis succinea* 2 and *H. axyridis spectabilis* contained less IPMP than *succinea* 1 when fed with *A. pisum* or on *E. kuehniella eggs* (LSD; P<0.05; Fig. 3). *C. septempunctata* contained significantly less IPMP than *H. axyridis* when fed on *A. pisum*. When fed additionally on grape juice, the IPMP content of *C. septempunctata* increased significantly (Fig. 3), and was higher than in all morphotypes of *H. axyridis*. When fed on *E. kuehniella*, no differences could be observed between *C. septempunctata* and *H. axyridis succinea* 1 and *H. axyridis spectabilis* (Fig. 3). No differences in IPMP contents between males and females of the same feeding group and with the same elytral color were detected (LSD; P>0.05). Likewise, no differences in IPMP contents could be measured between *H. axyridis* and *C. septempunctata* adults collected from grapes in September 2011 (Mann–Whitney-U test; P>0.05).

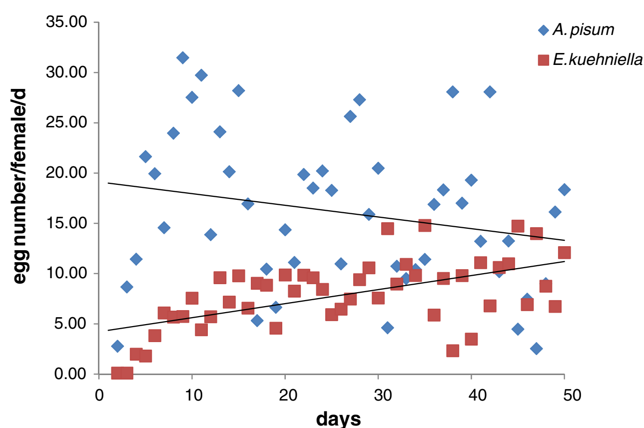

Fig. 1 Oviposition rate depicted as eggs/female/d laid under two different diet regimes, either live *Acyrthosiphon pisum* aphids or frozen eggs of *Ephestia kuehniella*, for 15 females of *Harmonia axyridis*. Eggs were counted for a total period of 49 days. Mann–Whitney U test, P<0.001. A linear trend line is given

Differences in IPMP Contents of Morphotypes of H. axyridis and C. septempunctata between the Feeding Groups and on Different Host Plants In beetles reared in the laboratory on *A. pisum, A. pisum*+grape juice and *E. kuehniella* eggs, significant differences in IPMP contents could be measured (Fig. 3). IPMP contents of all morphotypes of *H. axyridis* fed with *E. kuehniella* were higher than when fed on '*A. pisum*' or '*A. pisum*+grape juice' (LSD; P<0.05; Fig. 3). A diet of *A. pisum*+grape juice increased the IPMP contents of *C. septempunctata* in comparison to other diet treatments (LSD; *A. pisum*: P=0.03; *E. kuehniella*: P=0.016).

Significant differences in IPMP contents could be detected in adults that emerged from pupae of *H. axyridis* previously collected in the field on host plants with specific aphid prey: Larvae fed with aphids collected on *P. domestica* (*Brachicaudus helichrysi), P. mahaleb* (unknown aphid species), *C. avellana* (unknown aphid species), and *S. nigra (Aphis sambuci)* contained significantly less IPMP than larvae fed with aphids from *P. persica (Myzus varians)* (LSD; P<0.05; Fig. 4). In addition, larvae fed with *A. sambuci* (*S. nigra)* and aphids from *C. avellana* had lower IPMP contents than larvae fed on *D. platanoides* (*A. platanoides)* (LSD; P<0.05; Fig. 4).

No differences were found in IPMP contents when adults were fed on the respective host aphids for another five days after eclosion (Mann–Whitney-U-test; P>0.05).

Discussion

Under prevailing conditions in our climate chamber, we found a significant effect of diet on the fecundity of *H. axyridis* females. Fecundity of *H. axyridis* was higher under a diet of pea aphids compared to *E. kuehniella* eggs. Contrary to the

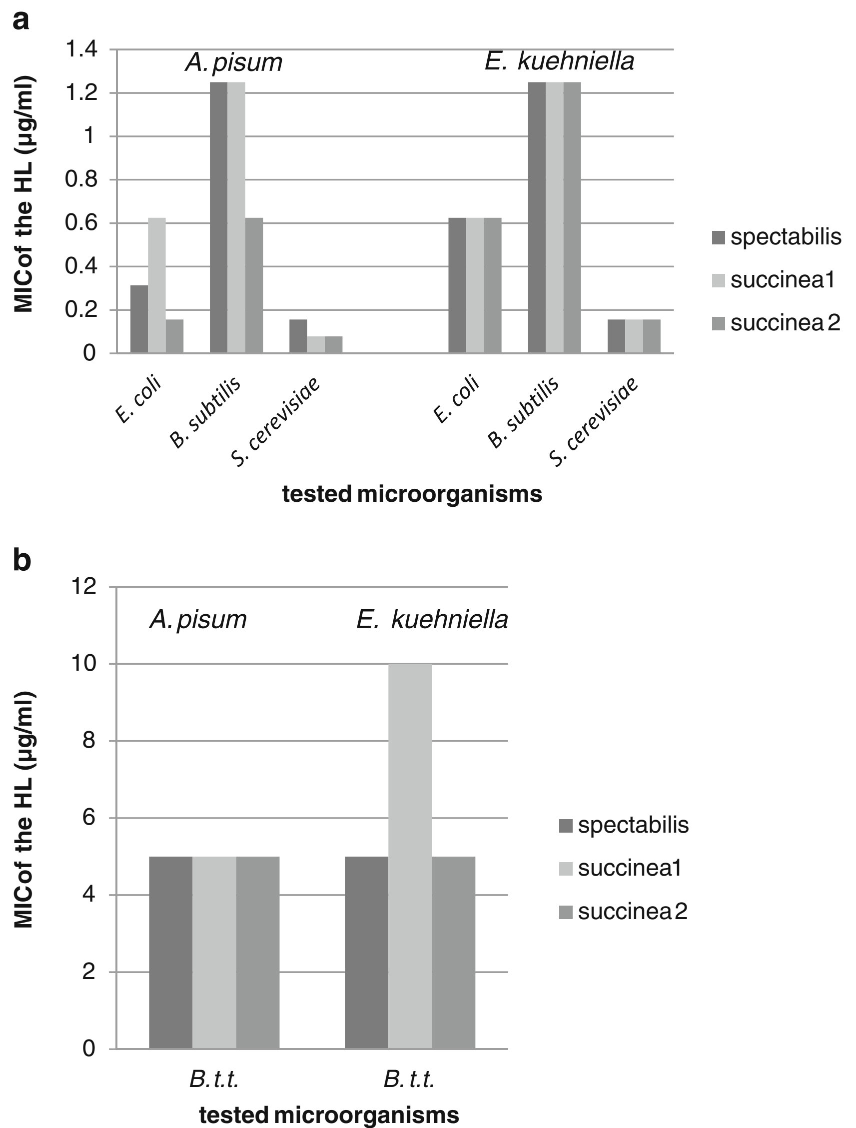

Fig. 2 Minimal inhibitory concentrations (MIC) in µg/ml found in the hemolymph of live and unchallenged *Harmonia axyridis* adults. Antibacterial standard gentamycin had a MIC value of 0.195 µg/ml against *Escherichia coli* and *Bacillus subtilis*, and of 1.563 µg/ml against *Bacillus t. thuringiensis*. The antifungal standard nystatin had a MIC value of 0.781 µg/ml against *Saccharomyces cerevisiae*. MIC values of the standards tested did not differ between diet treatments. Beetles were reared on a diet of live *Acyrthosiphon pisum* or frozen eggs of *Ephestia kuehniella*. **a**) Comparison of the MIC values of three morphotypes (*spectabilis, succinea* 1 and 2) from each diet regime against three different model microorganisms. **b**) Comparison of the MIC values of three morphotypes (*spectabilis, succinea* 1 and 2) for each diet regime against entomopathogenic *Bacillus thuringiensis tenebrionis*

results reported by Berkvens et al. (2008), we observed no differences in developmental parameters between the cohorts reared on either diet. Those authors concluded from their laboratory data that *E. kuehniella* eggs were a better food than live *A. pisum* aphids. Moreover, they related the observed differences to the distinct morphotypes. We did not compare morphotypes in this part of our study. While the preimaginal survival and fecundity was higher in our study than reported by Lanzoni et al. (2004), the oviposition rates were similar, and no differences in sex ratio were found in our experiments. A previous experiment by Specty et al. (2003) reported higher adult weight for female beetles after ingestion of *E. kuehniella* eggs compared to a diet of live *A. pisum*. The authors attributed their results to different amino acid and lipid contents in the food. Contrary to these authors, we observed no weight differences in the newly emerged adult beetles reared on either diet. Stathas et al. (2001) found similar fecundity and oviposition rates for *Aphis fabae* fed *H. axyridis* females as we did for *E. kuehniella* eggs fed beetles. However, the fecundity observed for female beetles fed on pea aphids was twice as high in our study. McClure (1987) obtained a similar number of eggs laid per female when the beetles were reared permanently at 27°C with a diet of *A. pisum* as we did by alternating rearing temperature between 20 and 25°C. Thus, our data confirm the observation previously reported by Stathas et al. (2001) that temperature seems to affect mainly the duration of the preovipositional period.

To date, we do not know what contributes to the observed significant differences in fecundity between the diet regimes we tested. Specty et al. (2003) detected higher amounts of lipids and amino acids in meal moth eggs than in aphids, but higher glycogen content in live aphids than in the eggs. The authors also found differences in mortality and weight based on diet, with *E. kuehniella* eggs being the better diet. Likewise, they also found no effect of diet on larval development time. Interestingly, like Berkvens et al. (2008), they observed

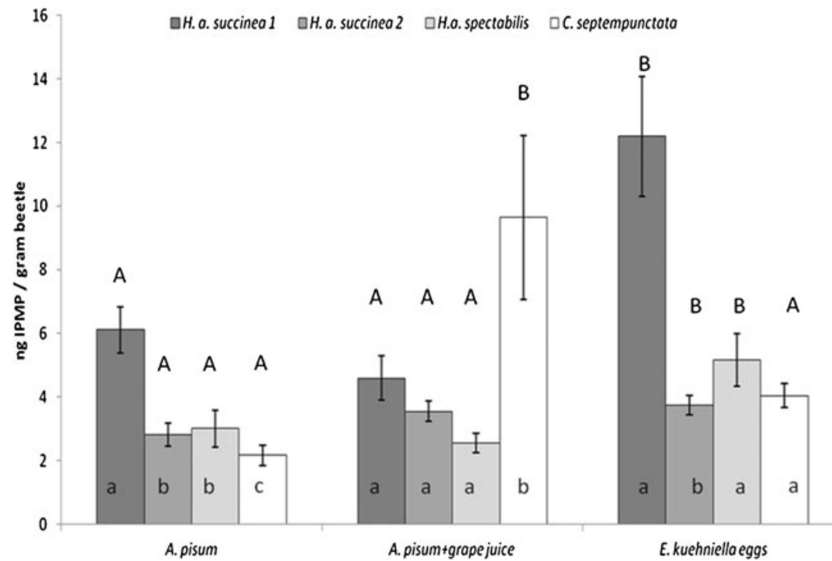

Fig. 3 Mean IPMP contents (ng/g fresh weight) +/− SE under different feeding conditions (*Acyrthosiphon pisum, A. pisum*+grape juice or *Ephestia kuehniella* eggs) of three morphotypes of *Harmonia axyridis* (*H. axyridis succinea 1* (dark grey), *succinea 2* (grey), and *H. axyridis spectabilis* (light grey) and *Coccinella septempunctata* (white). Small letters indicate significant differences between morphotypes of *H. axyridis* and *C. septempunctata* within one feeding group ($P<0.05$). Capital letters indicate significant differences of the two species between the feeding groups ($P<0.05$). 10 replications were done in every treatments

significantly higher fecundity of the beetles reared with *E. kuehniella* eggs. Our results contradict both of those previous studies. In comparison to our experiment, however, the study by Specty et al. (2003) evaluated the fecundity of *H. axyridis* females only for a period of 10 days, whereas we followed the females for a total period of 49 days. For both treatments, egg laying activity showed strong daily variation (Fig. 1) until the end of the observational period. It is striking, that in *H. axyridis* fed a diet of *A. pisum* the average number of eggs laid per female and day was much higher in the beginning of the egg laying period compared to a diet of *E. kuehniella*, and was then followed by a gradual decrease of egg numbers laid per female and day until the end of the observational period. By contrast, under a diet of *E. kuehniella*, the number of eggs

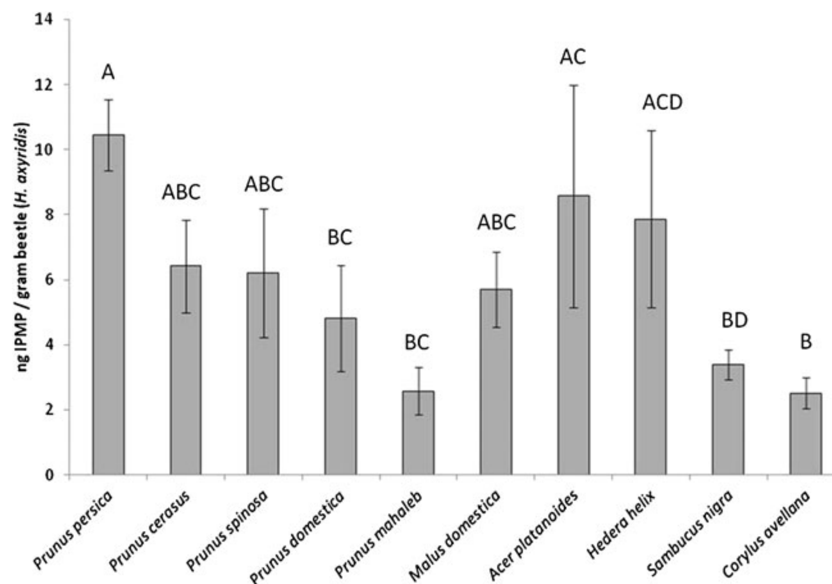

Fig. 4 Mean IPMP contents (ng/g fresh weight) +/– SE of three morphotypes of *Harmonia axyridis* 3 d after eclosion from field collected pupae on the respective plant species infested by given aphid species (*Myzus varians* on *Prunus persica* (N=5); *Brachycaudus helichrysi* on *Prunus domestica* (N=7); *Dysaphis plantaginea* on *Malus domestica* (N=9); *Drepanosiphon platanoides* on *Acer platanoides* (N=5); *Aphis hederae* on *Hedera helix* (N=5)*; Aphis sambuci* on *Sambucus nigra* (N=5); Unknown aphids on *Prunus spinosa, Prunus mahaleb, Prunus cerasus* and *Corylus avellana* (N=5)

laid per female and day started out at a low level and was followed by a constant increase in egg numbers. Since we have no data on the hatching rate of these eggs, we cannot conclude how the age of the beetles and egg quality might be related for either diet. However, in terms of the number of eggs laid during the course of our study, *A. pisum* can clearly be considered a better diet for *H. axyridis* females.

Little is known on the influence of host plant secondary compounds experienced by *H. axyridis* through prey aphids (Francis et al., 2000; Alhmedi et al., 2008). As presented in this paper, larvae fed on *Aphis sambuci* had lower IPMP concentrations than larvae fed on other aphid species. *A. sambuci* was shown to be toxic for ladybird beetles as *C. septempunctata* (Nedved and Salvucci, 2008), but was consumed as non-optimum food by *H. axyridis* (Ungerova et al., 2010). Further, it was shown that *H. axyridis* can survive on a diet of *A. sambuci* contrary to *C. septempunctata* (Francis et al., 2000). The ability to detoxify plant allelochemicals like the cyanogenic and phenolic glycosides produced by *Sambucus nigra* (D'Abrosca et al., 2011) and sequestered by the aphids might be responsible for this difference in the diet spectrum of both coccinellid species. *Prunus mahaleb* is known for its high levels of coumarins. Those secondary compounds are supposed to cause feeding deterrence against Japanese beetles (*Popillia japonica*) (Patton et al., 1997). *Corylus avellanae* contains high concentrations of phenolic compounds (Amaral et al., 2010) that remain to be tested for physiological effects on aphids and their predators.

On the plants mentioned above, we collected *H. axyridis* pupae that supposedly developed as larvae with a diet of aphids feeding on the respective host plants. Thus, we conclude that lower IPMP content in the beetles emerged from those pupae compared to beetles fed on aphids from other host plants (Fig. 4) could indicate an interaction of host plant compounds sequestered in the aphids and ingested by the predatory beetles with autogenously produced methoxypyrazines. *Myzus persica* fed on leaves of *P. persica* was shown to be the most suitable food for *H. axyridis*, which preferred this aphid over *A. fabae*, *Macrosiphum albifrons*, *M. pseudorosae,* and *M. euphorbiae* (Soares et al., 2004; Finlayson et al., 2010). Alyokhin and Francis (2005) described a significant reduction of *M. persicae* following the establishment of *H. axyridis*. In the present study, the IPMP contents in the hemolymph of *H. axyridis* were significantly higher when fed a closely related aphid species (*M. varians*) than when fed other species. We hypothesize that *H. axyridis* may detoxify allomones, but when feeding on chemically defended prey, it can not invest in high methoxypyrazine contents. Allelochemicals can thus influence the herbivore – predator relationship and the outcome of the biological control attempt or the prevalence of a predatory species on a certain host. Further studies on the interactions between chemically defended plants, sequestering insects and ladybird beetles are needed.

Adults fed for five days on the same prey as the larvae showed no higher IPMP contents than adults that were not fed after eclosion. Thus, in our study, larval diet seems to be the decisive factor for IPMP contents detectable in adult beetles. Under field conditions, larval mortality has been reported as the most important factor for the population dynamic of *H. axyridis* (Osawa 1993). Higher plasticity of larval IPMP content might be interpreted with regard to those field observations.

The results of our assays on the MIC values of the hemolymph of *H. axyridis* adults show evidence for an impact of beetle diet on the antimicrobial activity of their hemolymph. Similar to a previous study (Gross et al., 2010), we observed the highest susceptibility against the antimicrobial compounds in the hemolymph of *H. axyridis* for *E. coli* and *S. cerevisiae.* We found no differences in the antimicrobial activity of the hemolymph between male and female beetles, regardless of diet regime and elytral color. From our data, we cannot conclude that the three morphotypes of *H. axyridis* tested differ in the antimicrobial activity of their hemolymph. We found evidence, however, that a diet of pea aphids resulted in stronger growth inhibition of the most susceptible bacterium tested, the gram negative *E. coli*, when compared to the hemolymph activity of beetles fed on frozen *E. kuehniella* eggs. These results are congruent with the diet effects on fitness parameters observed here.

Within aphid fed beetles, we also observed higher activity against the growth of *E. coli* for the *succinea 1* morphs than for the less abundant *succinea 2* morphs. The *spectabilis* morphs had an intermediate growth inhibitory effect on this microorganism. Barnes and Siva-Jothy (2000) described a positive correlation of cuticle melanization with parasite resistance for *Tenebrio molitor* beetles. Since immune-function and melanin production use the same enzymatic pathway, increased melanization might interfere with immune defense (Carton and Nappi, 1997). Our results, however, do not provide evidence for this hypothesis. Based on our data, we cannot conclude that the rare melanic *spectabilis* morphs were less defended against microorganisms than the prevalent, less melanic *succinea 1* morphs of *H. axyridis*. In our assays, a growth inhibition of *B. t. tenebrionis* was only visible with the highest concentrations of hemolymph applied.

Furthermore, we found no evidence for antimicrobial properties of methoxypyrazines in our MIC tests. Thus, we conclude that the observed growth inhibition through the hemolymph from *H. axyridis* must be caused by other substances that remain to be identified. Pyrazines have long been described as major compounds in defensive odors emitted by different insect groups (Rothschild, 1961; Moore and Brown, 1981). Cudjoe et al. (2005) reviewed that *H. axyridis* contained a hundred times higher concentration of IPMP than *C. septempunctata*, which was described as the main compound of a so-called ladybird taint and can alter

the taste of wine (Pickering et al., 2004; Kögel et al., 2012a). This hypothesizes that *H. axyridis* could cause greater damage to viticulture than *C. septempunctata*. Recent studies have refuted this hypothesis: by using gaschromatography and olfactometer tests, it was found that *C. septempunctata* had the same amount of IPMP in its hemolymph as *H. axyridis*. Additionally, GC-nitrogen-phosphor detector analyses revealed nearly the same peak areas of IPMP in chromatograms of hemolymph headspace for both species (Kögel et al., 2012b). Additionally, Botezatu and Pickering (2010) described similar IPMP concentrations in wine produced after the addition of *H. axyridis* or *C. septempunctata* into crushed grapes. The differences in the concentrations of IPMP measured by Cudjoe et al. (2005), Botezatu and Pickering (2010), and our study might be due to the use of live beetles in our and Pickering's trials, while Cudjoe et al. (2005) analyzed frozen beetles.

In this study, several factors that may influence production of IPMP in ladybird beetles were investigated. IPMP contents of *H. axyridis* were significantly higher than of C. *septempunctata* in one out of three feeding treatments (*A. pisum*) in the laboratory. In one treatment (*A. pisum*+grape juice), *C. septempunctata* had higher IPMP contents than the Multicolored Asian ladybird beetle. In beetles fed on *E. kuehniella* eggs and in field-collected beetles, no significant differences between the two species could be observed. Thus, concerning methoxypyrazine contents, *H. axyridis* cannot be regarded as more hazardous for viticulture than *C. septempunctata*. In addition to development time, fresh body mass and fecundity, IPMP content can be used to evaluate food quality (Kalushkov and Hodek, 2004).

Elytral color apparently has an influence on IPMP content. Cai et al. (2007) previously described significant differences between the *succinea 1* and *succinea 2* morphs. In our study, *H. axyridis succinea 1* also contained significantly higher values of IPMP than *succinea* 2. Differences between *red* and *black color* morphs were studied before by Bezzerides et al. (2007). They showed that darker beetles were less defended against predators due to lower alkaloid contents. Slogett (2010) could not measure this, but they showed higher predation of non-melanic morphs by an orb-web spider. In our assays, significant differences in IPMP content were observed in one feeding group between the *H. axyridis succinea* 1 morphs and *H. axyridis spectabilis* morphs (Fig. 4). Grill and Moore (1998) suggested elytral coloration was an effect of diet. In our experiments, larvae fed on *A. pisum, E. kuehniella,* or other aphid species did not differ in variation of elytral pigmentation.

Currently, we have no explanations for the differences detected in methoxypyrazine content in relation to elytral color. Bezzerides et al. (2007) argued that lower defense levels in melanic beetles could be balanced by other advantages, such as increased activity rates through a thermal advantage during periods with lower temperature. The advantages of melanic beetles with respect to body temperature, activity range, and walking speed at lower temperatures have been shown for leaf beetles and ladybird beetles (De Jong et al., 1996; De Jong and Brakefield, 1998; Gross et al., 2004b; Michie et al., 2010).

In summary, our results provide further evidence for the influence of diet on fitness and defense chemistry in coccinellid beetles. It leads us to conclude that the predicted risk of a single specimen of *H. axyridis* for viticulture, with regard to tainting wine with IPMP, is not higher compared to specimens of other ladybird beetle species. However, *H. axyridis* will remain a threat for wine production due to huge population densities that may develop during some years, bearing the risk of tainting wine by getting harvested and processed in high numbers.

Our observations on factors influencing the IPMP content in both ladybird species examined show the need of further tritrophic level studies for *H. axyridis*. Field observations on prey species and the size of local sub-populations of this ladybird species, invasive in many countries, are important to understand the environmental factors that affect its competitive advantage over native ladybird species (Kindlmann et al., 2011).

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Direct Proof of Ingested Food Regurgitation by *Spodoptera littoralis* Caterpillars during Feeding on *Arabidopsis*

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Abstract Oral secretions of herbivorous lepidopteran larvae contain a mixture of saliva and regurgitant from the insect gut. Different compounds from the oral secretions can be recognized by the host plants and, thus, represent elicitors that induce plant defenses against feeding herbivores. Exogenously applied oral secretions can initiate the biosynthesis of jasmonates, phytohormones involved in the regulation of plant defense. However, it is not known (a) whether or not non-manipulated insects indeed release oral secretions including gut-derived compounds into a leaf wound during the natural feeding process, or (b) whether they adjust the release of gut components to the state of plant defense. We addressed these questions by using *Arabidopsis thaliana* as host plant and larvae of the generalist herbivorous insect *Spodoptera littoralis*. We investigated the conversion of the plant-derived jasmonate precursor, *cis*-12-oxophytodienoic acid (*cis*-OPDA), to *iso*-OPDA by the larvae. This enzymatic reaction is mediated by a specific glutathione-*S*-transferase in the insect gut, but not in the plant. Any presence of *iso*-OPDA in plant tissue, thus, indicated that gut content had been regurgitated into the plant wound. Our study demonstrates that the plant is the only source for the substrate *cis*-OPDA by using *aos* (allene oxide synthase) mutants that are unable to synthesize OPDA. The fact that *iso*-OPDA accumulated over time on feeding-damaged leaves shows that the feeding larvae are constantly regurgitating on leaves. Although the larvae provided the signaling compounds that were recognized by the plant and elicited defense reactions, the larval regurgitation behavior did not depend on whether they fed on a defensive wild type plant or on a non defensive *coi1-16* plant. This suggests that *S. littoralis* larvae do not adjust regurgitation to the state of plant defense.

Keywords Herbivory · *Arabidopsis thaliana* · *Spodoptera littoralis* · *iso*-OPDA · Regurgitant · Oral secretion · Plant defense induction

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Introduction

Plants infested by arthropod herbivores are able to recognize the attack and react accordingly with the induction of various direct and indirect defense responses against the feeding aggressors, such as the biosynthesis and accumulation of toxic compounds and volatile emission that attract predators of the herbivores (Kessler and Baldwin, 2001; Mithöfer et al., 2009). The specificity of the induced defenses can depend on the plant and the feeding herbivore species (De Moares et al., 1998; Stout et al., 1998; Leitner et al., 2005). Herbivore specificity of plant defense might be due either to the feeding behavior, e.g., chewing caterpillars, piercing-sucking spider mites, phloem feeders, or to the presence of specific herbivore-derived chemical signals, also referred to as herbivore-associated-molecular-patterns, HAMPs (Felton and Tumlinson, 2008; Mithöfer and Boland, 2008). Even more likely, a combination of mechanical wounding during the feeding process and signaling compounds derived from the herbivores are responsible to finally elicit a specific plant defense response. However, when using a mechanical larva,

MecWorm, it has been demonstrated that artificial plant damage mimicking insect feeding is sufficient to induce the emission of volatiles that are known to be elicited also by real herbivory (Mithöfer et al., 2005). In some plant species such as lima bean (*Phaseolus lunatus*), MecWorm treatment elicits the whole herbivory-related volatile blend; in other plants, for instance in potato (*Solanum tuberosum*), this occurs only partly (Maffei et al., 2007), suggesting that in the latter case the chemical signals are necessary for the onset of the full plant defense response.

In many lepidopteran species, larval oral secretion (OS) contains *N*-acyl-glutamine conjugates (or: fatty acid-amino acid conjugates, FACs). Among them, *N*-(17-hydroxylinolenoyl)-L-glutamine (volicitin) is the most prominent one (Alborn et al., 1997). Volicitin and other FACs represent typical HAMPs that have the capacity to induce defense response in plants when added to wounded tissue (Alborn et al., 1997; Landolt et al., 1999; Halitschke et al., 2001; Schmelz et al., 2001). In contrast, FACs are not generally active in lima bean and cotton (*Gossypium hirsutum*) for the induction of volatiles (Spiteller et al., 2001), and in *Arabidopsis thaliana,* FACs do not even induce enhanced levels of phytohormones (Schmelz et al., 2009).

Besides FACs, other OS components have been identified to activate plant defense. Caeliferins (sulfated α-hydroxy fatty acids) from the grasshopper species *Schistocerca americana* (Alborn et al., 2007) and a lipase from *S. gregaria* (Schäfer et al., 2011), inceptins (peptidic fragments of the chloroplastic ATP synthase γ-subunit), first isolated from *Spodoptera frugiperda* larvae feeding either on cowpea (*Vigna unguiculata*) or maize (*Zea mays*) (Schmelz et al., 2006), and a β-glucosidase from *Pieris brassicae* larvae (Mattiacci et al., 1995) are additional examples.

Lepidopteran OS is a mixture of secretions from salivary glands and gut reflux, referred to as regurgitant. Almost all studies on OS activities and composition have been performed with OS collected by gently squeezing the caterpillars with forceps. The larvae then immediately start to release all fluids from their mouth parts and foregut. Interestingly, although enzymes have been identified from salivary secretions, for example, glucose oxidase in *Helicoverpa zea* (Eichenseer et al., 1999), only components from the regurgitant show elicitor activities in plant tissue, and are apparently used by the plant to recognize and react to herbivory. Hence, regurgitation has a central function in plant - herbivorous insect interactions. Strikingly, saliva-derived glucose oxidase can even suppress rather than induce plant defense (Musser et al., 2002, 2005).

Up to now, only two studies have shown that during the feeding process of lepidopteran larvae on host plants, caterpillars can deposit compounds from the gut onto the leaf by regurgitation. First, *Spodoptera exigua* larvae were fed on a diet with radiochemically labeled volicitin introduced radioactivity at the feeding site (Truitt and Paré, 2004). Second, Peiffer and Felton (2009) offered a diet containing a fluorescent dye (Alexa 488) to larvae of *H. zea* and other lepidopteran species, and thus showed that the larvae released gut components on a leaf. However, in these studies, the physiology of the insect gut might have been affected by the manipulation of the food, and the manipulated food might have provoked regurgitation.

Therefore, we used an alternative approach that left the feeding larvae completely untreated in order to investigate whether a feeding insect releases gut components on a leaf. This approach is based on conversion of a phytohormone precursor in the insect gut. The phytohormone jasmonic acid (JA) is well-known to be involved in plant-induced herbivore defense. In plant tissue, insect leaf feeding induces the generation of defense-related phytohormones, jasmonates, including *cis*-OPDA (12-oxo-phytodienoic acid) as biosynthetic precursor of jasmonic acid (Wasternack, 2007). Uptake of *cis*-OPDA is toxic for an insect as it contains a reactive α,β-unsaturated carbonyl structure that easily adds cellular nucleophiles making *cis*-OPDA potentially toxic for the herbivore (Vollenweider et al., 2000). Moreover, *cis*-OPDA structurally resembles certain animal prostaglandins, which, for example, have been claimed to play a role in the activation of the insect innate immune system (Stanley et al., 2009). The plant-derived jasmonate precursor *cis*-OPDA is converted in the insect gut to *iso*-OPDA (Fig. 1) (Schulze et al., 2007) by the activity of an insect-derived glutathion-*S*-transferase, GST. *iso*-OPDA is less toxic to the insect when compared to *cis*-OPDA (Schulze et al., 2007; Dabrowska et al., 2009). The ability for the enzymatic isomerization is particularly widespread among generalist lepidopteran herbivores. However, this particular GST is the only known enzyme that can convert *cis*-OPDA into *iso*-OPDA (Dabrowska et al., 2009). In various lepidopteran species, *iso*-OPDA has been detected in larval frass and larval OS after feeding on lima bean (*Phaseolus lunatus*) plants (Schulze et al., 2007).

Hence, the presence or absence of *iso*-OPDA on infested leaves of the model plant *A. thaliana* was used in this study as a proof for the regurgitation of recently ingested and digested host plant tissues during the feeding process. The

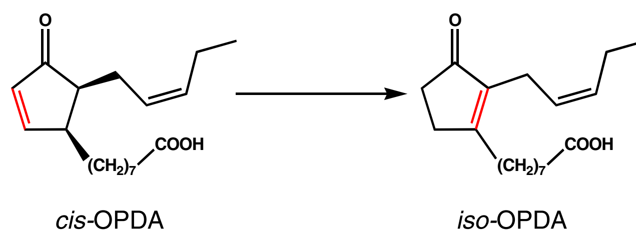

Fig. 1 **a** Isomerization of *cis*-12-oxophytodienoic acid (*cis*-OPDA) to *iso*-12-oxophytodienoic acid (*iso*-OPDA)

lepidopteran *S. littoralis* caterpillars is a generalist herbivore widely used in studies on insect herbivory.

In addition to the question whether or not *S. littoralis* releases *iso*-ODPA on infested leaves that is derived from the plant-produced *cis*-ODPA, we further addressed the question whether *S. littoralis* larvae adjust their regurgitation behavior and release of *iso*-ODPA on leaves according the defensive state of the plant. We used mutant plants that are able to produce *cis*-ODPA, but are JA insensitive (*coi* mutant) to study this latter question.

Methods and Materials

Insects and Plants In all experiments, *Spodoptera littoralis* (Boisduval, 1833) (Lepidoptera, Noctuidae) larvae were used. Larvae were reared on artificial diet at 23 °C with a light period from 7 a.m. to 9 p.m. The diet consisted of 500 g chopped beans, 9 g ascorbic acid, 9 g 4-ethylbenzoic acid, 0.7 g vitamin E, and 4 ml formaldehyde per liter water that were mixed with approx. 650 ml of a 7.5 % agar solution.

Wild type *Arabidopsis thaliana* (L.) Heynh., ecotype Columbia, seeds were obtained from the *Arabidopsis* Stock Centre (Nottingham, UK). A jasmonate insensitive *coi1-16* mutant (Xie et al.1998; Ellis and Turner, 2002) and an OPDA and jasmonic acid biosynthesis mutant (*aos*), that is defective in allene oxide synthase (Park et al., 2002), were obtained from J. G. Turner and G. Bonaventure, respectively. Seeds of all plants were sown on 10 cm round pots and stratified for 2 d at 4 °C. Thereafter, plants were moved to ventilated growth rooms with constant air flow at 23 °C and 40 % humidity. Plants were grown at a distance of 30 cm from fluorescent light banks with 6 bulbs of cool white and 2 bulbs of wide spectrum lights at a 10 hL/14 h D photoperiod. Plants were shifted to wide spectrum light source 3 wk after growth, and all experiments were done with 5-wk-old plants.

Collection of Oral Secretions Oral secretions (OS) were collected on ice from fourth instars, which were reared on artificial diet and fed on *A. thaliana* leaves for 24 h. OS were collected into glass capillaries by gently squeezing the larva with a forceps behind the head, which caused immediate regurgitation. Harvested samples were centrifuged at 13,000 x *g* for 2 min to obtain clear supernatant and diluted 1:1 with water. When necessary, secretions were stored at -20 °C until use. Under these conditions, *iso*-OPDA was stable over time.

Plant Treatments Experiments were performed with 5-wk-old *A. thaliana* plants at a vegetative growth stage and pre-bolting. For experiments with insect regurgitant, wounding was carried out with a pattern wheel (6 vertical motions on a single row) on either side of the leaf resulting in ~10-13 punctures. A volume of 20 µl of diluted regurgitant was spread across all the holes on a single leaf. In control plants, water was added. Insect feeding assays were carried out with three fourth instars per rosette, except for 1-wk-lasting feeding experiments that started with first instars. Before collecting plant material, leaves were carefully checked for remaining frass, which was completely removed by wiping with a moist paper towel when necessary. The plant leaf samples were harvested and stored in liquid nitrogen.

Liquid Chromatography–Mass Spectrometry Analyses of iso-OPDA After finishing the plant treatment, the material was weighed (250 mg) and frozen with liquid nitrogen. Samples were kept at -80 °C until used. For phytohormone analysis, finely ground leaf material was extracted with 1.5 ml methanol containing 60 ng of 9,10-D_2-9,10-dihydrojasmonic acid. This homogenate was mixed for 30 min on a shaker under gentle agitation, and finally centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant was collected. The homogenate was re-extracted with 500 µl methanol, mixed, and centrifuged. Supernatants were pooled. The combined extracts were evaporated in speed-vac at 30 °C and re-dissolved in 500 µl methanol.

Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies, Böblingen, Germany). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent, Waldbronn, Germany). Formic acid (0.05 %v/v) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was: 0-0.5 min, 5 % B; 0.5-9.5 min, 5-42 % B; 9.5-9.51 min 42-100 % B; 9.51-12 min 100 % B, and 12.1-15 min 5 % B. The mobile phase flow rate was 1.1 ml/min. The column temperature was maintained at 25 °C.

An API 3200 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbospray ion source was operated in negative ionization mode. The instrument parameters were optimized by infusion experiments with pure standards, where available. The ion spray voltage was maintained at -4500 eV. The turbo gas temperature was set at 700 °C. Nebulizing gas was set at 60 psi, curtain gas at 25 psi, heating gas at 60 psi, and collision gas at 7 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion→product ion: *m/z* 290.9→165.1 (collision energy (CE) -24 V; declustering potential (DP) -45 V; entrance potential (EP) -12 V for *cis*-(+)-12-oxophytodienoic acid (*cis*-OPDA) and *iso*-OPDA; *m/z* 213.1→56.0 (CE -24 V; DP -35 V; EP -10 V) for 9,10-D_2-9,10-dihydrojasmonic acid. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Linearity in ionization efficiencies was verified by analyzing dilution series of standard mixtures. The identity of *cis*-

OPDA and of *iso*-OPDA was confirmed by match of retention time in LC-MS compared to authentic standards.

For quantification of *cis*-(+)-12-oxophytodienoic acid (*cis*-OPDA) and of *iso*-OPDA, 9,10-D2-9,10-dihydrojasmonic acid was used as the internal standard applying an experimentally determined response factor of 2.24.

Results

iso-OPDA in Spodoptera littoralis Oral Secretions Depends on Plant cis-OPDA Synthesis To examine first whether or not *iso*-OPDA from larval OS was detectable when OS was transferred to plant tissues, we mimicked herbivory in *A . thaliana* leaves using a pattern wheel to injure leaves and applied artificially OS of *S. littoralis* larvae. Plant wounding plus OS treatment was done for different time intervals from 30 min to 3 h (Fig. 2). Within this period, the amount of OS-derived *iso*-OPDA on plant leaves did not significantly change, indicating its chemical stability.

To further prove that *iso*-OPDA present in leaf extracts was insect derived, we used two controls: i) when water instead of OS was added to wounded leaves, no *iso*-OPDA could be detected in the leaf extracts. This indicates that only the insect is able to generate *iso*-OPDA; ii) we used the *Arabidopsis* JA biosynthetic mutant, *aos*, which cannot synthesize *cis*-OPDA (Park et al., 2002). Here, the larvae feeding on *aos* mutants could not generate *iso*-OPDA due to non-availability of *cis*-OPDA (Fig. 2). By using OS collected from caterpillars that fed on artificial diet, also no *iso*-OPDA was detectable.

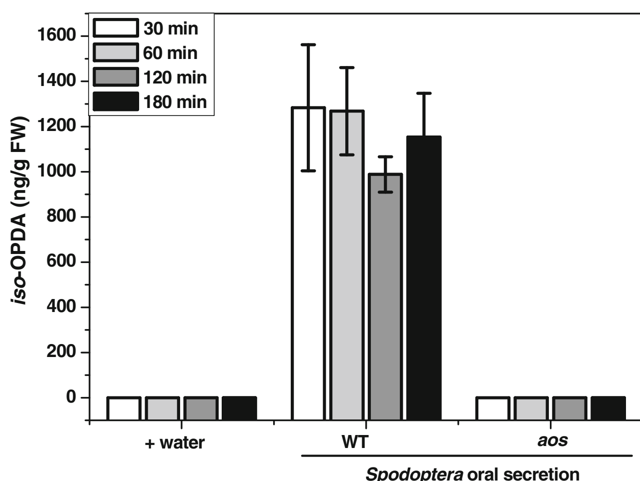

Fig. 2 Detection of *iso*-OPDA in *Arabidopsis thaliana* leaves wounded with a pattern wheel and subsequently treated with either *Spodoptera littoralis* oral secretions (20 µl leaf^{-1}, 1:1 diluted with water) or water as control, both placed on the punctures. *S. littoralis* larvae used for oral secretion collection fed for 24 h on *A. thaliana* wild type (WT) or *aos* mutant plants. Shown is the mean +/- SE (*N*=6)

Regurgitation by Spodoptera littoralis Caterpillars during Feeding From the plant´s perspective, insect herbivory includes both mechanical wounding and exposure to insect oral secretion. Real insect herbivory is far more complex than simply wounding plants and applying OS, as it involves continuous feeding with deposition of OS (Mithöfer and Boland, 2008). Therefore, we investigated the *iso*-OPDA deposition on *Arabidopsis* plants fed by *Spodoptera* larvae. Only in plant tissue that was in contact with feeding caterpillars both *cis*-OPDA and *iso*-OPDA were found. In contrast, in undamaged leaves, *cis*-OPDA exclusively was detected (Fig. 3a, b), suggesting that *iso*-OPDA originated from the feeding insect.

Since *iso*-OPDA is an insect-derived component, we used it as a marker to investigate whether insects indeed regurgitate during real feeding, and we traced the kinetics of *iso*-OPDA deposition on *A. thaliana* leaves fed by *S. littoralis. iso*-OPDA was first detected after 4 h of feeding and remained detectable for up to 1 wk (Fig. 3c). This correlated with the finding that in herbivore-challenged plants *cis*-OPDA levels increased over time (Fig. 4a). The insects need time to convert *cis*-OPDA into the *iso*-form, and they start doing so when after continuous feeding *cis*-OPDA levels in the gut are too high. The levels of *iso*-OPDA on *A. thaliana* leaves were heterogeneous, but tended to increase in course of time; however, the increases were statistically not significant, which might be due to the larval feeding pattern.

Regurgitation by Spodoptera littoralis Caterpillars while Feeding on Jasmonate -insensitive coi1 Mutants The amount of *cis*-OPDA significantly increased upon *Spodoptera* feeding within 1 h; a maximum response was reached after 1 d of feeding (Fig. 4a). Most JA responses are mediated through the coronatine insensitive 1 (COI1) F-box protein, which is part of a JA-receptor complex. JA perception occurs *via* binding of bioactive jasmonate, JA-Ile, to the jasmonate receptor, COI1. Hence, the *coi1-16* mutant cannot perceive jasmonic acid, although the JA biosynthetic pathway is unaltered, and therefore cannot activate jasmonate dependent plant defenses (Xie et al., 1998; Chini et al., 2009). The range of *cis*-OPDA increase within 24 h after *Spodoptera* feeding, which was observed in the jasmonate-insensitive mutant, *coi1-16,* was similar to the one observed after feeding upon wild-type *A. thaliana* leaves (Fig. 4b), Thus, the comparison of *coi1-16* with wild-type plants was ideal to address the question whether feeding *Spodoptera* larvae might recognize the presence of jasmonates and jasmonate-mediated plant defense and, as a consequence, reduce regurgitating to avoid further induction of defenses by providing HAMPs in the larval OS. As shown in Fig. 4c, the levels of *iso*-OPDA deposition on WT or *coi1-16* leaves were indistinguishable. This indicated

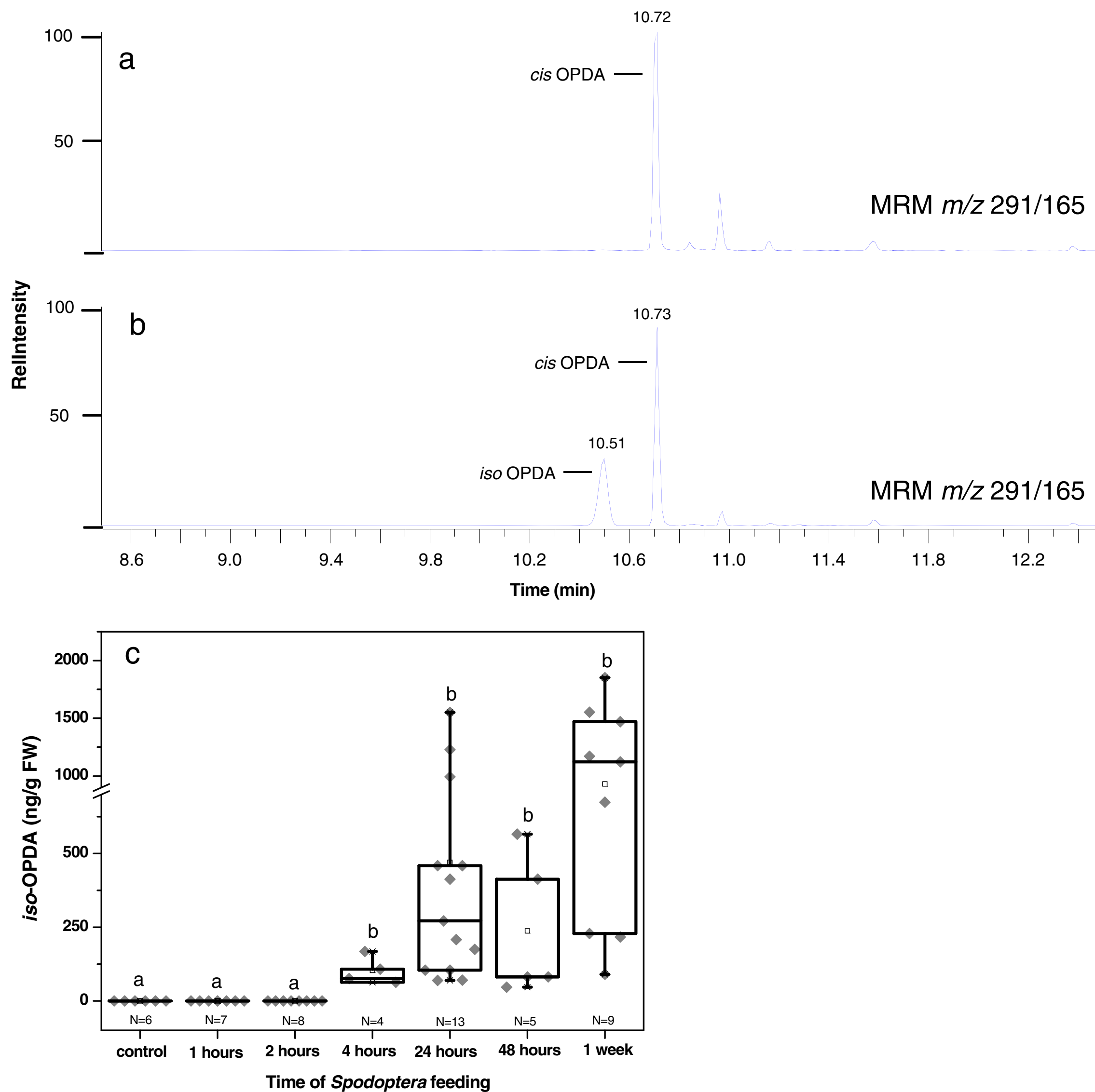

Fig. 3 **a, b** Chromatographic separation of OPDA isomers isolated from untreated *Arabidopsis thaliana* leaves (**a**) and leaves after *Spodoptera littoralis* feeding (**b**). Isomers of OPDA were identified by using authentic standards. The analysis was carried out using LC-Triple Quadrupole mass spectrometry in MRM modus in negative ionization mode; **c** Time-dependent *iso*-OPDA accumulation in *A. thaliana* wild type leaves during *S. littoralis* feeding. On 5-wk-old *A. thaliana* plants, 3*S. littoralis* larvae per rosette were placed and allowed to feed for different time periods. Injured leaves were collected and *iso*-OPDA levels were analyzed. Statistics were performed with one-way ANOVA. The rectangles represent individual data points and a single mean value

that insects are not able to regulate their regurgitation behavior depending on the plant defense state.

Discussion

Plants can detect the attack by caterpillars. Besides the mechanical wounding, insect-derived chemistry contributes to the process of recognition and, subsequently, to the induction of plant defense responses (Mithöfer et al., 2005; Mithöfer and Boland, 2008). So-called HAMPs are typical signals that "inform" the plant of herbivore attack. However, their defense-inducing activities have been investigated by collecting OS followed by exogenous application to the plant tissue, rather than by showing OS presence on the respective leaves during feeding. Strikingly, although it seems obvious, up to now it has not been directly demonstrated that non-treated larvae indeed regurgitate ingested plant material during the feeding process. Thus, the *iso*-OPDA formed in insect gut upon detoxification of plant

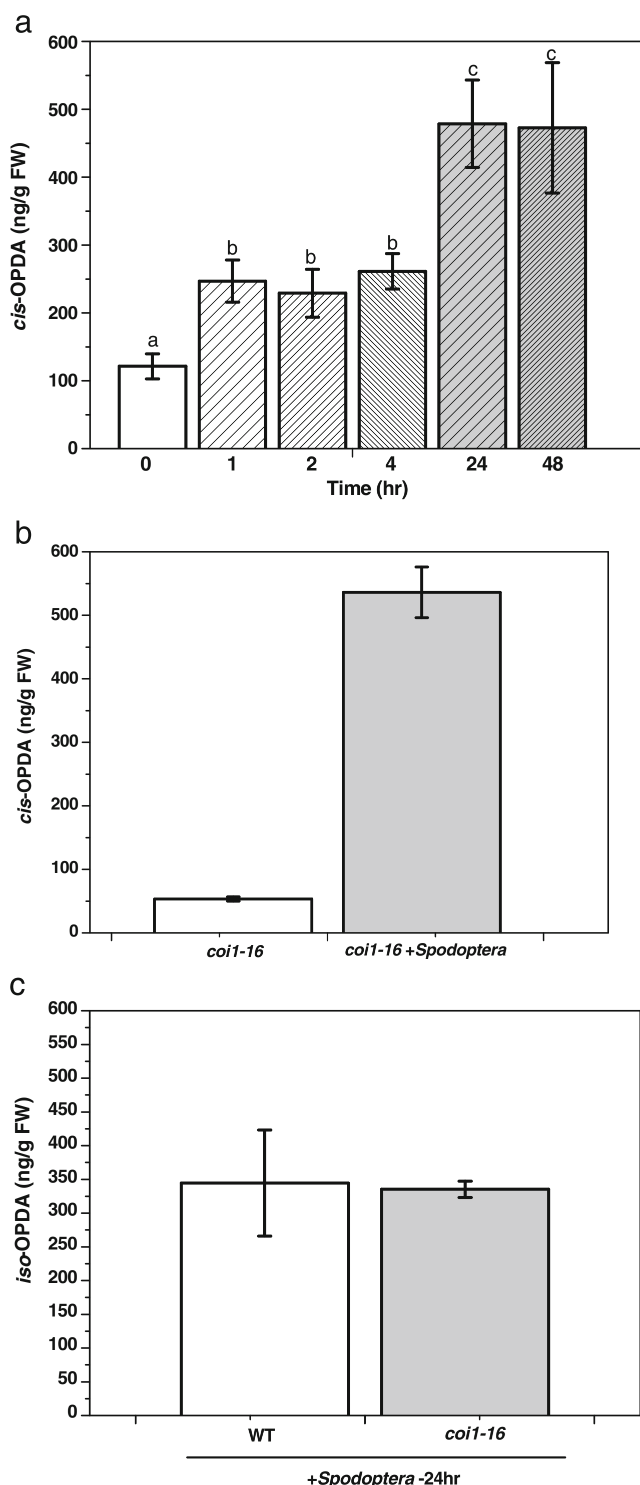

Fig. 4 Impact of *Arabidopsis thaliana coi1-16* plants on regurgitation of *Spodoptera littoralis*. Always shown is the mean +/- SE (N=10). Different letters indicate significant differences between treatments (P<0.05) according to one-way ANOVA. **a** Time-dependent *cis*-OPDA accumulation upon *S. littoralis* feeding in *A. thaliana* wild type leaves. *S. littoralis* larvae fed on *A. thaliana* for 1, 2, 4, 24 and 48 h. **b** *cis*-OPDA accumulation in *coi1-16* mutant upon *S. littoralis* feeding for 24 h. **c** *iso*-OPDA accumulation in *A. thaliana coi1-16* plants upon *S. littoralis* feeding for 24 h

cis-OPDA by insect glutathion-*S*-transferase activity was used as a proof for the presence of regurgitant on plant tissue.

iso-OPDA could be detected only if the larvae fed on wild type plants that are able to synthesize *cis*-OPDA, in contrast to *aos*-plants that are unable to synthesize OPDA (Fig. 2). This result clearly demonstrated the need for plant *cis*-OPDA as the biosynthetic precursor for *iso*-OPDA formation by the insect. Our finding also shows that the plant provides the only source of *cis*-OPDA. Furthermore, *iso*-OPDA is an extremely rare, unusual plant compound that has been described only twice for plants: in two moss species (Ichikawa et al., 1983) and as a minor component in osmotic stress-challenged barley leaves (Kramell et al., 2000). Moreover, neither leaf treatment with a pattern wheel nor treatment with *Spodoptera* OS obtained from non *cis*-OPDA-containing *aos* plants, were able to induce *iso*-OPDA (Fig. 2). These results showed that *iso*-OPDA is not produced in the plant itself. Interestingly, *iso*-OPDA remained detectable for at least 7 d on the plant without oxidation to *cis*-jasmone, a behaviorally active molecule that repels aphids and attracts parasitoids (Figs. 2, 3) (Birkett et al., 2000; Bruce et al., 2003).

Formation of *cis*-jasmone from *iso*-ODPA has been described for various plants when the methyl ester of *iso*-OPDA was applied (Dabrowska and Boland, 2007). In the present study, the free acid or the *iso*-OPDA salt as a charged compound apparently might not be able to penetrate the various hydrophobic layers of a leaf, the cuticula, plasma membrane, or the peroxisome membrane.

It is tempting to speculate that feeding larvae recognize induction of plant defenses. Because the caterpillars indirectly induce these defenses by providing HAMPs, the easiest way to stop this would be to stop the delivery of OS. After 1 d of feeding, the plant had begun to respond to the attack as indicated by the high level of *cis*-OPDA in *Arabidopsis* wild type and the jasmonate insensitive *coi1-16* plants (Fig. 4) in which jasmonate-mediated defenses are absent (Xie et al., 1998; Wasternack, 2007). However, the caterpillars did not change their regurgitating behavior on the actively defending wild type plants compared with the less defending *coi1-16* plants, as the amount of *iso*-OPDA on the leaves was the same (Fig. 4c). This strongly suggests that at least the regurgitation during feeding by *S. littoralis* caterpillars is not affected by plant defense. The insect continued to provide elicitors, thereby further inducing the defensive system of the host plant. However, insect oral secretions also contain effectors that are able to down-regulate plant defense (Musser et al., 2002; Bos et al., 2010; Consales et al., 2011). Thus, on one hand, insect regurgitation betrays the presence of an insect and induces plant defense, but on the other, it can also protect the insect from the plant´s defensive activities. The advantage of

regurgitation for the larvae is a matter of ongoing debate (Rhainds et al., 2011), but the presence of effectors in regurgitant that suppress plant immunity points to the fact that insect oral secretions have multiple roles in ecological interactions.

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Analysis and Optimization of a Synthetic Milkweed Floral Attractant for Mosquitoes

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Abstract A pentane extract of flowers of common milkweed, *Asclepias syriaca* (Asclepiadaceae), elicited significant orientation from both male and female *Culex pipiens* in a dual-port flight olfactometer. Analysis of the extract by gas chromatography–mass spectrometry revealed six major constituents in order of relative abundance: benzaldehyde, (*E*)-β-ocimene, phenylacetaldehyde, benzyl alcohol, nonanal, and (*E*)-2-nonenal. Although not all were collected from the headspace profile of live flowers, a synthetic blend of these six compounds, when presented to mosquitoes in the same levels and proportions that occur in the extract, elicited a response comparable to the extract. Subtractive behavioral bioassays demonstrated that a three-component blend consisting of benzaldehyde, phenylacetaldehyde, and (*E*)-2-nonenal was as attractive as the full blend. These findings suggest the potential use of synthetic floral-odor blends for monitoring or control of both male and female disease-vectoring mosquitoes.

Keywords *Asclepias syriaca* · *Culex pipiens* · Gas chromatography–mass spectrometry (GC-MS) · Milkweed · Nectar feeding · Northern house mosquito · Plant attractants · Subtractive bioassays · Synthetic blends · Asclepidaceae · West Nile virus · Mosquito vector control

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Introduction

The northern house mosquito, *Culex pipiens* L. (Diptera: Culicidae), is a member of a species complex that includes vectors of lymphatic filariasis and several arboviral diseases, such as St. Louis encephalitis (SLE), Rift Valley fever (RFV), and West Nile virus (WNV). In the northeastern quadrant of the United States, *C. pipiens* recently has become important because of its role in the transmission of WNV. The virus was introduced into New York City in 1999 and is currently the most widespread arboviral disease in the U.S. (CDC, 2010). The blood-feeding pattern of *C. pipiens* (shifting from avian to human feeding within the transmission season) (Edman and Taylor, 1968; Kilpatrick et al., 2006), coupled with its high vector competence (Turell et al., 2005) and ability to carry WNV through the winter, has allowed the virus to spread from its point of origin and maintain transmission throughout the mosquito's range. Thus, *C. pipiens* is one of the major targets for mosquito vector control and surveillance in the U.S. Current methods of monitoring by abatement districts and health departments include Centers for Disease Control (CDC) light traps and CO_2-baited traps, which mainly trap blood-seeking females, along with gravid traps, which target gravid females.

Plant-derived attractants have the potential to act as surveillance-trap lures and, when combined with poison-laced sugar solutions (Müller et al., 2010), to control mosquito populations. Mosquitoes are attracted to various plant species, from which they obtain nectar and other juices. Sugar feeding by mosquitoes has a significant influence on dispersal (Hocking, 1953; Magnarelli, 1977) and vectorial capacity (Gary and Foster, 2001; Gu et al., 2011), and, contrary to long-held conjecture, is required by both males and females throughout the adult stage (Downes, 1958; Yuval, 1992; Foster, 1995). Both sexes typically first visit

plants soon after emergence. Males then require sugar at frequent intervals to maintain their energy reserves in order to join nightly mating swarms (Yuval et al., 1992). Females take sugar between blood meals, when they are digesting blood, or when they are gravid (Clements, 1999; Foster, 2008). Furthermore, females of most temperate-climate *Culex* and *Anopheles* species enter reproductive diapause in late summer, after which they no longer are attracted to blood hosts, but engage heavily in plant-sugar feeding (Bowen, 1992a).

The potential for plant volatiles to lure mosquitoes has been demonstrated in several laboratory behavioral assays that have used stimuli such as natural plant extracts (Vargo and Foster, 1982; Jepson and Healy, 1988; Mauer and Rowley, 1999) or single floral compounds (Jhumur et al., 2006). In field studies, Bates (1949) reported the successful trapping of anopheline mosquitoes with plant and fruit baits; fruit also proved to be an effective attractant in CDC traps for *C. tarsalis* (Reisen et al., 1986). Sandholm and Price (1962) observed that mosquitoes in the field were attracted to light-colored flowers with distinct fragrances; odor appears to be primarily responsible for long-range attraction, with visual cues playing a role at shorter range (Thorsteinson and Brust, 1962; Healy and Jepson, 1988; Jepson and Healy, 1988). Orientation to commercially obtained floral extracts and honey has been demonstrated for various mosquito species (Thorsteinson and Brust, 1962; Hancock and Foster, 1997; Foster and Takken, 2004).

Schlein and Müller (2008) and Müller et al. (2008, 2010) reported dramatic population reductions of *C. pipiens* and other mosquito species by spraying a fruit-based sugar bait containing insecticide on vegetation surrounding larval habitats. Light-less CDC traps baited with the blossoms of *Tamarix jordanis* were highly effective in trapping *C. pipiens*, and populations were reduced where these blossoms were treated with spinosad insecticide (Schlein and Müller, 2008).

Flower species that elicit the highest attraction probably vary by region. During mid-summer in the U.S., mosquitoes have been observed probing blossoms of common milkweed, *Asclepias syriaca*, at rates disproportionate to their abundance relative to other flowering plants, with one study reporting 54 species occurring in the study area that did not serve as nectar sources (Grimstad and Defoliart, 1974). Another study collected 25 *Aedes vexans* from one milkweed cluster in just 15 min (Sandholm and Price, 1962). The observed preference of insects for milkweed flowers, both day and night, possibly is due to its strong and distinctive fragrance, lighter-colored flowers, and greater nectar production (Sandholm and Price, 1962). Common milkweed is indigenous to eastern and midwestern North America, where its principal native pollinators are mainly large Hymenoptera and Lepidoptera (e.g., Jennersten and Morse, 1991). Mosquitoes are incapable of transferring milkweed pollinia and therefore function as nectar thieves, appearing to co-opt the attractive properties used by the pollinators to locate the flowers (Foster, 1995).

Solvent extracts of common milkweed flowers also are attractive to mosquitoes (Vargo and Foster, 1982; Mauer and Rowley, 1999), confirming that volatile chemicals are at least partly responsible for that attraction. Mauer and Rowley (1999) determined the headspace profile of common milkweed flower to be predominantly 2-phenylethanol and benzyl alcohol; however, they found that a synthetic blend of these two compounds was not attractive to *C. pipiens*. Due to the multiple demonstrations of *C. pipiens* attraction to milkweed, both in the laboratory and the field, we used a solvent extract of the flower as a potential model for synthetic mosquito lures.

Methods and Materials

Mosquitoes Experiments were conducted with *C. pipiens* from a colony established in 2009 from larvae collected near Columbus, OH, USA. Larvae were identified at L4 by siphonal hair tufts (Vinogradova, 2000). Adults were maintained in 41-l clear acrylic cages on a diet of 10 % sucrose, water, and weekly blood meals from the legs of a rooster (ILACUC permit No. 2005A0054). Oviposition water was prepared by soaking grass clippings in aged tap water and allowing fermentation over a 3-d period. Three days after each blood meal, oviposition cups were placed with caged adults, and eggs were collected the following day. Two hundred first-instar larvae were placed into 22.8× 33.0 cm aluminum pans with 450 ml of aged tap water. The larvae were provided finely ground TetraMin® flakes, increasing the quantity daily from 50 mg for first instars to 500 mg for final instars until pupae appeared on the 8th and 9th day post-hatching. Pupae then were counted and transferred to plastic cups and placed in a 41-l cage supplied with water wicks. Emerging adults were given *ad libitum* access to water, but were deprived of sugar. Experiments were conducted 36±12 h after emergence. The mosquito rearing and maintenance conditions were 27±1 °C, 85±5 % RH, and 16:8 (L:D), with 30-min gradual crepuscular transitions between photophase and scotophase.

Chemicals Phenylacetaldehyde (>90 %), benzaldehyde (≥99.5 %, purified by redistillation), nonanal (≥95 %), (*E*)-2-nonenal (97 %), and an alkane-standards mixture (C_8 - C_{20}) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Benzyl alcohol (99.9 %) was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA), and β-Ocimene [(75 % (*E*)-β-ocimene)] was synthesized by CHEMOS GmbH (Regenstauf, Germany). Synthetics were

diluted with HPLC-grade *n*-pentane (Fisher Scientific, Pittsburgh, PA, USA).

Extract Preparation Flowers of common milkweed, *A. syriaca*, were collected in early summer from The Ohio State University campus in Columbus (40º00′18.95" N, 83º02′47.11" W), placed in an ice cooler, and transported 10-min to the laboratory. Single florets were plucked from the main milkweed umbel and separated from the calyx and other green parts of the flower, weighed, and placed into a 500 ml narrow-mouth glass Erlenmeyer flask. Milkweed florets were submerged in HPLC-grade *n*-pentane (Fisher Scientific) in a 1:8 ratio (w/v) for a total solvent volume of 480 ml and held for 24 h at room temperature, at which time the extract was decanted into 21-ml borosilicate glass vials, capped with Teflon-lined screw caps and stored at -20 °C.

Headspace Analysis The volatile profile of milkweed flower was characterized by placing a single floret into a 21-ml borosilicate glass vial with a Teflon-lined rubber septum. Volatiles were allowed to equilibrate in the vial headspace at 30 °C for 10 min before collections were made. Volatiles were collected by using a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (50/30 μm) solid phase micro-extraction (SPME) fiber (Supelco, Bellefonte, PA, USA). This mixed-chemistry fiber provides affinity for chemicals with a far broader range of polarity and volatility than PDMS alone. The fiber was introduced to the vial through the septum and exposed for 30 min. The collected volatiles were analyzed immediately by using an Agilent Technologies 6890 series gas chromatograph interfaced to an Agilent Technologies 5973 quadrupole mass selective detector (GC-MS). The SPME fiber was desorbed at 275 °C for 3 min with a splitless injection onto a Zebron™ ZB-1 ms column (30 m×0.25 mm, 0.25 μm film thickness) (Phenomenex, Torrance, CA, USA). The oven temperature was held at 25 °C for 3 min, then ramped at 15 °C/min to 250 °C, where it was held for 2 min. The carrier gas was helium at a flow rate of 1 ml/min. The mass selective detector was operated in EI mode at 70 eV, scanning 19-350 *m/z* with a quadrupole temperature of 180 °C and source temperature of 240 °C.

Extract Analysis To guide the construction of a synthetic floral blend, pentane extracts of milkweed florets were analyzed by GC-MS on a Zebron™ ZB-50 column (15 m× 0.25 mm, 0.25 μm film thickness) (Phenomenex), with a 1-μl splitless injection at 210 °C. The temperature program began at 25 °C for 3 min and increased at 13 °C/min to 200 °C, followed by a 25 °C/min increase to 280 °C to remove high-boiling lipids. Other parameters were as described above. Peak identities were validated by re-injection on a Finnegan Trace GC/MS (Thermo Fisher Scientific Inc., Waltham, MA, USA), with a 1:10 split at 180 °C and a Rtx®-5MS column (30 m×0.25 mm, 0.25 μm film thickness) (Restek, Bellefonte, PA, USA). The helium flow was 1 ml/min, and temperature program was 40 °C for 1 min, ramped at 15 °C/min to 275 °C, with a 2-min final hold time. Retention indices (RI) of compounds were determined on both columns relative to *n*-alkanes (C_8–C_{20}). Components of floral headspace and extract were identified by matching their mass spectra to NIST/EPA/NIH Mass Spectral Library 2005 and comparison of retention indices to published values (Adams, 1995). Identity of major components was confirmed by comparison of spectra and retention times to authentic standards. Peak purity was determined by using AMDIS deconvolution software (NIST, Gaithersburg, MD, USA) to assure the absence of any coeluting components. A synthetic mimic of the extract was constructed by blending authentic standards of the major components identified in the extract. Quantities of each component were individually adjusted until the overall concentration and component proportions closely approximated those of the floral extract (Fig. 1**a**).

Behavioral Assays All behavioral assays were conducted in a clear acrylic dual-port flight olfactometer, modified from Hancock and Foster (1993), with three main parts: an introduction/release chamber, flight chamber, and trapping ports. The introduction/release chamber was located at the downwind end, measuring 30×40 cm wide and 30 cm long. A sliding gate separated the release chamber from the main flight chamber (30×40 cm wide, 90 cm long), which had two cylindrical glass jar trapping ports (15 cm long by 7 cm diameter) on its upwind end. Ports were fitted with borosilicate glass funnels, with the wide end opening into the flight chamber and the narrower (3 mm diam) end pointing into a glass jar. Thus, mosquitoes entering the wide end of the funnel from the flight chamber were channeled into the jar, where they were retained. Trapping ports were located 11 cm above the flight chamber floor and were separated by 21 cm. The funnel and glass jar were held together by Parafilm® sealing film (Pechiney Plastic Company, Menasha, WI, USA), which was replaced between treatments. We recognized the pitfalls associated with the use of Parafilm® in solvent-based behavioral assays (Millar and Haynes, 1998), and we ensured that this sealant would not come in contact with any chemicals in the olfactometer. A 7.5-mm diam hole in the upwind end of each jar allowed the introduction of purified/humidified air after it had passed from an oil-free air pump through an activated carbon canister, and then through a water column. Air flow was maintained at 50 ml/s into each port, providing a velocity of 72 mm/s in the center of the flight chamber. A black cotton cloth, covering a dampened layer of cotton wool, covered the

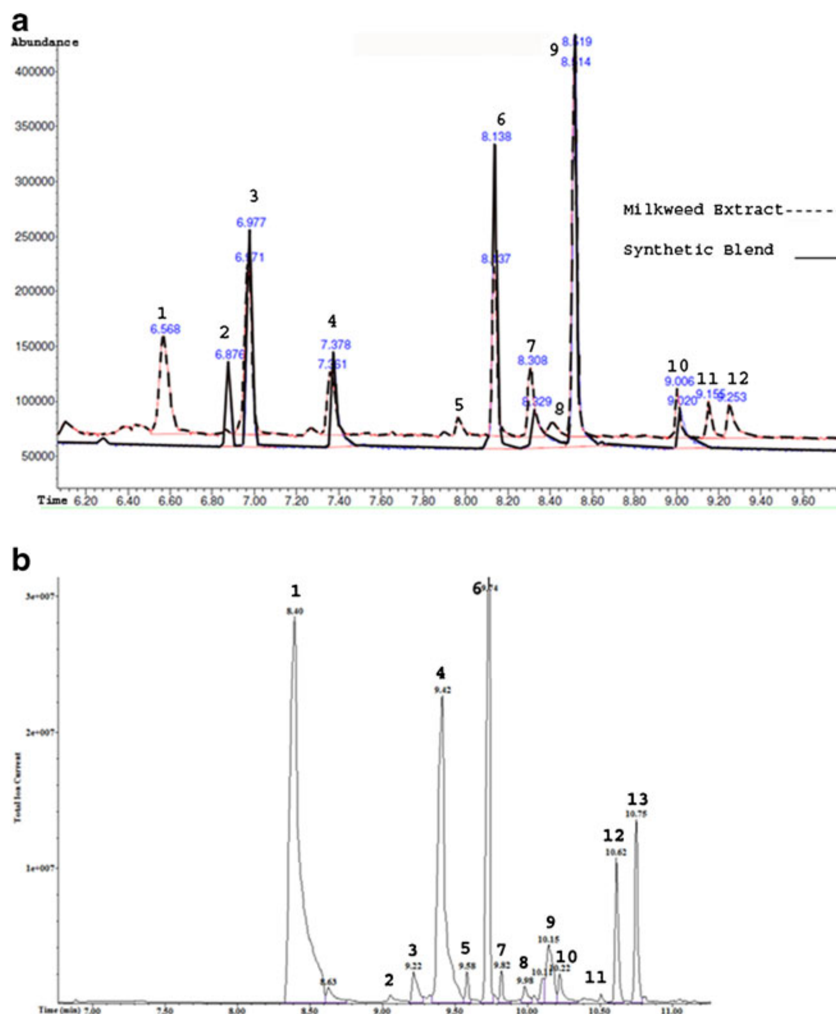

Fig. 1 Total ion chromatograms (**a**) of florets of milkweed, *Asclepias syriaca*, pentane extract (broken line), and a synthetic blend (solid line) used to assay upwind attraction of *Culex pipiens* (Peak # 1. unknown, 2. (*Z*)-β-ocimene, 3. (*E*)-β-ocimene, 4. benzaldehyde, 5. unknown, 6. nonanal, 7. benzyl alcohol, 8. unknown, 9. phenylacetaldehyde, 10. (*E*)-2-nonenal, 11. 2,6-nonadienal, and 12. phenylethanol; column = Zebron™ ZB-50) and total ion chromatogram (**b**) of the head-space profile of a single *A. syraica* floret captured on a divinylbenzene/carboxen/polydimethylsiloxane SPME fiber (Peak # 1. benzaldehyde, 2. myrcene, 3. unknown monoterpene, 4. phenylacetaldehyde, 5. (*Z*)-β-ocimene, 6. (*E*)-β-ocimene, 7. γ-terpinene, 8. unknown monoterpene, 9. dimethylstyrene, 10. unknown monoterpene, 11. unknown monoterpene, 12. (*E,Z*)-allocimene, and 13. (*E,E*)-allocimene; column = Zebron™ ZB-1)

entire floor of the flight chamber, to maintain a level of humidity similar to that within the choice ports. Data loggers (HOBO®, Onset Computer Corporation, Bourne, MA, USA) recorded temperature and humidity in the olfactometer, which was 25.0-27.5 °C and 75-95 % RH. An exhaust duct, connected at the downwind end of the release chamber, directed the effluent air out of the building through an exhaust hood. To test for positional bias of the ports, mosquitoes were released into the chamber after either baiting both ports with honey or leaving both empty. Mosquitoes were divided equally between left and right ports when baited with honey and were found rarely in ports when both were empty.

Response to whole milkweed flowers was determined in the flight olfactometer with 2 g of florets placed in an aluminum weighing boat in one of the trapping jars, and compared with an empty control jar. Extracts and synthetic chemicals were applied to 15-cm long cotton wicks (TIDI Products, Neenah, WI, USA) and compared to a pentane control. The positions of the treatment and control ports were alternated between bioassays as a further safeguard against positional bias. The olfactometer parts were cleaned with 70 % ethanol followed by water after each experiment; gloves were used at all times to avoid contamination with human-related kairomones. The 16:8 (L:D) light cycle used for mosquito rearing was maintained in the bioassay room. At 2 h prior to scotophase, approximately 200-300 mosquitoes of both sexes in similar numbers were released through a sleeve connection, directly from an acrylic plastic cage (30×30×30 cm) into the holding/release chamber, where they were held for 15 min to acclimatize before the release gate was opened. After 12 h, the numbers of mosquitoes in the treatment port, the control port, and remaining in the flight chamber were recorded. Use of a 12-h period captured the bimodal nocturnal-crepuscular sugar-feeding rhythm of *C. pipiens*, which includes some sugar feeding throughout the night (Yee and Foster, 1992).

Subtractive bioassays were conducted on the synthetic blend to determine the relative activity of the identified components. The full six-component blend was presented in one of the paired olfactometer ports alongside a reducted five-compound blend, in which one of the components was removed. Each reducted blend was tested 4-6 times. After establishing the significance of each chemical on mosquito attraction, we compared the minimal attractive blend against the full six-component blend. This was followed by a dose–

response study of the minimal blend to determine the optimal concentration for response. Doses were presented in a randomized block design ($N=5$), with percentage response calculated as the number in the synthetic blend port minus the number in the control port and expressed relative to the number of mosquitoes released.

Video Recording Mosquito flight behavior was recorded by using three infrared RCA closed-circuit video cameras (Lancaster, PA, USA) onto which was mounted a TC1824B wide-angle ES 25 mm 1:1.4 lens for flight-chamber recordings or a TC1874C ES 75 mm 1:1.8 lens for each of the choice ports. The cameras were controlled in series with a Burle Security Products TC8108 (Lancaster, PA, USA) eight-channel switcher, with video output to two RCA TC1109 video monitors. Video output was recorded with an Emerson EWV404 VCR (Parsippany, NJ, USA) onto VHS tape in time-lapse mode, 20 s each min.

Statistical Analysis Mosquito response in the flight olfactometer was analyzed by comparing numbers caught in the two ports by a goodness-of-fit *chi-squared* test with SPSS v. 17 (SPSS Inc, Chicago, IL, USA). The threshold for significance was $\alpha=0.05$. In the dose–response experiment, a regression model of mosquito response to log-transformed blend dose was determined by using the Fitted Line Plot module of Minitab v. 16 (Minitab Inc., State College, PA, USA).

Results

Chemical Identification Six compounds comprised >90 % of the relative abundance of the milkweed floret components in the pentane extract: (*E*)-β-ocimene, benzaldehyde, nonanal, benzyl alcohol, phenylacetaldehyde, and (*E*)-2-nonenal (Fig. 1a, Table 1). The volatile profile collected by DVB/CAR/PDMS SPME directly from fresh florets was dominated by three of these compounds: (*E*)-β-ocimene, benzaldehyde, and phenylacetaldehyde (>75 % of the total), but there were also substantial differences compared to the pentane extract (Fig. 1b). Most notably, (*E,Z*)- and (*E,E*)-alloocimene were collected from the flower headspace, but were almost completely absent from the extract, whereas nonanal, a major constituent of floret extract, was missing in the headspace sample. These disparities can be explained partly by variation in volatility among the components, but also likely reflect differences between the chemical composition in flower tissue and what is released. The extract also contained higher-boiling hydrocarbons and fatty acids, but due to their low volatility, they were not included in behavioral studies. Based on the quantities of synthetic standards used to mimic the extract GC profile (Table 1), the total floral concentration of the six major volatiles was estimated at 32 μg/g floret fresh weight.

Flight Olfactometer Response Infrared video recordings showed *C. pipiens* in the flight chamber engaged in a zigzagging flight that was directed upwind towards ports baited with florets, extracts, or synthetic blends. The angle of the turns progressively decreased as the mosquitoes approached the target. In most cases, mosquitoes flew into the ports, but in a few cases they landed on the entrance of the port and walked in. There was no observed oriented flight towards the control port.

In the first behavioral assay, mosquitoes showed a significant response to whole milkweed florets, where 67 % of released mosquitoes were captured in the floret-baited port, compared to only 6 % in the control port (Fig. 2a; $\chi^2=515.02$ $df=1$, $P<0.001$). Despite the chemical differences measured between the headspace and extract profiles of milkweed florets, mosquitoes showed a similarly strong response to a pentane extract of milkweed florets (52 %) compared to pentane alone (11 %) (Fig. 2b; $\chi^2=61.44$, $df=1$, $P<0.001$). Moreover, mosquitoes were observed in video recordings to probe the extract-treated cotton wick during the early scotophase and early photophase, suggesting that the extract also stimulated a feeding response. This behavior was never observed on control wicks.

Table 1 Major volatile constituents of a pentane extract of the common milkweed, *Asclepias syriaca*, as determined by gas chromatography–mass spectrometry, and quantities used in a synthetic mimic

COMPOUND[a]	Relative retention index[b]	Diagnostic EI-MS fragment ions (% intensity)	Synthetic blend (μg/ml)
(*E*)-β-Ocimene	1124	77(36), 79(42), 91(53), 93(100)	6.14[c]
Benzaldehyde	1155	51(54), 77(100), 105(88), 106(88)	3.27
Nonanal	1220	29(66), 41(100), 56(61), 57(94)	4.14
Benzyl alcohol	1236	77(85), 79(100), 107(59), 108(75)	4.58
Phenylacetaldehyde	1257	65(22), 91(100), 92(24), 120(16)	11.25
(*E*)-2-Nonenal	1304	29(56), 41(100), 55(80), 70(58)	2.12

[a] Identity established by comparison to authentic standard of each compound

[b] GC retention times relative to *n*-alkane standards on a Phenomenex ZB-50 column phase

[c] Synthetic β-ocimene contained a 3:1 (*E:Z*) isomer mixture

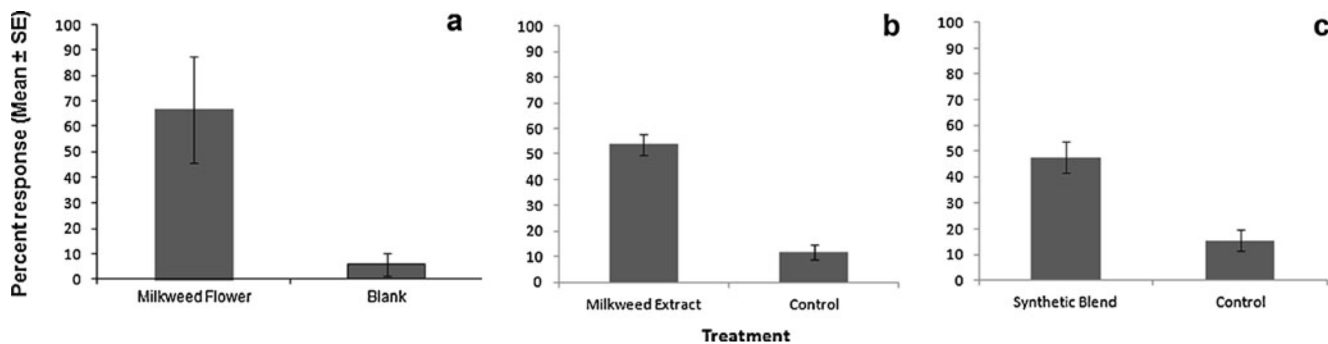

Fig. 2 Percentage of *Culex pipiens* flying upwind in a dual-port flight olfactometer to: **a** whole milkweed, *Asclepias syriaca*, florets; **b** pentane extract of *A. syriaca* florets; and **(c)** synthetic *A. syriaca* floret blend compared against a water **a** or solvent (**b, c**) control. *** $P<0.001$ by *chi-squared* test

Based on the positive response to the milkweed floret extract, a six-component synthetic blend was formulated to simulate the concentrations and relative proportions of major constituents of the pentane extract (Fig. 1a). One discrepancy in the GC profiles was caused by the presence of the (*Z*)-isomer in synthetic (*E*)-β-ocimene, resulting in a synthetic blend that exceeded the levels of this isomer in the floret extract. In the olfactometer, the synthetic floret blend performed similarly to the natural extract, with 48 % of released mosquitoes trapped, compared to 16 % for the control (Fig. 2c; $\chi^2=120.6$, $df=1$, $P<0.001$). Again, video-recordings showed a directed upwind flight response to ports containing the synthetic blend, accompanied by vigorous probing of the treated wicks, but not to the controls. Males and females responded to milkweed floret odors in similar numbers in all three experiments as the sex ratio of mosquitoes captured in either the treatment or control ports did not deviate significantly from 1:1.

The subtractive bioassay of the synthetic blend indicated a significant role for three compounds in *C. pipiens* response to milkweed: benzaldehyde ($\chi^2=13.22$, $df=1$, $P<0.001$), phenylacetaldehyde ($\chi^2=8.25$, $df=1$, $P=0.004$), and (*E*)-*2*-nonenal ($\chi^2=3.62$, $df=1$, $P=0.05$) (Fig. 3). When any of these compounds was removed, mosquitoes showed a significant preference for the full blend over the reducted blend. A nearly significant preference was seen for blends missing either β-ocimene ($P=0.078$) or benzyl alcohol ($P=0.082$) compared to the full blend. The activity of benzaldehyde, phenylacetaldehyde, and (*E*)-*2*-nonenal was confirmed when the three were combined: the three-component blend captured 31 % of mosquitoes compared to only 7 % for the control (Fig. 4a; $\chi^2=196.56$, $df=1$, $P<0.001$) and was as active as the full blend (Fig. 4b**,** 4; $\chi^2=3.02$, $df=1$, $P=0.082$).

Because quantity as well as quality can determine the intensity of chemically mediated behavior, *C. pipiens*

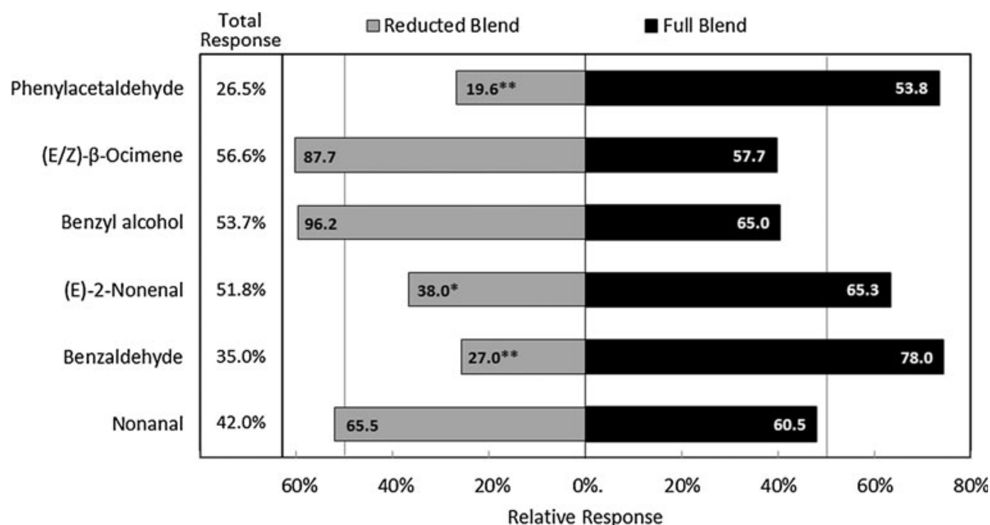

Fig. 3 Choice response of *Culex pipiens* in a dual-port flight olfactometer to a six-component (full) synthetic blend of milkweed, *Asclepias syriaca,* floret volatiles compared to blends in which each component has been removed individually (reducted). Total response indicates the percentage of *C. pipiens* released that were captured by one of the blends (no significant differences by 1-way ANOVA). Relative response shows the proportional response to the reducted (gray bars) and full (black bars) blends. Numbers in bars indicate the mean number of *C. pipiens* responding ($N=4$–6). Asterisks denote significant differences in mean response to reducted *versus* full blend by chi-squared test (* $P<0.05$, ** $P<0.005$)

response was measured in the flight olfactometer to different doses of the three-component blend compared to a solvent control. There was a significant positive quadratic response [*log*(dose) ($y=-20.527x^2+73.476x-43.709$; $R^2=57.6$ %], with a response maximum predicted at 62 μg (Fig. 5). The response flattened out at both the lowest and highest doses; removal of the two most extreme doses produced a stronger-fitting regression model ($R^2=93.3$ %), but with a similar predicted optimal dose of 65 μg.

Discussion

A positive upwind orientation by *C. pipiens* to floret volatiles of *A. syriaca* was demonstrated in this study, suggesting a new potential source of attractants for use in the field as lures. Males and females showed significant response to milkweed florets, a pentane extract, and a synthetic blend of the extract's six major constituents: benzaldehyde, (*E*)-β-ocimene, benzyl alcohol, phenylacetaldehyde, nonanal, and (*E*)-2-nonenal. The first three of these compounds are among the most common floral odor constituents in plants (Knudsen et al., 2006). Subsequent

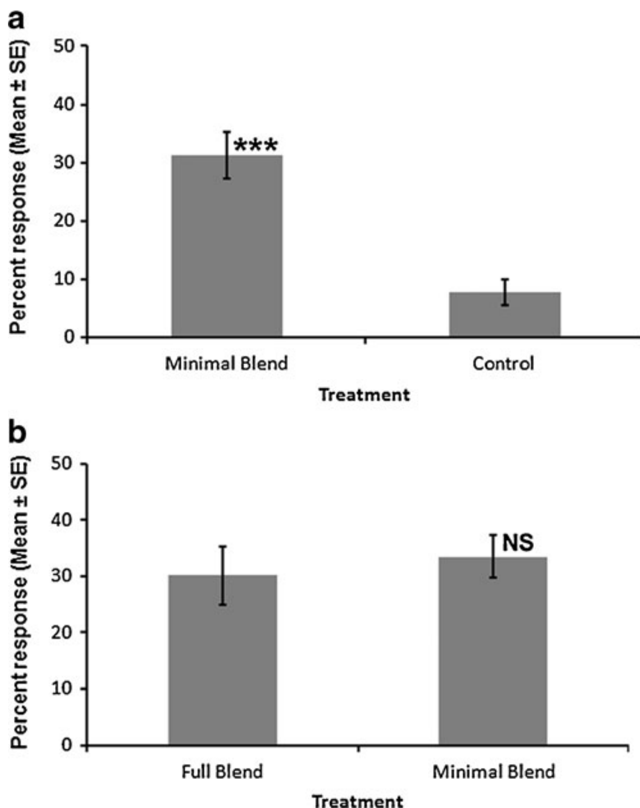

Fig. 4 **a** Percentage of *Culex pipiens* flying upwind in a dual-choice flight olfactometer in response to a three-component blend, consisting of benzaldehyde, (*E*)-*2*-nonenal, and phenylacetaldehyde; *** *P*< 0.001 by *chi-squared* test. **b** Percentage of *C. pipiens* flying upwind in response to the full (six-component) and minimal (three-component) synthetic blends of *A. syriaca* floral odor. NS = no significant difference

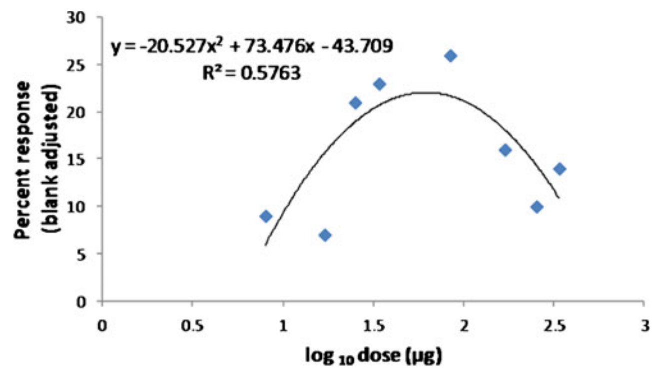

Fig. 5 Percentage of *Culex pipiens* flying upwind in a dual-choice flight olfactometer in response to different concentrations of a three-component kairomone blend (benzaldehyde, (*E*)-*2*-nonenal, and phenylacetaldehyde) after subtracting the response to a paired solvent (pentane) control

subtractive bioassays of the synthetic blend demonstrated that only three of its components contributed to attraction: benzaldehyde, phenylacetaldehyde, and (*E*)-2-nonenal.

The attraction of mosquitoes to plant odors has been demonstrated in numerous field and laboratory studies, but few studies have shown significant attraction to synthetics. Mauer and Rowley (1999) observed orientation of *C. pipiens* to a methylene chloride extract of *A. syriaca* in a dual port olfactometer. However, a synthetic blend of the two dominant compounds in the extract headspace, benzyl alcohol and 2-phenylethanol, failed to attract the mosquitoes. We did not test 2-phenylethanol, but benzyl alcohol was not active in our flight olfactometer bioassays. Jhumur et al. (2007) also found benzyl alcohol to not be very attractive to *C. pipiens* var. *molestus*, although Puri et al. (2006) recorded significant upwind response by *C. quinquefasciatus* to the compound.

Flowers of *Tanacetum vulgare* also attract *Culex* species in the field (Andersson and Jaenson, 1987), and Bowen (1992b) discovered a high proportion of both broadly- and narrowly-tuned antennal receptor neurons of *C. pipiens* sensitive to thujone, the primary constituent of *T. vulgare* fragrance. However, thujone only elicited a close-range dose-dependent probing response in *C. pipiens*; it did not stimulate upwind flight in a wind-tunnel olfactometer (Bowen, 1992b). The only previous flower-based synthetic blend causing mosquito orientation was developed from *Silene otites*, whose flowers attract mosquitoes in the field (Brantjes and Leemans, 1976). The synthetic blend mimic consisted of phenylacetaldehyde, veratrole, and 2-methoxyphenol, the first of which elicited the strongest attraction in *C. pipiens* var. *molestus* when presented individually (Jhumur et al., 2006). Phenylacetaldehyde also was a prominent component of our *A. syriaca* headspace and solvent extract, and it was essential for maximum attraction to the synthetic blend.

Although nonanal was a major component of *A. syriaca* floret extract, we saw no evidence that it played a role in

attraction in our bioassays. In contrast, from single-cell recordings, Syed and Leal (2009) determined that ca. 40 % of all antennal olfactory receptors of *C. quinquefasciatus* were acutely sensitive to nonanal, and it produced a synergistic effect on trap catch when presented with CO_2. This apparent discrepancy likely is explained by differences in mosquito physiological state. Nonanal is a major skin odorant of birds and mammals, including humans, resulting from the oxidation of sebaceous fatty acids (Haze et al., 2001). This fact, along with the significant behavioral interaction with CO_2, suggests that nonanal may play an important role in mediating *Culex* host-finding, but does not attract sugar-seeking mosquitoes.

We found notable differences between profiles of the pentane extract and the SPME-collected headspace of *A. syriaca* florets. Pentane extracts were dominated by phenylacetaldehyde, (*E*)-β-ocimene, and nonanal, whereas the headspace profile contained primarily benzaldehyde, ocimene isomers, and phenylacetaldehyde. Disparities between extracts and chemicals collected in the headspace might be explained by differences in the physico-chemical properties of the constituents, deep penetration by solvents to extract compounds that are not normally released by the tissue, and/or selective trapping of chemicals by SPME. In this study, differences between the methods are largely consistent with chemical differences in vapor pressure. Benzaldehyde, β-ocimene, and allоocimene have the highest vapor pressures of all the chemicals identified from milkweed, and they make up most of the volatile profile. The vapor pressure of benzaldehyde is almost twice that of nonanal, almost four times that of (*E*)-2-nonenal, and more than six times that of benzyl alcohol. The latter three compounds were either absent or found in very low levels in the headspace analysis. Phenylacetaldehyde is intermediate in its volatility, but was also the component found in the highest levels in the pentane extract.

Volatile collection analyses can produce misleading results, as they may not accurately reflect the actual proportionality of constituents in the headspace. We do not believe that this was a major factor for explaining the differences in the headspace and extract profiles of milkweed due to our choice of SPME phase. Although PDMS has been the most widely used SPME phase, it is actually a poor choice for characterizing plant volatile profiles that contain constituents with a range of volatilities and functional groups. In preliminary studies (not shown), we found a broader array of volatile compounds was trapped from various flowers by DVB/CAR/PDMS compared to PDMS alone. This mixed-bed fiber not only employs a broader range of phase polarity, but also incorporates both adsorption and partitioning as mechanisms of collection (Koziel and Novak, 2002). A number of recent studies have demonstrated the higher recovery efficiency and linearity by DVB/CAR/PDMS compared to other SPME phases for all of the compound classes that we identified from milkweed (Cui et al., 2009; Ferreira et al., 2009; Zhang et al., 2009). For example, Zhang et al. (2009) found that volatile profiles of longan fruit extracted by a 50/30 μm DVB/CAR/PDMS fiber were 3x higher in terpenes, 5x higher in alcohols, and 14x higher in esters than those produced by a 100 μm PDMS fiber, and revealed volatile carbonyls and acids, which were completely absent from PDMS profiles.

This study points to floral odors as a potential new model for chemical lures useful for mosquito sampling or control. If similar attraction can be demonstrated at the field level, as has already been demonstrated with human kairomone blends (Mukabana et al., 2012), synthetic floral blends could potentially be used in trapping devices to sample adult populations. Relative to animal-derived odors (Mukabana et al., 2012), floral odors have the advantage of attracting both sexes of mosquitoes in proportional numbers, and females in all gonotrophic states and in reproductive diapause. Given that *C. pipiens* visits a variety of flowers for nectar-feeding, the three compounds identified here are likely not the only floral components attractive to them, and more effective blends may remain to be discovered. It also remains to be seen whether different mosquito species use similar chemical cues. Future research should seek additional attractants and determine optimal blend release rates, delivery systems, and trap designs for maximizing capture in the field.

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Insect Egg Deposition Induces Indirect Defense and Epicuticular Wax Changes in *Arabidopsis thaliana*

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Abstract Egg deposition by the Large Cabbage White butterfly *Pieris brassicae* on Brussels sprouts plants induces indirect defense by changing the leaf surface, which arrests the egg parasitoid *Trichogramma brassicae*. Previous studies revealed that this indirect defense response is elicited by benzyl cyanide (BC), which is present in the female accessory reproductive gland (ARG) secretion and is released to the leaf during egg deposition. Here, we aimed (1) to elucidate whether *P. brassicae* eggs induce parasitoid-arresting leaf surface changes in another Brassicacean plant, i.e., *Arabidopsis thaliana*, and, if so, (2) to chemically characterize the egg-induced leaf surface changes. Egg deposition by *P. brassicae* on *A. thaliana* leaves had similar effects to egg deposition on Brussels sprouts with respect to the following: (a) Egg deposition induced leaf surface changes that arrested *T. brassicae* egg parasitoids. (b) Application of ARG secretion of mated female butterflies or of BC to leaves had the same inductive effects as egg deposition. Based on these results, we conducted GC-MS analysis of leaf surface compounds from egg- or ARG-induced *A. thaliana* leaves. We found significant quantitative differences in epicuticular waxes compared to control leaves. A discriminant analysis separated surface extracts of egg-laden, ARG-treated, untreated control and Ringer solution-treated control leaves according to their quantitative chemical composition. Quantities of the fatty acid tetratriacontanoic acid (C34) were significantly higher in extracts of leaf surfaces arresting the parasitoids (egg-laden or ARG-treated) than in respective controls. In contrast, the level of tetracosanoic acid (C24) was lower in extracts of egg-laden leaves compared to controls. Our study shows that insect egg deposition on a plant can significantly affect the quantitative leaf epicuticular wax composition. The ecological relevance of this finding is discussed with respect to its impact on the behavior of egg parasitoids.

Keywords Tritrophic interactions · Plant defense · Insect eggs · *Pieris brassicae* · *Trichogramma brassicae* · Brassicaceae · Epicuticular wax · Leaf surface

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Introduction

Plants are well-known to respond to herbivorous insect egg deposition by direct and indirect defense (Hilker and Meiners, 2006, 2010, 2011). Direct plant defenses induced by egg deposition harm the eggs directly by (a) a hypersensitive response that can lead to detachment of eggs from leaves (known for *Solanum* and *Brassica*, Shapiro and De Vay, 1987; Balbyshev and Lorenzen, 1997; Petzold-Maxwell et al., 2011), (b) production of ovicidal substances that kill the eggs (known for *Oryza* lines, Seino et al., 1996; Suzuki et al., 1996), (c) formation of neoplasms that hamper access of the hatching larvae to leaf tissue (known from *Pisum* lines, Doss et al., 1995, 2000 and *Physalis*, Petzold-Maxwell et al., 2011), or (d) production of egg-crushing plant wound tissue (Desurmont and Weston, 2011).

Indirect oviposition-induced defenses are detrimental to eggs or hatching larvae either because they attract parasitoids to egg-induced plant odor or because they arrest parasitoids on the egg-induced leaf surface (reviewed by Hilker and Meiners, 2011). Oviposition on leaves of *Pinus*, *Ulmus*, *Vicia*, and *Phaseolus* induces plant volatiles that attract egg and/or larval parasitoids (reviewed in Hilker and Meiners, 2010; *Zea:* Bruce et al., 2010; Tamiru et al., 2011). Egg-induced changes of the leaf surface have been shown for *Brassica oleracea* var. *gemmifera* and var. *sabauda* (Fatouros et al., 2005, 2009; Conti et al., 2010). The parasitoid species arrested on *Pieris* egg-induced surface of *B. oleracea* var. *gemmifera* leaves is a generalist (*Trichogramma brassicae*). The changes in the leaf surface caused the egg parasitoids to spend more time on an egg-induced leaf, thereby enhancing the parasitoid's chance to locate host eggs (Fatouros et al., 2005).

The molecular responses of plants to egg deposition have been studied in *Pisum, Pinus, Brassica*, and *Arabidopsis* (Cooper et al., 2005; Doss, 2005; Little et al., 2007; Fatouros et al., 2008; Koepke et al., 2008, 2010). Taken together, these studies showed that egg deposition can significantly affect transcript levels of a wide range of genes involved both in the plant's primary and secondary metabolism. The egg-induced molecular changes in *Pisum, Pinus*, and *Brassica* are known to be reflected by egg-induced changes of the plant's phenotype that can defend directly or indirectly against the eggs (see above for references). However, no information is available on the ecological relevance of the egg-induced molecular changes in *A. thaliana*. So far, it is unknown whether the egg-induced molecular responses shown for *A. thaliana* result in defense against the eggs.

Hence, we here investigated whether egg deposition by *Pieris brassicae* on *A. thaliana* induces indirect defense. We tested the hypothesis that egg deposition by this butterfly on *A. thaliana* has similar effects to those shown for *Brassica* leaves, i.e., that eggs induce changes of the *A. thaliana* leaf surface that arrest the generalist egg parasitoid *T. brassicae*.

If we could obtain evidence supporting this hypothesis, we further aimed to examine the chemical changes of the *Arabidopsis* leaf surface in response to egg deposition. The impact of abiotic and biotic stresses on leaf surface chemistry is well known (Kerstiens, 1996; Shepherd and Griffiths, 2006; Riederer and Müller, 2006 and references therein), but there have been no studies specifically examining how leaf surface chemistry might be altered after egg deposition.

Parasitoid-arresting leaf surface changes in Brussels sprouts (*B. oleracea* var. *gemmifera*) have been shown to be induced by a secretion released with *P. brassicae* eggs (Fatouros et al., 2008). This secretion is produced in the accessory reproductive glands (ARG) of the females and glues clusters of 20–50 eggs on the lower leaf surface (Feltwell, 1982). In *P. brassicae*, males transfer the anti-aphrodisiac benzyl cyanide (BC) to females during mating, which prevents further mating (Andersson et al., 2003). Traces of BC have been detected in the ARG secretion of mated *P. brassicae* females. When applied onto Brussels sprouts leaves, BC itself was sufficient to act as an elicitor of the plant's indirect defense and to induce changes in the leaf surface that arrested *T. brassicae* wasps (Fatouros et al., 2008).

Here, we investigated (a) whether *P. brassicae* egg deposition on *A. thaliana* induces leaf surface changes that arrest the egg parasitoid *T. brassicae*, and (b) whether the ARG secretion of mated females of *P. brassicae* or BC elicit the same arrestment effect on the parasitoid. In order to elucidate egg-induced chemical changes of the epicuticular leaf wax pattern, we analyzed leaf surface extracts of egg-laden and ARG-treated *A. thaliana* leaves by GC-MS and compared them with appropriate controls.

Methods and Materials

Plants and Insects We used *Arabidopsis thaliana* ecotype Col-0 for bioassays and chemical analysis. After 3 d of stratification at 4 °C, plants were grown in soil (soil type P, Kausek GmbH & Co. KG, Mittenwalde, Germany) in a climate chamber under short day conditions (photoperiod 10 hr, photosynthetic photon flux 100 μmol m^{-2} sec^{-1}, temperature 22 °C, r.h. 70 %). While working with the plants, gloves were used to avoid contamination of the plants with skin lipids. All experiments were done with ca. 8–10-wk-old, non-flowering plants.

We used Chinese cabbage (*Brassica rapa* var. *pekinensis*) to feed larvae of the Large Cabbage White butterfly *Pieris brassicae* L. (Lepidoptera: Pieridae). Plants were grown in soil in a greenhouse under long day conditions (photoperiod 16 hr, photosynthetic photon flux 175 μmol m^{-2} sec^{-1} temperature 22±4 °C, r.h. 35±10 %).

Pieris brassicae was reared in a climate chamber under long day conditions (photoperiod 18 hr, photosynthetic photon flux on average 220 μmol m^{-2} sec^{-1}, temperature 23 °C/ 15 °C, r.h. 70 %). Butterflies (60–80 adults) were kept in cages (25×62×62 cm). For egg deposition, an *A. thaliana* plant was placed into a cage for a period of 24 hr. Hatching caterpillars were transferred to leaves of Chinese cabbage that were placed in a small plexiglass box (15×26×42 cm) and kept in a climate chamber under long day conditions (photoperiod 18 hr, photosynthetic photon flux 160 μmol m^{-2} sec^{-1}, temperature 20 °C, r.h. 70 %). Caterpillars were supplied with fresh Chinese cabbage leaves until pupation (larval development from L1 to L5: 21 d). Pupae were separated by sex. Only female pupae were transferred to cages in order to obtain virgin females,

and pupae of both sexes were transferred to cages in order to obtain mated females laying fertilized eggs.

The egg parasitoid *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae) was reared on eggs of the grain moth *Sitotroga cerealella* Olivier (Lepidoptera: Gelechiidae) (AMW Nützlinge GmbH, Pfungstadt, Germany). About 400–600 eggs of this moth were glued onto a small paper card (4 cm^2) and exposed to 300–400 parasitoids in a glass tube (20 ml). Tubes were closed with cotton wool and stored in a climate chamber (photoperiod 18 hr, photosynthetic photon flux 160 µmol m^{-2} sec^{-1} across the tubes, temperature 20 °C, r.h. 70 %). Ten days later, emerging adult parasitoids were sexed based on their antennal morphology (Pinto and Stouthammer, 1994) and kept in a glass tube (20 ml) under the same abiotic conditions as used for the rearing. Only females were used for the bioassays.

Prior to a bioassay, ca. 200 freshly emerged parasitoid females per glass tube were exposed for 24 hr to one egg cluster with >20 eggs (3-d-old) of *P. brassicae*. The egg masses were cut from *A. thaliana* leaves. During this exposure time to host eggs, parasitoid females were supplied with an aqueous honey solution offered on a small piece of filter paper. The supply of food together with host presence has been shown to be important to increase behavioral response levels of the parasitoids (Fatouros et al., 2005; Schroeder et al., 2008). Thus, only 1 to 2-d-old, oviposition-experienced, and honey-fed parasitoid females were used. We used parasitoid females of this age since these young females are actively foraging for host eggs (Knutson, 1998).

Plant Treatments We placed one *A. thaliana* plant in a cage with about 30–40 male and 30–40 female butterflies for egg deposition. The cages and abiotic conditions were the same as those used for *P. brassicae* rearing. Plants were removed from the cages after 24 hr. Only plants with at least four leaves laden with an egg cluster of at least 20 eggs were used as test plants. Egg-laden plants were stored for further 48 hr in another climate chamber under long day conditions (photoperiod 18 hr, photosynthetic photon flux 160 µmol m^{-2} sec^{-1} across the plants, temperature 20 °C, r.h. 70 %). Similarly, egg-free control plants were stored for 72 hr under the same abiotic conditions.

In order to treat plants with secretion of accessory reproductive glands (ARG) from *P. brassicae* females, we dissected glands from 3 females and transferred the reservoirs to 100 µl isotonic insect Ringer solution (1 l distilled water with 2.25 g NaCl, 0.0105 g KCl, 0.08 g $CaCl_2 \times 2H_2O$, 0.01 g $NaHCO_3$, and 0.0105 g NH_4Cl, Merck KGaA, Darmstadt, Germany). We differentiated between glands from mated and virgin females because glands from virgin females lack benzyl cyanide (BC) (Fatouros et al., 2008). Glands from mated females were dissected 1–2 d after mating; all butterfly females were at least 5 d-old. The samples with glands in Ringer solution were stored at −80 °C when not used immediately. For application, the samples were defrosted and then homogenized with a micropistill (Eppendorf AG, Hamburg, Germany). Prior to centrifugation of the sample (13000 rpm for 5 min), we added an additional 100 µl Ringer solution. After centrifugation, the supernatant (100 µl, i.e., equivalent of 1.5 glands of mated or virgin females) was applied onto *A. thaliana* leaves. For application, the entire plant was turned upside down. We carefully paid attention not to damage any part of the plant. The supernatant was applied with a pipette (100 µl) on the outer edge of the lower surface of a leaf, and the droplet was carefully and slowly dispersed onto an area of about 2 cm^2 with a smooth brush. When the solution had dried after about five min so that it no longer dripped off, the plants were turned upright again. Three leaves per plant were treated in this way, each with 100 µl of the test solution. Control plants were treated in the same way with 100 µl Ringer solution per leaf (3 leaves per plant).

In order to treat plants with benzyl cyanide (BC; Fluka Chemie AG, Buchs, Switzerland), the compound was dissolved (a) in Ringer solution (1 ng BC/100 µl), or (b) in a sample with ARG secretion from virgin females that was prepared as described above. A volume of 100 µl was applied per *A. thaliana* leaf on an area of about 2 cm^2. The application procedure was the same as the one mentioned above for application of ARG homogenate. The elicitor amounts applied to the plant were equivalent to those used in the studies with *B. oleracea* var. *gemmifera* (Brussels sprouts) where treatment with about 1 ng BC per leaf was shown to induce a leaf surface change arresting *T. brassicae* 3 d after application (Fatouros et al., 2008). Furthermore, 1 ng BC was found to be present in the ARG secretion of a mated *P. brassicae* female (Fatouros et al., 2008). The *P. brassicae* strain used in the present study originated from the same population as used by Fatouros et al. (2008). Therefore, we assumed that the ARG of mated females contained similar amounts of BC. Three leaves per plant were treated with the BC-containing samples. Again, control plants were treated with 100 µl Ringer solution per leaf on an area of about 2 cm^2. After treatment, the plants were stored for 72 hr and 96 hr, respectively, in a climate chamber (photoperiod 18 hr, photosynthetic photon flux 120 µmol m^{-2} sec^{-1}, temperature 20 °C, r.h. 70 %).

Dual Choice Contact Bioassays We compared the behavioral response of the egg parasitoid *T. brassicae* to treated and control leaves in a two-choice bioassay set-up allowing contact with the leaves. We used a set-up similar to the one described by Fatouros et al. (2005, 2008). A leaf square (1 cm^2) was cut from a treated test and a control leaf prior to

the bioassay. The test and control leaf square were placed 1.5 cm apart in a small glass Petri dish (5.5 cm diam.). Both local and systemic effects of the treatments on the parasitoid's response were tested. Local effects were tested after 72 hr, and systemic effects were tested 96 hr after treatment.

To test local effects, test leaf squares (about 2 cm^2) were cut adjacent to an egg cluster or adjacent to the ARG- or BC-treated edge. Hence, the test leaf squares *per se* were left untreated. These test leaf squares are referred to here as "locally induced" leaf area.

To test systemic effects of the treatments, squares of untreated leaves (systemic leaves) from treated plants were used. Control leaf squares were taken from untreated plants from a position on the leaf similar to the test leaf squares.

A female wasp was released halfway between the leaf squares. The time spent on each of the squares was observed for 600 sec. We used the Observer software v. 3.0 (Noldus Information Technology, 1993©, Wageningen, The Netherlands) and recorded the behavior of actively foraging wasps that walked on the leaf squares for at least half of the observation period (300 sec), whereas inactive wasps (mainly sitting during observation) were discarded. Each wasp was tested only once. After each observation period, the Petri dish was rotated 180° to avoid directional biases due to side preferences. Three wasps were tested per test and control leaf square; thereafter, the Petri dish was cleaned with ethanol, and freshly cut squares were used. The total number of wasps and plants used for the different treatments are given in Fig. 1. The time spent by the parasitoids walking on treated test leaf squares and the respective controls were used for statistical data analysis (see below).

Preparation of Leaf Surface Extracts Since wasps contact only the outermost leaf surface, we intended to selectively isolate and analyze epicuticular leaf wax compounds. We used gum arabic for detachment of the epicuticular waxes (Jetter and Schäffer, 2001) of locally induced leaves 3 d (72 hr) after egg deposition or treatment with ARG secretion from mated females (i.e., leaves with a surface that significantly arrests the parasitoids); for control, waxes of untreated leaves or leaves treated with Ringer solution were extracted. We did not analyze waxes of leaves treated with BC since we wanted to focus our analyses here on those treatments that also occur in nature (egg deposition) or mimic natural egg deposition most (contact of leaf with ARG secretion, the egg glue).

Prior to the extraction of the leaf epicuticular wax layer, gum arabic (Fluka, Steinheim, Germany) was purified by soxhlet extraction with heated dichloromethane (24 hr–40 °C) and subsequently air-dried. An aqueous solution of gum arabic (50 % w/w) was applied spot-like (initial spot size about diam. 0.5 cm) on the lower side of a leaf right next to the treated leaf area (i.e., 0.5 cm apart from an egg mass, or the ARG-, or Ringer-treated leaf area). After 1 to 2 hr, the dry gum arabic film containing epicuticular waxes was carefully removed with forceps, and attention was paid to avoid damage of epidermal cells and exudation of intracellular compounds. Each gum arabic piece with the attached leaf epicuticular waxes was extracted in 500 µl dichloromethane (DCM) at room temperature for 1 hr. Extracts from 16 gum arabic-wax spots obtained from 3 to 5 plants were combined and considered as a single sample. After separation of the water and DCM phase, 10 µl of *n*-eicosane (0.1 mg/ml hexane) were added as internal standard to the DCM sample. This DCM extract sample was derivatized with N,O-bis-trimethylsilyl-trifluoroacetamide and trimethylchlorosilane (BSTFA-TMCS) (99:1; SUPELCO, Bellefonte, PA, USA), evaporated under nitrogen, and redissolved in 50 µl DCM. In total, 10 epicuticular wax extract samples were obtained each from egg-laden leaves and untreated controls, 8 extracts each from ARG-treated leaves and respective Ringer-treated controls.

The aqueous gum arabic solution spread differently over the surface after application of the initial spot of about 0.5 cm diam. After removal of the gum arabic film, the leaves were scanned to measure the sampled leaf area for later precise quantification of epicuticular wax compounds relative to the leaf area extracted.

Chemical Analysis of Leaf Surface Extracts The quantitative and qualitative composition of epicuticular waxes was analyzed by a coupled gas chromatography–mass spectrometry system (7890 GC-5975C MSD; Agilent Technologies, Waldbronn, Germany) equipped with a programmable temperature vaporization inlet (CIS-4; Gerstel, Mühlheim a. D. Ruhr, Germany). The sample volume (1 µl) was injected in splitless mode at 150 °C, and the CIS-4 was immediately heated to 300 °C at 12 °C/sec. A fused silica column (DB-5MS, 30 m×0.32 mm ID×0.25 µm, J and W Scientific, Folsom, CA, USA) was used for separation with a constant helium flow of 1 ml/min. The oven was programmed from 100 °C to 300 °C (50 min isotherm) at a rate of 5 °C/min. Electron impact ionization was 70 eV.

For identification, retention indices of the epicuticular wax compounds were calculated by co-injection of *n*-alkanes (Kováts, 1965). Compounds were identified by comparing mass spectra and linear retention indices with those of authentic standards or literature data. Hydroxyl-containing reference compounds were analyzed as trimethyl silylated derivatives. All *n*-alkanes, fatty acids, 1-alcohols, and phytosteroids were obtained from Sigma (Sigma-Aldrich Chemie GmbH, Munich, Germany). Single compounds were quantified against the internal standard (*n*-eicosane; 1 µg per sample) by integrating peak areas and reference to the removed surface area. For those compounds for which reference compounds were available, specific correction factors were

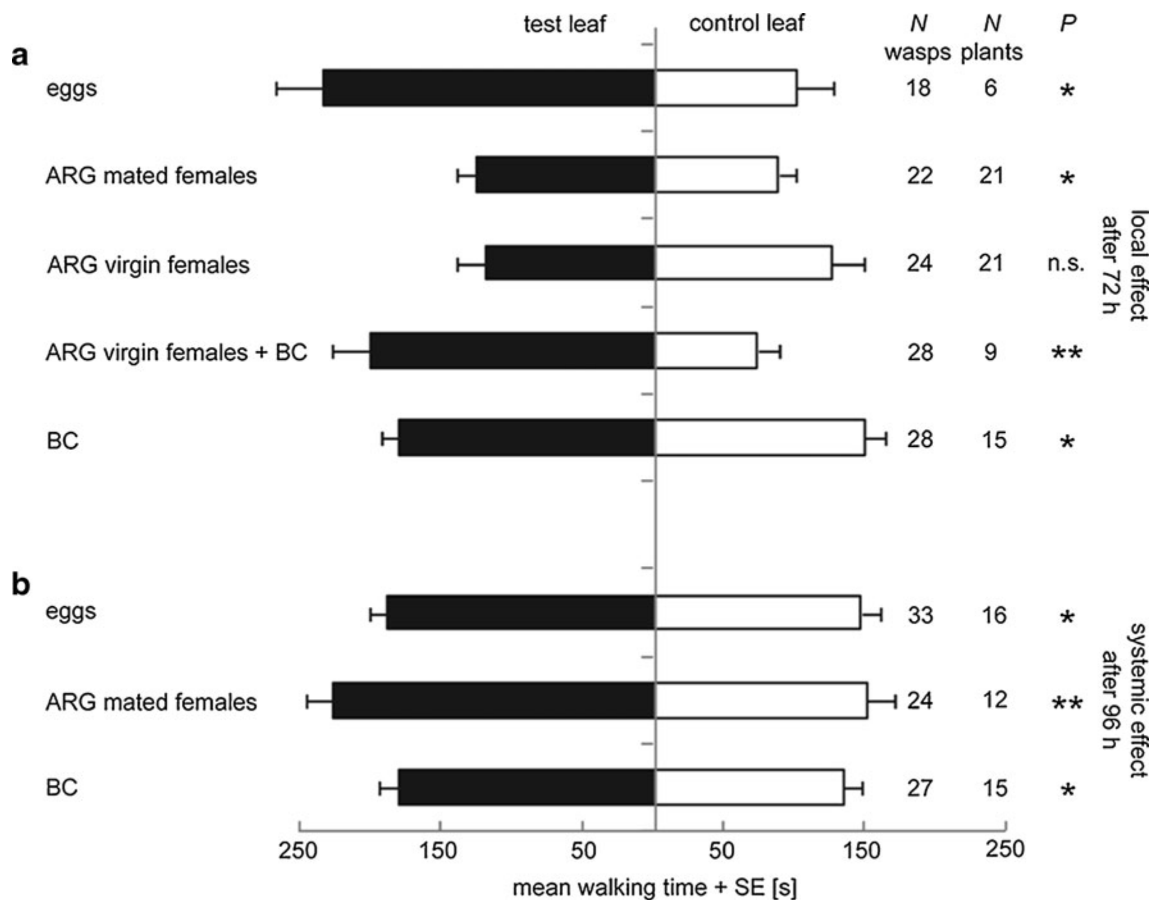

Fig. 1 Response of *Trichogramma brassicae* females to leaf squares of differently treated *Arabidopsis thaliana* leaves in a dual choice contact bioassay. Mean walking time (+ SE) on test and control leaf squares is given. **a** Locally induced test leaf squares were cut adjacent to the treated leaf area and tested 72 h after treatment (local effect); **b** Systemically induced test leaf squares were cut from an untreated leaf of treated plant and tested 96 h after treatment (systemic effect). ARG = secretion of accessory reproductive glands; BC = benzyl cyanide. *N* = number of tested females and plants per treatment. Asterisks indicate significant differences: **P*≤0.05, ***P*<0.01, n.s. = not significant with *P*>0.05 (Wilcoxon's signed rank matched pairs test)

calculated by comparing peak areas of defined amounts of internal standards and those of reference compounds: *n*-alkanes (×1.00), fatty acids (×0.69), alcohols (×0.68) and phytosteroids (×0.92). The quantities of epicuticular wax compounds are given in μg/dm² leaf surface (compare e.g., Kosma et al., 2009).

Statistical Analysis All statistical analyses were conducted using Statistica 7.0 (StatSoft Inc., Tulsa, USA) or SPSS 17 (SPSS Inc., Chicago, IL, USA).

For bioassays, the walking time spent by parasitoids on test- and control leaf squares were statistically compared with the Wilcoxon matched pairs signed rank test (Sokal and Rohlf, 1969).

For comparison of epicuticular wax compounds obtained from differently treated leaves, we considered only those compounds that were detected consistently in at least 60 % of the samples. Differences of the total amounts of all epicuticular wax profiles were analyzed by an ANOVA. Quantitative differences of epicuticular waxes among the four types of samples (egg-laden leaves and untreated controls; ARG-treated leaves and Ringer-treated controls) were statistically analyzed by a MANOVA. Prior to the MANOVA, the data were arcsin transformed. Furthermore, differences in the absolute quantitative composition of epicuticular wax profiles of the four types of samples were evaluated by a canonical discriminant analysis (DA) based on 16 compounds present in extracts of all treatments. Wilks' λ and the percentage of correctly classified individuals by a leave-one-out cross validation were used to assess the quality of the DA. Student's *t*-test was used to determine significant quantitative differences of each compound between the different treatments.

Results

Egg-Infested Arabidopsis Arrests Trichogramma Egg deposition by *Pieris brassicae* on *Arabidopsis thaliana* leaves had significant effects on the behavioral response of the egg

parasitoid *Trichogramma brassicae* to leaf area adjacent to the eggs. Our dual choice bioassays revealed that 72 hr after egg deposition, the parasitic wasp spent significantly more time walking on locally egg-induced leaf area when compared to leaf area of egg-free control plants ($P=0.012$, Wilcoxon matched pairs signed rank test, Fig. 1a). Leaf squares obtained from systemic leaves of egg-infested plants also significantly arrested the wasps when compared to leaf squares of control plants 96 hr after egg deposition ($P=0.016$, Wilcoxon matched pairs signed rank test, Fig. 1b).

ARG- or BC-treated Arabidopsis *Leaves Arrest Trichogramma* In a subsequent step, we tested whether treatment of leaves with ARG secretion of *P. brassicae* females had the same effects on parasitoid behavior as egg deposition. The time parasitoids spent walking on local squares of leaves treated with ARG secretion of mated females was significantly longer than the time walking on Ringer-treated controls ($P=0.012$, Wilcoxon matched pairs signed rank test, Fig. 1a). Systemic leaves of plants treated with ARG secretion of mated females had similar effects on the parasitoid arrestment ($P=0.005$, Wilcoxon matched pairs signed rank test, Fig. 1b). In contrast, local leaves treated with ARG secretion of virgin females (containing no BC) did not arrest the parasitoids ($P=0.650$, Wilcoxon matched pairs signed rank test, Fig. 1a).

In contrast, when the local leaf areas of plants were treated with a mixture of 1 ng of BC plus the ARG secretion of a virgin female, the parasitoids were arrested ($P=0.004$, Wilcoxon matched pairs signed rank test, Fig. 1a). Finally, local and systemic leaves of plants treated with only BC also arrested the parasitoids ($P=0.020$ local, $P=0.011$ systemic, Wilcoxon matched pairs signed rank test, Fig. 1a, b).

Chemical Composition of Leaf Surface Extracts All compounds detected in the extracts of egg-laden *A. thaliana* leaves and ARG-treated leaves also were detected in extracts of the control plants except for tetratriacontanoic acid. This compound was not detected in epicuticular wax profiles of Ringer-treated leaves, but was present in untreated controls (Table 1). We detected 16 compounds that were present in at least 60 % of the samples of all treatments. These profiles comprised phytosteroids, *n*-alkanes, fatty acids, aldehydes, and alcohols, with chain lengths of acyclic compounds ranging from C24 to C34. Extracts of all treatments were dominated by phytosteroids (untreated: 18.4 μg/dm²; egg-laden: 16.6 μg/dm²; Ringer: 10.4 μg/dm²; ARG: 13.3 μg/dm²) followed by *n*-alkanes (untreated: 11.8 μg/dm²; egg-laden: 12.3 μg/dm²; Ringer: 3.0 μg/dm²; ARG: 3.5 μg/dm²), fatty acids (untreated: 9.8 μg/dm²; egg-laden: 4.9 μg/dm²; Ringer: 2.0 μg/dm²; ARG: 1.8 μg/dm²), aldehydes (untreated: 3.1 μg/dm²; egg-laden: 5.2 μg/dm²; Ringer: 2.0 μg/dm²; ARG: 1.3 μg/dm²), and alcohols (untreated: 0.7 μg/dm²; egg-laden: 0.7 μg/dm²; Ringer: 1.8 μg/dm²; ARG: 1.5 μg/dm²). The major compounds were (in descending order by quantities) *β*-sitosterol, *n*-hentriacontane, hexacosanoic acid, and cholesterol.

When comparing total amounts of those epicuticular leaf wax compounds we consistently found that in at least 60 % of the samples, there were no significant differences between egg-laden leaves (48.1 μg/dm²) vs. those of the respective untreated controls (50.6 μg/dm²), and between ARG-treated leaves (24.1 μg/dm²) vs. Ringer-treated control leaves (23.3 μg/dm²) (ANOVA egg-laden vs. untreated control: $MS=30.04$, $F=0.09$, $P=0.76$; ANOVA ARG-treated *vs*. Ringer-treated: $MS=2.05$, $F=0.01$, $P=0.91$). However, the total amounts obtained from leaves subjected to natural situations (egg-laden and untreated control leaves) were twice as high as the amounts from artificially treated leaves (ARG- and Ringer-treated control leaves) (ANOVA egg-treated plus untreated control vs. ARG-treated plus Ringer-treated: $MS=1959.13$, $F=7.93$, $P=0.001$).

We analyzed the epicuticular wax profiles according to their quantitative chemical composition. We classified the leaf treatments in two ways. One classification, based on effects on parasitoid behavior, grouped those with significant defense-eliciting activity (egg-laden and ARG-treated leaves) vs. those without eliciting activity (untreated and Ringer-treated control leaves). A second classification was based on artificial/natural character, and grouped those for which we used Ringer (treatment with ARG secretion in Ringer and Ringer-treated control leaves) vs. those with fully natural character (egg-laden and untreated leaves). In both analyses, differences according to treatment classifications were found: defense-eliciting activity vs. non-eliciting activity: Wilks' $\lambda=0.197$, $F=4.32$, $P=0.002$ (MANOVA); artificial vs. natural character of treatment: Wilks' $\lambda=0.033$, $F=30.55$, $P=0.001$ (MANOVA).

The discriminant analysis based on the quantities of the 16 compounds that were found in at least 60 % of the samples clearly separated all four types of *A. thaliana* leaves tested (egg-laden, untreated control, ARG-treated, Ringer-treated) according to the chemical composition of their epicuticular waxes (Wilks' $\lambda=0.008$, $\chi^2=119.3$, $P<0.001$, Fig. 2a), and 63.9 % of the cross-validated cases were correctly classified. The first canonical root accounted for 73.6 % of the total variance of the data and separated the profiles according to the natural (egg-laden or untreated) and artificial treatment (ARG-secretion in Ringer, Ringer only). The third canonical root, explaining 10.7 % of the total variance, separated the profiles of egg-laden and ARG-treated leaves from those of the respective controls. The corresponding loading plot points out which epicuticular waxes contributed strongly to separation of *A. thaliana* leaves (Fig. 2b). The fatty acids tetracosanoic acid and tetratriacontanoic acid are the compounds responsible for

Table 1 Quantities (mean±SE) of epicuticular wax compounds extracted from *Arabidopsis thaliana*

Compound[a]	RI[b]	Compound class	Amount (µg/dm²)			
			Treatments[c]			
			Untreated	Egg-laden	Ringer	ARG
Tetracosanoic acid[d]	2836	Fatty acid	**1.68±0.49**	**0.61±0.18**	0.51±0.08	0.33±0.07
n-nonacosane	2900	*n*-alkane	2.76±0.50	3.03±0.42	0.76±0.23	1.22±0.21
Hexacosanoic acid[d]	3034	Fatty acid	7.82±2.22	3.48±0.84	1.48±0.35	1.05±0.24
n-hentriacontane	3100	*n*-alkane	8.70±1.92	8.81±1.53	1.81±0.54	2.16±0.45
Cholesterol[d]	3120	Phytosteroid	5.03±1.20	5.22±0.59	3.56±0.49	2.83±0.69
1-octacosanol[d]	3140	1-alcohol	0.28±0.09	0.34±0.09	1.32±0.23	0.96±0.26
Unidentified[d]	3179	–	0.44±0.12	0.40±0.08	0.32±0.16	0.15±0.10
n-dotriacontane	3200	*n*-alkane	0.39±0.10	0.42±0.09	0.45±0.26	0.15±0.10
Campesterol[d]	3276	Phytosteroid	0.20±0.06	0.28±0.08	1.38±0.28	2.09±0.49
Unidentified	3300	–	3.79±0.73	4.40±0.62	1.86±0.43	1.28±0.19
β-sitosterol[d]	3309	Phytosteroid	12.73±1.74	10.68±0.94	5.12±0.57	8.19±2.29
1-triacontanol[d]	3336	1-alcohol	0.44±0.15	0.32±0.08	0.48±0.14	0.49±0.17
Dotriacontanal	3446	Aldehyde	1.47±0.42	2.34±0.44	0.80±0.38	0.39±0.20
Unidentified	3498	–	2.89±0.74	4.05±0.56	2.33±0.60	1.42±0.40
Tetratriacontanal	3648	Aldehyde	1.67±0.44	2.90±0.53	1.15±0.64	0.95±0.27
Tetratriacontanoic acid[d]	3827	Fatty acid	**0.26±0.14**	**0.82±0.24**	–	**0.40±0.15**

[a] Compounds that were detected consistently in at least 60 % of the samples are given

[b] RI = retention index

[c] Untreated control plants (N=10), plants laden with eggs from *Pieris brassicae* (N=10), Ringer-treated control (N=8), and plants treated with secretion of accessory reproductive glands (ARG) of mated *P. brassicae* (N=8)

[d] Determined as corresponding TMS derivatives

Bold numbers indicate significant differences ($P \leq 0.05$; t-test) between untreated control vs. egg-laden leaves or Ringer-treated control vs. ARG-treated leaves

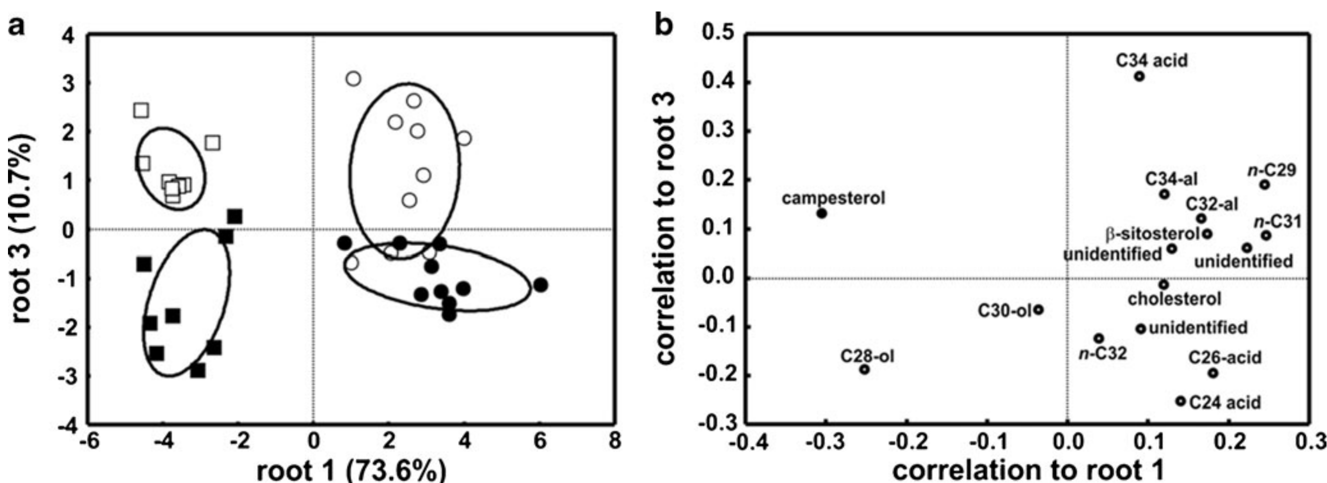

Fig. 2 Discriminant analysis of the quantitative pattern of epicuticular wax profiles of differently treated *Arabidopsis thaliana* (local) leaves based on 16 compounds common to all treatments shown. **a** Scatterplot of canonical root 1 vs. 3; profiles of control leaves (*black circle*), leaves treated with Ringer solution (*black square*), egg-laden leaves (*white circle*), and plants treated with secretion of accessory reproductive glands (ARG) of mated *Pieris brassicae* females (*white square*). Number of analyzed plants: untreated control and egg-laden (N=10 for each treatment); Ringer-treated control and ARG-treated plants (N=8 for each treatment). **b** Corresponding loading plot; C24 acid (tetracosanoic acid); *n*-C29 (*n*-nonacosane); C26 acid (hexacosanoic acid); *n*-C31 (*n*-hentriacontane); C28-ol (1-octacosanol); *n*-C32 (*n*-dotriacontane); C30-ol (1-triacontanol); C32-al (dotriacontanal); C34-al (tetratriacontanal); C34 acid (tetratriacontanoic acid)

the observed separation of indirect defense-eliciting activity (egg-laden and ARG-treated leaves) vs. the treatments without eliciting activity (untreated and Ringer-treated control leaves).

When comparing quantities of single compounds between treatment classes, significant differences were detected for the same compounds that were identified by the discriminant analyses. The quantities of the fatty acids tetracosanoic acid (C24) differed between egg-laden and untreated control leaves (*t*-test: $t=-2.19$, $df=18$, $P=0.041$); this fatty acid was present in reduced quantities in egg-laden leaves. Quantities of the fatty acid tetratriacontanoic acid (C34) were higher in both egg-laden and ARG-treated epicuticular wax extracts when compared to untreated control leaves and Ringer-treated control leaves, respectively (*t*-test: egg vs. untreated control: $t=-2.34$, $df=18$, $P=0.030$; ARG vs. Ringer control: $t=-2.61$, $df=14$, $P=0.020$).

Discussion

Our study showed that egg deposition by *Pieris brassicae* on leaves of *Arabidopsis thaliana* induces indirect defenses by changes at the leaf surface that significantly arrest the egg parasitoid *Trichogramma brassicae*. Treatment of leaves with ARG secretion from mated butterfly females or with the egg-associated elicitor BC had the same effects as natural egg depositions. The parasitoid's response to these artificially treated leaves shows (a) that arrest of the parasitoid on the naturally egg-laden leaves was not due to kairomonal cues left by the butterfly on the leaves (scales, footprints), but to plant cues induced by egg deposition, and (b) that the elicitor of the egg-induced effects on parasitoid behavior is BC, present in the ARG secretion of mated *P. brassicae* females and released with the eggs in order to glue them to the leaf surface.

These results of our bioassays with egg parasitoids are in accordance with previous behavioral studies that investigated how *P. brassicae* egg deposition on leaves of *Brassica oleracea* var. *gemmifera* affects foraging behavior of *T. brassicae* (Fatouros et al., 2005, 2008); these studies also showed that egg parasitoids were arrested on leaves that carried eggs or had been treated with ARG secretion and BC. Both in the present study using *A. thaliana* and in the studies with *B. oleracea*, the egg-induced effects on parasitoid behavior were detectable 72 hr after egg deposition. Fatouros et al. (2005) suggest that the induction of a plant's response about 3 d (72 hr) after egg deposition is timely since parasitization success of *T. brassicae* is highest in 72 hr-old *P. brassicae* eggs, significantly lower in younger ones, and close to zero in older ones. Since insect host eggs (or the host embryos inside the eggs) may defend against parasitism (Abdel-latief and Hilker, 2008), a parasitoid needs to attack host eggs at the time when the host is most vulnerable.

The results of our present study add a tritrophic ecological perspective to previous studies that investigated the effects of *P. brassicae* eggs on expression of genes involved in primary and secondary metabolism of *A. thaliana* leaves. In a whole-genome analysis, Little et al. (2007) found significant changes in transcript levels of numerous genes, thus clearly indicating strong effects of butterfly eggs on *A. thaliana*. Here, we demonstrated that for *A. thaliana*, egg-induced plant responses mediate indirect defense by arresting egg parasitoids on egg-induced leaf surfaces.

In addition to our behavioral results, our chemical analyses revealed that insect egg deposition induces a significant change in the quantitative pattern of leaf epicuticular wax compounds when compared to untreated controls, although qualitative changes were not detected. The quantitative change is not due to altered total amounts of epicuticular waxes, but due to quantitative changes in single compounds, causing a shift in ratios of the components. Total amounts of wax compounds extracted from Ringer-treated leaves (with or without ARG secretion) were much lower than those from egg-laden leaves and untreated controls. Our data suggest that application of Ringer moistened the surface, which might have impaired the extraction of waxes with DCM and thus reduced extractability of waxes. Nevertheless, leaf surfaces that significantly arrested the parasitoid (leaves laden with eggs and leaves treated with ARG-Ringer-solution) showed similar chemical differences compared to their respective controls; both types of these arresting surfaces are characterized by significantly enhanced quantities of the fatty acid tetratriacontanoic acid (C34). In addition, the leaf surface of egg-laden leaves showed significantly reduced quantities of the fatty acid tetracosanoic acid (C24) when compared to untreated controls.

The enhanced quantities of tetratriacontanoic acid and the reduced amounts of tetracosanoic acid in samples from egg-laden or ARG-treated leaves might be due to changes in transport of these compounds from the epidermal cells through the cell wall to the outer cuticular leaf wax layer. This suggestion is based on the finding that insect egg deposition alters transcription of genes involved in cell wall metabolism (Little et al., 2007; Fatouros et al., 2008). Hence, the permeability of the wall matrix of epidermal cells of egg-laden leaves might differ from that of egg-free leaves. Another explanation for the differences in quantities of tetratriacontanoic acid and tetracosanoic acid in egg-laden or ARG-treated leaves when compared to control leaves might be that these samples differ in expression of some enzymes required for fatty acid biosynthesis. Indeed, Little et al. (2007) found that expression of some genes involved in cutin and wax biosynthesis of *A. thaliana* leaves were repressed 2 to 3 d after *P. brassicae* egg deposition (an aldehyde decarbonylase CER1, an acyl transferase-like

protein CER2, a fatty acid elongase 3 ketoacyl-CoA synthase KCS1, a long-chain fatty acid-CoA ligase LACS2, and a very-long-chain fatty acid condensing enzyme β-ketoacyl-CoA synthase FDH). Knowledge of biosynthesis of plant cuticular wax and of wax transport through the cell wall has been reviewed by, e.g., Kunst and Samuels (2003) and Samuels et al. (2008), but the question how insect egg deposition affects these processes has not yet been addressed.

The qualitative pattern of epicuticular wax compounds identified here in *A. thaliana* leaves (wild type Col-0) matches in part the patterns found in previous work; in previous studies, compounds with chain lengths of C16 to C33 were detected in epicuticular leaf wax extracts of untreated *A. thaliana* (e.g., wild type Wassilewskija WS, Landsberg erecta Ler) (Jenks et al., 1995, 2002; Wen and Jetter, 2009). In our study, compounds with C24 to C34 were detected (C24: fatty acid; C34: fatty acid and aldehyde). The quantitative composition of the leaf wax extracts of *A. thaliana* Col-0 detected in our analyses differed from compositions found in other studies. Our extracts were dominated by triterpenoid derivatives (i.e., phytosteroids), followed by *n*-alkanes and fatty acids, whereas in *A. thaliana* WS and Ler leaf wax extracts, Jenks et al. (1995) found *n*-alkanes as the most dominant compounds, followed by alcohols and triterpenoids.

The differences between the results of our chemical analyses and those of Jenks et al. (1995, 2002) and Wen and Jetter (2009) may be due to several factors. It has been shown for several plant species that data on the composition of leaf wax patterns depend on the plant variety, plant age (Edwards, 1982; Avato et al., 1990; Jenks et al., 1996b), abiotic parameters (Riederer and Schneider, 1990; Jetter et al., 2006), and extraction methods. The latter factor - the extraction method - probably contributed most to the differences between our analysis data and those published previously by others (Jenks et al., 1995, 2002). In preliminary analyses, we validated our GC-MS program and sensitivity by comparing the chemical pattern of an *A. thaliana* leaf wax extract obtained by briefly dipping egg-free leaves in dichloromethane for 30 sec with the wax pattern obtained by Jenks et al. (1995) who used a similar extraction and GC-MS method. When using this dipping method, we detected almost all the compounds described by Jenks et al. (1995; 2002) and some additional ones (see Supplementary Table 1).

However, in our study, we used gum arabic for extraction of leaf surface compounds for the following reasons: (a) this method allowed us to limit the extracted waxes to a distinct, quantifiable area of the lower leaf surface (where the parasitoid would come into contact with locally egg-induced leaf tissue); such restriction to a distinct area of the lower leaf surface is difficult when rinsing the leaf with a solvent or dipping leaves with their upper and lower surface in solvent (Riederer and Schneider, 1990; Jenks et al., 1995, 1996a; Jetter et al., 2000, 2006); (b) gum arabic removes only the outermost surface of a leaf that is in contact with the parasitoid. We used only those samples where removal of gum arabic succeeded without disrupting epidermal cells. That allowed us to avoid extraction of intracellular compounds, which might dissolve when applying pure solvent onto the leaf surface (e.g., Jetter and Schäffer, 2001; Reifenrath et al., 2005).

The ecological relevance of plant cuticular waxes for plant–insect interactions has been addressed in numerous studies. Leaf surface waxes can affect feeding and oviposition behavior of herbivorous insects and foraging behavior of predatory and parasitic insects searching for prey or hosts on a plant (e.g., Espelie et al., 1991; Eigenbrode and Espelie, 1995; Bernays and Chapman, 1997; Eigenbrode, 2004; Müller, 2006, 2008). For example, parasitoids of aphids spent more time actively searching for hosts on plant varieties with lower amounts of wax and parasitized more aphids on these plants (Chang et al., 2004). Some parasitoids use lipidic footprints left by their herbivorous hosts on the plant surface as kairomones (Colazza et al., 2007; 2009; Rostás and Woelfling, 2009; Lo Giudice et al., 2010); the chemical composition of the plant's cuticle may affect foraging success of parasitoids using this type of kairomones (Rostás et al., 2008). Egg parasitoids can even use volatile host sex pheromones that are adsorbed by plant surface waxes and function as kairomones (Noldus et al., 1991). We could not find any hints that egg-laden *A. thaliana* leaves release volatile host or volatile plant cues that attract *T. brassicae* (unpublished data). The impact of contact cues of the plant surface waxes *per se* on host foraging behavior of *T. brassicae* could be tested in future studies by using mutant plants with reduced waxes or by full removal of leaf waxes, as has recently been described in an elegant behavioral study of egg parasitoids by Lo Giudice et al. (2010).

So far, little knowledge is available on the relevance of specific plant cuticular wax compounds or plant cuticular compound classes on parasitoid foraging behavior (but see e.g., Dutton et al., 2002). A summary of host insect kairomones and especially volatile plant synomones provided by Rutledge (1996) shows that parasitic wasps are able to respond to a wide range of chemicals. For example, parasitoids of fruit flies are attracted to short-chain volatile organic compounds such as acetaldehyde released from rotting fruits (Greany et al., 1977). Examples of long-chain contact cues include. 13,17-dimethylnonatriacontane ($C_{41}H_{84}$), a kairomone isolated from scales of the pyralid moth *Ostrinia nubilalis* that is used by the egg parasitoid *Trichogramma nubilale* (Shu et al., 1990). In addition to the ability to respond to a wide range of host- and plant-derived

infochemicals, parasitoids adjust their behavior to fine-tuned ratios of compounds associated with a resource (e.g., Beyaert et al., 2010).

The present study shows that egg deposition by *P. brassicae* on *A. thaliana* plants induces indirect defense to arrest egg parasitoids and that the egg-induced leaf area changes its quantitative pattern of epicuticular waxes. Whether the arrest of the egg parasitoid *T. brassicae* in the vicinity of host eggs on *A. thaliana* leaves is mediated by a crucial ratio of tetratriacontanoic acid (C34) and tetracosanoic acid (C24) or other compounds remains to be analyzed in future behavioral studies with defined quantitative mixtures of tetratriacontanoic acid and other leaf surface compounds of *A. thaliana*. To our knowledge, this is the first study providing detailed information about the chemical changes of a leaf surface induced by insect egg deposition.

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Attenuation of the Jasmonate Burst, Plant Defensive Traits, and Resistance to Specialist Monarch Caterpillars on Shaded Common Milkweed (*Asclepias syriaca*)

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Abstract Plant responses to herbivory and light competition are often in opposing directions, posing a potential conflict for plants experiencing both stresses. For sun-adapted species, growing in shade typically makes plants more constitutively susceptible to herbivores via reduced structural and chemical resistance traits. Nonetheless, the impact of light environment on induced resistance has been less well-studied, especially in field experiments that link physiological mechanisms to ecological outcomes. Accordingly, we studied induced resistance of common milkweed (*Asclepias syriaca,* a sun-adapted plant), and linked hormonal responses, resistance traits, and performance of specialist monarch caterpillars (*Danaus plexippus*) in varying light environments. In natural populations, plants growing under forest-edge shade showed reduced levels of resistance traits (lower leaf toughness, cardenolides, and trichomes) and enhanced light-capture traits (higher specific leaf area, larger leaves, and lower carbon-to-nitrogen ratio) compared to paired plants in full sun. In a field experiment repeated over two years, only milkweeds growing in full sun exhibited induced resistance to monarchs, whereas plants growing in shade were constitutively more susceptible and did not induce resistance. In a more controlled field experiment, plant hormones were higher in the sun (jasmonic acid, salicylic acid, abscisic acid, indole acidic acid) and were induced by herbivory (jasmonic acid and abscisic acid). In particular, the jasmonate burst following herbivory was halved in plants raised in shaded habitats, and this correspondingly reduced latex induction (but not cardenolide induction). Thus, we provide a mechanistic basis for the attenuation of induced plant resistance in low resource environments. Additionally, there appears to be specificity in these interactions, with light-mediated impacts on jasmonate-induction being stronger for latex exudation than cardenolides.

Keywords Cardenolide · Herbivory · Latex · Monarch butterfly · Plant defense · Plant-insect interactions · Shade-avoidance response · Signal cross-talk · Specialist herbivore

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Introduction

Because plant traits that maximize light capture and reduce herbivory are almost universally phenotypically plastic (i.e., induced by low light and herbivory, respectively), both have been the subject of intensive study as forms of adaptive plasticity (Dudley and Schmitt, 1996; Agrawal, 1998; Callaway et al., 2003; Auld et al., 2010; Salgado-Luarte and Gianoli, 2011). A relatively consistent aspect of these responses in sun-adapted species is that plasticity to shade causes greater susceptibility to herbivores, and conversely, induced responses to herbivory often make plants less competitive (Dudt and Shure, 1994; Jansen and Stamp, 1997; Kurashige and Agrawal, 2005; Van Dam and Baldwin, 2001). In their simplest form, such tradeoffs are driven by the dual impact of particular traits. For example, shaded leaves typically have reduced trichome densities and higher nitrogen content (Morgan and Smith, 1981; Rozendaal et al., 2006), which are responses thought to reduce self-shading and increase allocation to RuBisCO, respectively. These same trait changes are predicted to make plants more palatable for herbivores (Agrawal and Fishbein, 2006). Similarly, when plants exhibit induced resistance to herbivores,

allocation to resistance traits is often associated with plants having a reduced ability to capture resources (Van Dam and Baldwin, 2001; Salgado-Luarte and Gianoli, 2011). In such cases, resource limitation often has been invoked to explain the tradeoff.

Although most early models of plant responses to light availability and herbivory were based on the concept of resource allocation (Herms and Mattson, 1992; Dudt and Shure, 1994), more recent mechanistic approaches have sought to address the biochemical basis of interactions between these plant responses (Table 1, Cipollini, 2004; Roberts and Paul, 2006). Advances have come, in part, because of the recognition that plastic signaling pathways in plants may interact in multiple ways (i.e., at the level of precursors, receptors, hormones, etc.). In particular, it has been suggested that the inducibility of either response (to light or herbivores) may be impaired by the activation of the other. For example, a recent study found that the light environment impacted both the biosynthesis of, and plant responses to, jasmonates, which are critical hormones involved in induced responses to herbivory (Radhika et al., 2010). Nonetheless, much of the initial mechanistic work on the interaction between light availability and induced responses to herbivory was conducted in highly controlled environments. Thus, an important next step is to conduct field studies, which typically allow for more realistic light levels, natural patterns of insect attack, and involvement of other natural stressors. Although several field studies have emerged recently, (Boege, 2010; Salgado-Luarte and Gianoli, 2011), they have yet to take a mechanistic approach linking plant hormonal regulation with ecological outcomes.

Table 1 Progress points over the last decade in understanding how a plant's light environment impacts induced resistance to herbivores

Study	Key Finding
Cipollini, 2004	Summarized the conceptual motivation and biochemical basis for why plant responses to shade and herbivory may interact
Kurashige and Agrawal, 2005	Directly found some evidence for reciprocal interactions between induced plant resistance to herbivory and the shade-avoidance response in *Chenopodium album*
Izaguirre et al., 2006	Showed that exposure of a wild tobacco (*Nicotiana longiflora*) to reflected far-red light impaired resistance to herbivores and some induced phenolic compounds. This effect was likely mediated by phytochrome B.
Mooney et al., 2009	A shade-adapted woody shrub (*Lindera benzoin*) showed stronger induction of peroxidase and resistance to caterpillars in shaded compared to sun leaves.
Moreno et al., 2009	Using *Arabidopsis* mutants, convincingly showed that attenuated induced responses in the shade are mediated by phytochrome and reduced sensitivity to jasmonates.
Radhika et al., 2010	Demonstrated that shade conditions reduce biosynthesis and responses to jasmonates, both of which modulate herbivore-induced extrafloral nectar in lima bean (*Phaseolus lunatus*).

Accordingly, our goal was to assess natural patterns of plant responses to light competition and herbivory, and then to experimentally assess the mechanistic basis of any potential conflict between these responses. We have been studying defense and competition in native common milkweed (*Asclepias syriaca* L), which typically occurs in full sun (open fields), although it also is found on forest edges, where it has a typical shade phenotype (Agrawal and Van Zandt, 2003; Agrawal, 2004; Mooney et al., 2008; Bingham and Agrawal, 2010). Shaded milkweeds appear to have larger, darker green, and floppy leaves compared to plants growing in the sun. Here, we addressed the impact of shade on the well-characterized induced responses to herbivory in milkweed. Specifically, we tested the following hypotheses: 1) Plants naturally growing in the shade will have lower levels of resistance traits and enhanced light capture traits compared to paired plants growing in full sun; 2) induced resistance to specialist monarch caterpillars will be impaired in naturally shaded plants; and 3) foliar hormones and resistance traits (i.e., cardenolides, latex) will show attenuated induction following herbivory in shaded compared to non-shaded plants.

Methods and Materials

Study System Asclepias syriaca is a native perennial plant that occurs throughout eastern North America, typically in open habitats but frequently extending to forest edges. When growing in competition, *A. syriaca* exhibits a shade-avoidance response, including stem elongation (Agrawal and Van Zandt, 2003). It reproduces both clonally and sexually, and is largely self-incompatible (Kephart, 1981). *Asclepias syriaca* employs a variety of heritable defense traits, including the production of toxic cardenolides, gummy latex, and non-glandular leaf trichomes (Agrawal, 2005). Cardenolide concentrations and latex exudation are both inducible following herbivory (Van Zandt and

Agrawal, 2004; Mooney et al., 2008; Bingham and Agrawal, 2010), the jasmonate pathway is involved in this induction (Rasmann et al., 2009; Agrawal, 2011), and specialist herbivores including monarch butterfly caterpillars (*Danaus plexippus*) are negatively impacted by induction (Van Zandt and Agrawal, 2004).

Natural Population Surveys To characterize the phenotypic differences between *A. syriaca* naturally growing in the sun and the shade that might be relevant to plant-herbivore interactions, we conducted a survey of natural populations. In July 2009, across four field sites we identified 24 pairs of naturally occurring mature *A. syriaca* plants in Tompkins County, NY (USA), with one individual occurring under natural shade at the forest edge and the other occurring in full sun in an open field (plants were typically≤10 m apart). Forests were mature, eastern deciduous forests (primary or secondary growth) next to open old-fields. We employed a paired design to minimize potential microclimatic, soil, and genetic differences. Although we have no information indicating that stems in the same pair were part of the same genet, this is certainly possible. Nonetheless, because microclimatic, soil, and genetic differences were not of direct interest in this study, all were considered part of the blocking factor (pair). Care was taken to include pairs where the shaded plant was facing each of the four compass directions. On average, the intensity of peak photosynthetically active radiation (PAR, 400-700 nm) was reduced by 91 % in the shade (mean±SE μmol/m^2/s, sun: 1784, shade: 168, $F_{1,23}$= 673, $P<0.001$).

Using standardized methods detailed elsewhere (Agrawal, 2005), we measured leaf number, leaf size, leaf toughness, specific leaf area (SLA, area/dry mass), water content, trichome density, foliar carbon-to-nitrogen ratio, plant height, and latex exudation. Cardenolide concentrations were assessed by HPLC, following Bingham and Agrawal (2010). Briefly, 50 mg dried leaf tissue from each plant were ground to a fine powder and extracted with 1.8 ml methanol (MeOH), spiked them with 20 μg digitoxin as an internal standard, and sonicated for 20 min at 55°C in a water bath. After centrifugation, the supernatant was collected, dried, resuspended in 1 ml MeOH, and filtered through a 0.45 μm syringe driven filter unit. Fifteen μl of extract were then injected into an Agilent 1100 series HPLC, and compounds were separated on a Gemini C18 reversed phase column (3 μm, 150 x 4.6 mm, Phenomenex, Torrance, CA, USA). Cardenolides were eluted on a constant flow of 0.7 ml/min with an acetronile-0.25 % phosphoric acid in water gradient as follows: 0-5 min 20 % acetonitrile; 20 min 70 % acetonitrile; 20-25 min 70 % acetonitrile; 30 min 95 % acetonitrile; 30-35 min 95 % acetonitrile. UV absorbance spectra were recorded from 200 to 400 nm by diode array detector. Peaks with absorption maxima between 217 and 222 nm were recorded as cardenolides and quantified at 218 nm. Concentrations were calculated and standardized by peak areas of the known digitoxin concentration.

All measures were taken from the youngest fully expanded leaves (avoiding severely damaged leaves); total N=48. We measured internode length as the distance between the youngest fully expanded pair of leaves and the node below. Finally, we also recorded the number of aphids (*Myzocallis asclepiadis*) found on each plant, and estimated leaf herbivory as the fraction of the total number of leaves with clearly identifiable chewing damage (typically imposed by *D. plexippus* or *Tetraopes tetraophthalmus*). Analyses were conducted with two-way analysis of variance (with pair and light environment as the main effects). We first conducted a MANOVA, to assess effects across our 13 response variables, followed by univariate analyses.

Induced Resistance in Natural Populations In July of 2010 and 2011, we visited the same natural populations as above and selected clusters of four mature plants (two in the sun and two in the shade) to test for induced resistance. Again, these clusters were selected (plants≤10 m apart) as a blocking factor to minimize microclimatic, soil, and genetic differences. Plants with minimal natural herbivory were selected, and then the top portion of plants (8 leaf pairs in 2010, 4 pairs in 2011) was enclosed in a spun polyester sleeve. Our bagging treatment did not impact leaf temperatures ($F_{1,176}$=0.003, P=0.955), although shading reduced temperature by 18 % (mean sun temperature °C: 24.8 +/- 0.3; in the shade: 20.4 +/- 0.3, $F_{1,79}$=135.0, $P<0.001$).

Plants in each light environment were assigned randomly to be either damaged (<5 % herbivory imposed by early instar *D. plexippus* larvae) or left as controls. Damage was controlled so as not to be different between the two light environments. After 3-5 d of feeding, the damaging caterpillars were removed, and pre-weighed bioassay caterpillars were introduced to all plants to assess their growth. In 2010, the bioassay consisted of two 1st instar caterpillars (20 plant clusters), whereas in 2011 we introduced a single 2nd instar caterpillar (25 plant clusters). We changed the procedure in 2011 to reduce mortality of the bioassay caterpillars (mortality was 18 % and 7 % in 2010 and 2011, respectively). We employed ANOVA to assess the impacts of shade, induction, shade-by-induction interaction, and block on the relative growth rate (final mass minus initial mass divided by initial mass) of the surviving caterpillars (total N=178). Block was each group of four plants, with one replicate in each treatment combination; in this way, we combined spatial variation (microclimatic, soil, and genetic differences) and variation across years in one blocking term. In this analysis, no interaction term between block and the other main effects is possible because a single replicate of each of the four treatment combinations is in each block. Mortality

was essentially random in 2010 and minimal in 2011. For the 2010 data, if two caterpillars were collected, their mass was averaged to produce a single datum. We took two approaches to assess differential induced resistance to caterpillars in the sun and shade: we first examined the statistical interaction between the shade and damage treatments, and second, we independently conducted analyses of the impact of damage treatment on monarch performance in the sun and shade environment.

Common Garden Study In a more controlled set of experiments, we exposed milkweed plants to natural sun and shade environments, moved them to a common "neutral" environment (see below), and then assessed traits associated with shade avoidance and induced resistance. In particular, our goal was to link plant hormonal responses to herbivory and the induction of defense traits in plants that had been grown in different light environment histories, but were assayed in a common environment. In 2009, we grew 10 full sibling families of *A. syriaca* (from a local natural population, all from a full sun habitat), randomized in a growth chamber in 500 ml pots filled with potting mix (mean of 15 plants per family, total $N=149$). Genetic families were used simply to control for variation. After 2 mo of growth, in October, the plants were moved out of doors, hardened in a lath house, clipped, and mulched to overwinter. In May 2010, we uncovered the plants and transplanted them into 4 l pots containing a 1:1:1 mixture of topsoil, compost, and sand. These second year plants were not fully mature (i.e., did not flower in 2010). Half of the plants from each family were randomized in a block under the canopy of a forest edge and the rest were placed 5 m away in a randomized block in the full sun. Both plots were fenced, and plants were watered as needed. This site was one of our original paired sun-shade sites and exhibited a 94 % reduction in PAR under the canopy. Our main goal in using this design was to have plants emerging from perennial root stocks in the contrasting light environments from the beginning of the growing season.

Three weeks after emergence, we non-destructively measured the number of stems in each pot (because milkweeds are clonal, some pots had several stems), the height of the tallest stem, and internode length (2 nodes below the apex) on all plants. All plants then were moved and fully randomized in a common neutral enclosure. Pots were spaced at least 5 cm apart and had no leaf overlap or light competition. This enclosure made of Lumite insect screen fabric (Baldwin, GA, USA) reduced ambient light by≈50 %, and importantly served as a neutral filter (i.e., did not impose reflected far red light, as is the case under the canopy of leaves). We used this enclosure to provide intermediate (neutral) light levels between the two extremes experienced during the early part of the growing season. Tall, leaning plants were staked as needed. On the same day, we introduced a single freshly hatched monarch caterpillar to the apex of half of the plants, both to initiate induced responses to herbivory and to conduct a bioassay of performance on plants with different light environment histories. Critically, the bioassay was conducted in a common environment (neutral enclosure) where the plants had been fully randomized. After 4 d of feeding (<5 % damage), we measured latex exudation and destructively harvested the apical tissue (usually 2 pairs of leaves, 600-800 mg fresh mass) for measures of hormones (jasmonic acid (JA), salicylic acid, abscisic acid, indole acidic acid) and cardenolides ($N=149$). Nearly all of the monarch herbivory was on apical leaves, and thus all samples for chemistry contained local damage.

In addition to total HPLC-determined cardenolides (as above), we analyzed data individually for the five major cardenolide peaks (see Table 2b). We include one early, highly polar peak (eluting at 3.45 min) that was likely a cardenolide (given its absorbance spectra), but co-eluted or attached to a phenolic compound (again, based on the absorbance spectra). Hormones were quantified by using an established liquid chromatography – mass spectrometry procedure, modified from Thaler et al. (2010). Briefly, frozen samples were transferred into 2-ml screw cap tubes containing 900 mg zirconia/silica beads (BioSpec, Bartelsville, OK, USA) and 1 ml extraction buffer. d_4-SA, d_5-JA, d_6-ABA, d_5-IAA (CDN isotopes, Point-Claire, Canada) were added as internal standards, and samples were homogenized in a FastPrep homogenizer (MP Biomedicals, Solon, OH, USA) at 6 m/s for 45 s. Samples were dissolved in 200 μl methanol after extraction with dichloromethane and solvent evaporation and 15 μl were analyzed on a triple-quadrupole LC-MS/MS system (Quantum Access; Thermo Scientific, Waltham, MA, USA). Analytes were separated on a C18 reversed-phase HPLC column (Gemini-NX, 3 μ, 150 X 2.00 mm; Phenomenex, Torrance, CA, USA) using a gradient of 0.1 % formic acid in water (solvent A) and 0.1 % formic acid in acetonitrile (solvent B) at a flow rate of 300 μl/min. The initial condition of 10 % B was kept for 2 min and increased to 100 % solvent B at 20 min. Phytohormones were analyzed by negative electrospray ionization (spray voltage: 3.5 kV; sheat gas: 15; auxiliary gas: 15; capillary temperature: 350°C), collision-induced dissociation (argon CID gas pressure 1.3 mTorr [1.3 μm Hg], CID energy 16 V) and selected reaction monitoring (SRM) of compound-specific parent/product ion transitions: SA 137→93; d_4-SA 141→97; JA 209→59; d_5-JA 214→62; ABA 263→153; d_6-ABA 269→159; IAA 174→130; d_5-IAA 179→135.

Statistical analyses were conducted with analysis of variance (ANOVA) using light environment and monarch induction as main effects, their interaction term, and plant genetic family included as a blocking factor. Residuals were

Table 2 Analyses of variance (*F*-values) for effects of light environment, damage by monarch caterpillars, and full sibling genetic family of *Asclepias syriaca* on hormone and defense expression

A.	MANOVA	JA	SA	ABA	IAA		Latex
Light (L)	10.038***	4.575*	7.093**	32.681***	10.771**		94.132***
Induction (I)	24.856***	98.650***	0.251	6.078*	0.504		1.665
LxI	1.674	4.915*	0.295	0.175	0.317		2.878†
Genetic family	1.511*	0.651	1.845†	1.647†	1.646		2.465*
B.	MANOVA	Cardenolide 3.45	Cardenolide 5.1	Cardenolide 13.8	Cardenolide 14.6	Cardenolide 18.4	Total cardenolide
Light (L)	4.574***	8.885**	6.060*	3.619†	7.366**	0.346	10.650**
Induction (I)	2.181†	7.095**	0.002	0.004	2.597	<0.001	3.901*
LxI	0.341	0.216	0.046	0.025	1.027	0.466	0.013
Genetic family	1.802**	1.036	1.687†	2.154*	1.991*	2.753**	1.146

a) Analysis of four plant hormones (*ja* jasmonic acid, *sa* salicylic acid, *aba* abscisic acid, and *iaa* indole acidic acid). Latex exudation was tested separately. b) A similar analysis on the five cardenolide peaks that were present in most samples (represented by their hplc retention time, the first two peaks made up 83 % of the total), with a MANOVA followed by univariate analyses. Total cardenolide concentration was tested separately. All measures were taken on a fresh mass basis. ***$P<0.001$, **$P<0.01$, *$P<0.05$, AND †$P<0.1$

distributed normally unless otherwise noted. In addition, to address the role of jasmonic acid in driving the patterns of induction in other traits, we conducted an additional set of analyses that examined the impacts of three factors (light environment, monarch damage, and JA concentrations) on the other hormone concentrations as well as latex and cardenolide values using analysis of covariance (ANCOVA). In other words, in this analysis, JA shifts from being a response variable to being a predictor as a means to address its role in the induction process. Indeed, the impact of JA on hormones and defensive end-products is well-established, and thus considered an *a priori* expectation. Although we started with the fully factorial model, all interaction terms with JA (which were never significant) were removed from this analysis to simplify and maximize power. Genetic family was included as a blocking factor in all models. We interpret a significant effect of JA in this set of analyses as an indication of a physiological correlation between JA and the traits, irrespective of treatment effects on JA or the response variable.

Results

Natural Population Surveys In our assessment of plant traits and leaf damage in natural populations, most of the 13 variables measured showed differences between the sun and shade, as well as some site differences between the 24 pairs (MANOVA: light environment: exact $F_{13,11}=24.301$, $P<0.001$; pair: Wilks' *lambda*<0.001, $F_{299,164}=1.478$, $P=0.003$; Fig. 1). Shaded plants produced less tough, larger leaves that were less dense, had fewer trichomes, a lower carbon-to-nitrogen ratio, and less concentrated cardenolides than their paired plants from the sun (statistics provided in Fig. 1). Plants from the shade also had about half the natural leaf herbivory of sun plants (although there was no difference in latex exudation or aphid abundance). Finally, although shaded plants had internode lengths almost 10 % longer than sun plants, this effect was not significant (Fig. 1H). We repeated the survey of insect damage on 20 of the pairs in 2010 and found no difference in leaf damage between plants in the sun and shade ($F_{1,19}=0.068$, $P=0.797$).

Induced Resistance in Natural Populations Across the two years of the experiment to examine induced resistance to monarch caterpillars in the sun and shade, we found that caterpillars grew 27 % faster in the shade ($F_{1,135}=10.821$, $P<0.001$) (Fig. 2). We did not detect an overall effect of previous monarch damage (induction, $F_{1,135}=0.611$, $P=0.441$), and the interaction between light environment and induction was suggestive, but not significant ($F_{1,135}=2.256$, $P=0.139$). Nonetheless, inspection of the means indicated a 16 % impact of induction (decrease in relative caterpillar growth) in the sun, with a reversal in the direction (3 % increase in caterpillar mass) caused by induction on shaded plants. To further contrast induction effects in the sun vs. shade, we conducted separate analyses for the two light environments. Indeed, in the sun, previous monarch damage significantly reduced monarch mass ($F_{1,49}=7.870$, $P=0.007$), but there was no effect in the shade ($F_{1,49}=0.179$, $P=0.674$) (Fig. 2).

Common Garden Study Our experimentally shaded plants differed substantially from sun plants, with a strong induction of the shade-avoidance response (Fig. 3). Shaded plants were taller, with longer internode lengths, and produced fewer stems compared to sun plants (Figs. 3, 4). Consistent with results from the natural populations, monarch caterpillars grew≈10 % faster on shade plants ($F_{1,69}=4.248$, $P=0.043$);

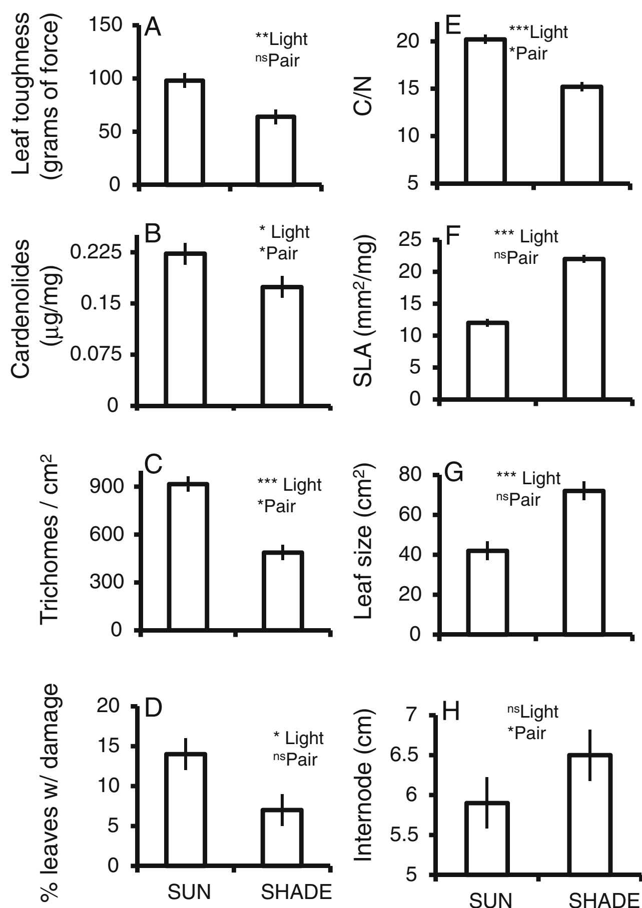

Fig. 1 A summary of phenotypic differences (mean ± SE) between paired *Asclepias syriaca* in full sun and shade (91 % reduction in photosynthetically active radiation at mid-day, N=24 pairs) from natural populations around Ithaca, NY (USA). Traits primarily involved in defense are on the left (panels A-D), while functional and structural traits are on the right (panels E-H). Significance of light environment and plant pair is indicated by * $P<0.05$, ** $P<0.01$, *** $P<0.001$, and ns $P>0.1$. C/N is the ratio of leaf carbon to nitrogen on a dry mass basis, SLA is specific leaf area (area per dry mass) and cardenolides are reported on a fresh mass basis. Data not shown for aphids per plant, plant height, number of leaves, latex exudation, and foliar water content (all factors not significant except pair for latex, and light environment for water content: $F_{1,23}$=15.15, $P<0.001$)

because this bioassay was conducted in a common environment, this result was due clearly to differences in plant quality (Fig. 4D).

Induction of hormones and resistance traits in the neutral environment was impacted by the plants' light environment history. Shaded plants showed a nearly 50 % attenuated jasmonate burst following monarch herbivory (see interaction term in Table 2 , Fig. 5A), and this was concordant with latex induction, which showed a 17 % increase in sun plants, but no effect in shade plants (see marginally significant interaction in Table 2, Fig. 5B). All other hormones (salicylic acid, abscisic acid, and indole acidic acid) were higher in sun compared to shade plants, and only abscisic acid

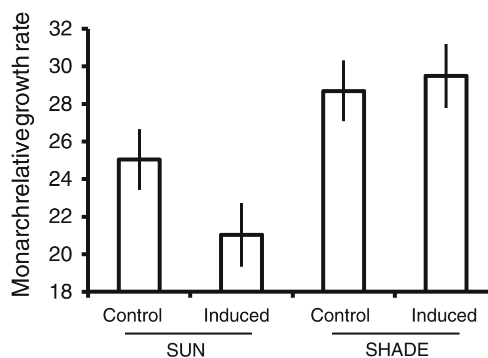

Fig. 2 Effects of experimentally imposed caterpillar damage (induced resistance) on relative growth rate (final minus initial mass divided by initial mass) of subsequently feeding monarch caterpillars (*Danaus plexippus*) on naturally occurring common milkweed (*Asclepias syriaca*) in the sun and shade. Shown are mean ± SE from experiments conducted over two years

showed a main effect of induced response (20 % increase) following herbivory (Table 2). For cardenolides, sun plants had 27 % higher concentrations than shaded plants, which again was consistent with our results from natural populations. Total cardenolide concentrations were inducible by herbivory, and the earliest peak to elute appeared to drive this pattern (a significant 14 % increase following damage, Table 2); light environment did not impact this induction. Nearly all hormones and defenses measured showed variation among the ten genetic families tested (Table 2).

In follow-up analyses, we treated JA as a predictor variable (alongside light environment and monarch damage treatment), to address directly the *a priori* prediction that JA impacts other hormones and the production of defensive end-products. A significant effect of JA in this set of analyses would indicate a linear correlation between JA and the traits, irrespective of treatment effects on JA or the response

Fig. 3 Representative full-sibling common milkweed (*Asclepias syriaca*), germinated and grown under the same conditions in the first year of life. Just before stem emergence in their second growing season, the plant on the left was placed at the forest edge under a tree canopy, whereas the plant on the right (note 3 stems) was placed in full sun, 5 m away

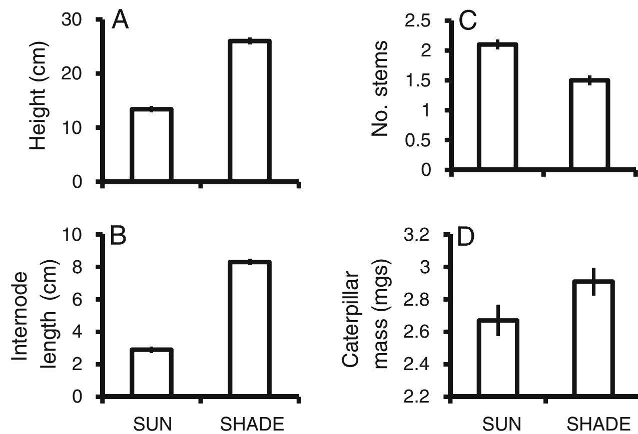

Fig. 4 A summary of phenotypic differences (mean ± SE) of *Asclepias syriaca* grown in full sun or shade from an experimental population: A height, B internode length, C number of stems, D and mass of a monarch caterpillar used as a bioassay. Caterpillar mass was assessed after plants were moved and randomized into a neutral environment (see Methods). All traits were significantly different in the two environments, ANOVAs, all *P*s<0.05

variable. Across treatments, jasmonate levels were positively correlated with latex, salicylic acid, and abscisic acid

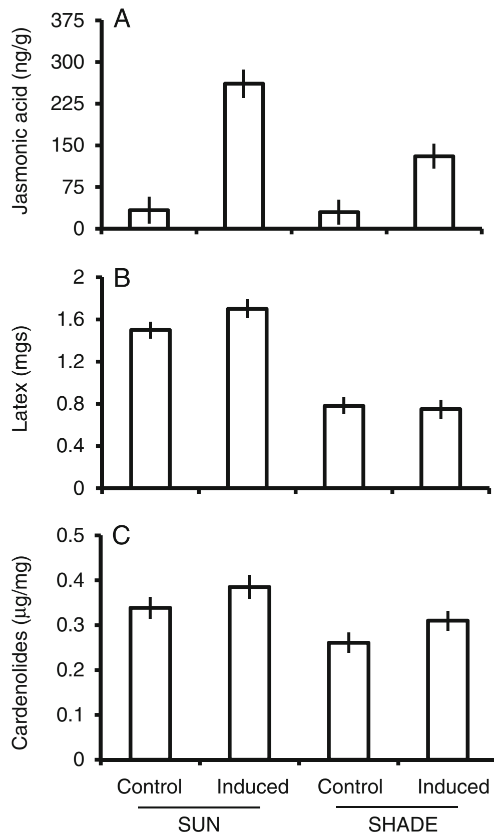

Fig. 5 Effects of previous damage (induced responses) on common milkweed (*Asclepias syriaca*) grown in the sun or shade, but then transported to a neutral environment prior to the experiment (see Methods for details). Shown are means ± SEs for A jasmonic acid, B latex exudation, and C total cardenolides, all taken on a fresh mass basis

(Table 3). Light environment had an additional impact on all response variables (Table 3), potentially indicating a resource effect independent of JA. Despite the jasmonate burst following damage being qualitatively concordant with cardenolide induction (Fig. 5), there was no quantitative relationship between the two. In other words, the cardenolide induction effect was not impacted by the inclusion of JA in the model (Table 3). Although we do not interpret this result to mean that cardenolide induction is independent of JA, there was not a linear relationship between JA and cardenolides.

Discussion

In this study we demonstrated that common milkweed, a plant that typically grows in full sun, has substantially altered resistance traits and hormonally-mediated interactions when growing in shaded habitats. Although the enhanced susceptibility of shaded plants has been reported in a wide array of species (Table 1), the means by which these changes occur have only recently been studied. For *A. syriaca*, naturally shaded plants produced leaves that were less defended by mechanical traits (less tough, fewer trichomes) and leaf chemistry (lower carbon-to-nitrogen ratio, reduced cardenolides) traits than plants from full sun. We further evaluated induced resistance in shaded and full sun habitats. For both *Arabidopsis* and lima bean, laboratory experiments have demonstrated that shading effects on induced resistance likely occur through phytochrome pigments, and can affect both the production of jasmonates as well as endogenous plant responsiveness to these jasmonates (Moreno et al., 2009; Radhika et al., 2010). For milkweeds, not only did monarch caterpillars grow better on shaded plants, but a strong attenuation of the jasmonate burst was concordant with reduced induction of latex and reduced induced resistance to caterpillars. Despite overall reductions of cardenolides in shaded plants, their induction was proportional in plants from both light environments (Fig. 5C). These results have two major implications. As

Table 3 Partial coefficients for the effects of jasmonic acid levels on five plant traits

	Estimate	t	Other factors that remain significant
Latex	0.001	2.261*	Light***, Family**
Cardenolides	<-0.001	-1.76	Light***, Induction*
Salicylic acid	0.063	2.620**	Light***, Family*
Abscisic acid	0.031	3.207**	Light***
Indole acidic acid	<0.001	0.122	Light**

These results are from five separate ANOVA models that each additionally included light, induction treatment, light-by-induction interaction, and genetic family as predictors. ***$P<0.001$, **$P<0.01$, *$P<0.05$

with previous studies, both latex and cardenolides likely contribute to resistance to specialist monarchs, although their relative importance appears to vary (Zalucki et al., 2001; Agrawal, 2005), and negative effects of latex are more consistent. Second, the two traits appear to be somewhat uncoupled in their regulation among genotypes and in their jasmonate-mediated phenotypic responses to the environment (Bingham and Agrawal, 2010).

The linkage of plant hormones, resistance traits, and insect performance may be impacted by the way in which they are measured. Here, we considered two aspects: the environment in which they are measured and whether plant traits are reported on a fresh or dry mass basis. First, because the light environment impacts the temperature, resource availability, and potentially other local interactions, common environment assays are critical for assessing the impacts of shading (Sipura and Tahvanainen, 2000). We achieved this by moving our plants, which were under divergent light conditions, to a common (neutral) environment the day before induction treatments and caterpillar bioassays were conducted. Our results confirmed that the jasmonate burst, latex exudation, and caterpillar performance were impacted by past light conditions, even when intermixed and identically treated for the duration of the experiment. Although we did not see this effect for cardenolides, it is conceivable that cardenolide induction would also be attenuated in shaded plants when plants are maintained in the shade during the induction process.

Because light environments often influence plant water content, or said another way, leaves in the shade often have reduced dry mass (Morgan and Smith, 1981), calculations of the impacts of induction treatments may be sensitive to whether they are calculated on a fresh or dry mass basis (Koricheva, 1999; Agrawal et al., 2012). In our study, milkweed leaves in the shade were 81.5 % water, vs. 79.3 % in the sun, a small but statistically significant effect (Fig. 1 legend). This potentially could be problematic because the change in water content is confounded with light environment, and it is unclear whether plant hormones or resistance traits are functionally impacted by water content. For our study, we calculated cardenolide values on both a fresh- and dry mass basis, yet the results were qualitatively the same (data not shown). Nonetheless, future work, especially in systems where there is a strong impact of environment on plant tissue water content, should consider drawing conclusions based on both types of calculations.

In this study, we focused on sun-loving plants and the impact of shading or growing on forest edges. Ultimately, the impacts of shading on herbivory will be the sum of changes in plant resistance, the probability that herbivores attack plants in the shade, and the per capita damage imposed by herbivores in the different environments. For example, Guerra et al. (2010) reported that *Aristotelia chilensis* (Elaeocarpaceae) saplings received more damage in the shade than the sun. This effect was concordant with laboratory bioassays of tissue quality, and was driven by leaf thickness (not secondary chemistry, water content, or insect abundance in the respective habitats). Similarly, Muth et al. (2008) report greater levels of plant damage in shaded habitats despite equal herbivore abundances in both habitats. Nonetheless, many herbivores are known to avoid entering shaded habitats, which could leave higher quality foliage in the shade unattacked. In particular, we found that despite lower mechanical and chemical resistance traits in shaded plants, levels of herbivore damage in the field were either equal between habitats or reduced in the shade. We speculate that milkweed herbivores are less abundant and behaviorally avoid shaded habitats. Future work on *A. syriaca* would benefit from a focus on herbivore behavior and more precise measures of plant damage.

Does the fact that shaded leaf tissues are often of higher quality represent a tradeoff for plants, or an adaptive strategy (Agrawal et al. 2010)? A few studies suggest that that there may be no ecological tradeoff realized in the field. For example, beetles on willows were primarily found in the sun in the field, although their performance was enhanced on leaves of shaded plants in laboratory experiments (Sipura and Tahvanainen, 2000). Similar results were found for South American *Embothrium coccineum* (Proteaceae), where herbivory levels in the field were highest in the sun, but palatability in the laboratory was highest for shaded leaves (Salgado-Luarte and Gianoli, 2010). Again, our results for milkweed also showed this pattern. Despite bioassays consistently showing greater susceptibility to monarch caterpillars in the shade over the sun, over two years of field observations, damage was never higher in the shade.

Thus, we hypothesize that impaired constitutive and induced resistance in the shade could actually be an adaptive strategy of common milkweed plants. In shaded plants, not only are some plant responses likely to enhance light capture (few trichomes, higher nitrogen, larger thinner leaves), but investment in induced defense is also less necessary given the low probability of attack. In our experiments and those using mutant lines (Moreno et al., 2009), it appears that limiting resource availability is not the sole cause of higher susceptibility in shaded plants.

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Genetic and Environmental Factors Behind Foliar Chemistry of the Mature Mountain Birch

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Abstract Previous studies of mountain birch (*Betula pubescens* spp. *czerepanovii*) repeatedly have found differences between individual trees in herbivory-related traits, but rarely have yielded estimates of the additive genetic variation of these traits or of their relationship to habitat. We used thirty-year-old birch half-sibs in a northern common garden to estimate the effect of genetics and local microhabitat on resistance-related traits. Genetic estimates of foliar chemistry have been studied only rarely with trees as old as these. Moth performance (*Epirrita autumnata*), rust (*Melampsoridium betulinum*) incidence levels, and the general level of natural herbivory damage to individual trees were used as direct measures of birch resistance. Chemical resistance-related traits in plant chemistry included 15 individual phenolics, 16 amino acids, and phenoloxidase activities in the foliage. We also followed birch phenology and growth. Our results show that the genotype of the birch was the most important determinant of phenolic composition and phenoloxidase activity, but that amino acid levels were best explained by the microhabitat of the birch. We also found that the phenology of the birch had a high heritability, although its variation was low. Our results reveal rich genetic variation in birch chemistry.

Keywords Microhabitat · Additive genetic variation · Heritability · Woody plant · Phenotypic correlation · Phenotypic plasticity · Insect outbreaks

Introduction

Long-living, "apparent" trees are especially vulnerable to herbivory (Feeny, 1976), as insect life cycles and potential population growth rates are faster than those of trees. Thus, insects should be able to adapt to the defensive chemistry of the trees. Nevertheless, plant-herbivore interaction models have shown that variance in plant quality has the potential to influence herbivore population dynamics (Shelton, 2004;

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Underwood, 2004), and thus to create an obstacle to herbivore evolution (Karban, 2011).

Forest trees show extensive phenotypic and genetic heterogeneity (reviewed in Linhart and Grant, 1996). This variability is expressed at many levels—not only among populations and individuals, but also among individual branches (Suomela et al., 1995). Phenotypic plasticity, including inducible responses due to herbivory, increases variability and aids trees in resisting herbivory (Adler and Karban, 1994; Pigliucci, 2001). Phenotypes may change temporally with phenology, making trees even more heterogeneous: "moving targets" for selection over evolutionary time scales (Adler and Karban, 1994; Roslin et al., 2006).

Outbreaks of *Epirrita autumnata* (Borkhausen) (Lepidoptera, Geometridae, autumnal moth) in Northern Europe, and the resulting widespread forest defoliation of *Betula pubescens* spp. *czerepanovii* ((Orlova) Hämet-Ahti, mountain birch) have been the subject of intensive study (e.g., Haukioja, 2003, 2005). Variation among birch individuals has been detected in the foliar chemistry of phenolics (Riipi et al., 2004; Haviola et al., 2006) and in activites in foliar oxidases (Yang et al., 2007). Variable chemical composition has been suggested to be a significant factor in the evolutionary relationship between birch and herbivores (Ruusila et al., 2005).

Individual birch trees differ in their suitability as food for their main herbivore, the autumnal moth, *E. autumnata* (Ayres et al., 1987; Haukioja, 2003). A tree's herbivore resistance status is affected by a large number of chemical compounds, all of them closely tied to phenology. Foliar water content is part of plant phenology, and is the best single predictor of larval performance (Ayres and MacLean, 1987; Senn et al., 1992; Kause et al., 1999a). In addition to phenology and water, the main candidates for determining herbivore performance, are phenolics, defensive phenoloxidase enzyme activities, and amino acids (Haukioja, 2005). The phenology index reflects chemical changes as the leaf matures. Maturation changes leaf traits drastically: water content (Riipi et al., 2002) and amino acid levels fall (Ruuhola et al., 2003), individual sugars (Riipi et al., 2002) and phenolics (Nurmi et al., 1996; Salminen et al., 2001; Riipi et al., 2002) peak at different times, phenoloxidase activities decrease (Yang et al., 2007), and leaves become tough (Haukioja et al., 2002). Moth eggs hatch in synchrony with the bud burst of birches. For proper growth and survival, it is imperative for the larvae to follow birch phenology closely (Haukioja et al., 2002).

Whether the basis for variability in birch foliage is primarily genetic or environmental is unclear. The environment commonly affects a plant's foliar chemistry, via such factors as soil properties, water availability and light, and wind and temperature conditions. These changes in chemistry indirectly affect herbivory (Bryant et al., 1983). In addition to chemical variability as such, the rate at which the phenology proceeds during the spring and summer may also have a genetic basis, and this too may play an important part in herbivory defense (Carmona et al., 2011).

Our goal was to determine the role played by genetic and environmental factors in individual herbivory-related traits in *B. pubescens* spp. *czerepanovii*. The study was conducted on 72 mature trees belonging to three half-sib families planted in their native area more than thirty years ago on the slope of a river valley at 69° N latitude. Birch individuals growing at the same elevation share a relatively homogeneous micro-environment with similar herbivore pressure, water and nutrition levels, and exposure to sunshine. We, therefore, assumed that most of the differences among trees would be associated with the topography of the garden. The resistance status of the birches was determined by conducting feeding experiments with *E. autumnata* on the trees, and by recording levels of natural herbivory damage and of *Melampsoridium betulinum* (Fr.) Kleb (rust) infection. We also recorded the phenology and senescence of the trees, since phenotypic chemical variability is known to change as the summer advances, whereas herbivore performance deteriorates at the same pace. As growth and reproduction may compete for the resources needed to produce the chemicals necessary to cope with herbivores, we also estimated the number of long and short shoots (Macdonald and Mothersill, 1983) and female flowers. The relative proportions of family and microhabitat components out of total variation were used to measure genetic and environmental variability.

Methods and Materials

Study Species Mountain birch, *B. pubescens* spp. *czerepanovii*, is a northern subspecies of the downy birch (*B. pubescens* Ehrh.) (Jonsell, 2000a), and is the dominant (and frequently the only) tree species in northernmost Finland (Kallio and Mäkinen, 1978). The birch forms the forest line in NW Europe, Fennoscandia, the Kola Peninsula, Iceland, and southern Greenland (Kallio et al., 1986). The birch is adapted to a harsh environment, with short subarctic summers, bitter winters, and soils that are generally poor in nutrients (Karlsson and Weih, 1996; Sveinbjörnsson et al., 1996; Aerts et al., 2006).

These birch forests suffer from repeated defoliations caused by *E. autumnata* outbreaks. Large areas of former birch forest are now treeless, due to severe defoliations by moth larvae (Tenow, 1972), coinciding with high seedling mortality due to *Rangifer tarandus* (reindeer) browsing and fungus infection (Kallio and Lehtonen, 1973). There also are other herbivorous species of Geometridae larvae that can cause cyclic outbreaks in birch forests, but *E. autumnata* is the most common (Tenow, 1996).

Kevo Tree Line Gardens and Experimental Setup The experiment was conducted in one of the Kevo tree-line gardens (Rassiniva) at the Kevo Subarctic Research Station (69°45′N, 27°01′E, 90 m above sea level) belonging to the University of Turku. The tree-line gardens were established in order to study the trees close to the limits of their habitats: trees do not grow beyond the tree line. In the Kevo region, the location of the tree line is a function of elevation and latitude. The area of the garden is approximately 4 ha, and it is fenced to keep out large mammalian herbivores. When the tree-line garden was established in the 1970s, native trees were removed and the humus layer was mixed with sand and gravel by ploughing with a chain rototiller. Since then, the trees have grown naturally, without watering or protection from insect herbivores (Kallio et al., 1986).

The trees in the garden originate from the seeds of single trees, and due to the open wind pollination of birch they form half-sib families (hereafter referred to as "families"). The trees were raised in a nursery for 3 yr. In 1977, the seedlings were planted in the garden in 6 blocks, each block including individuals from each birch family. The trees grow in lines from top to the river at an even distance from each other to ensure a similar exposure to winds and sunlight (the sun does not set in about 70 d in these latitudes). The Rassiniva garden is located on the eastern slope of a forested river valley, 90 m above sea level, where the cold winters usually kill most moth eggs and natural herbivory is low (Kallio and Lehtonen, 1973). The garden ranges from the high, dry ridge downwards towards a moister and more fertile valley. The site of a tree was interpreted as a measure of its local environment or microhabitat (Kallio et al., 1986; Elamo et al., 2000).

For this study, conducted in 2005, we randomly selected three half-sib families, comprising a total of 72 birch trees. The mother trees of the selected families were sampled in the vicinity of the Kevo Subarctic Research Station.

Fertilization treatments were applied to these families during the early phase of the growing seasons of 2003–2006, comprising a total of six nitrogen treatments (50 or 100 kg nitrogen/ha/year with NH_4Cl, NO_3Na, or NH_4NO_3) and a control treatment. Plants can absorb both ammonium and nitrate from the soil, but nitrate needs to be converted to ammonia by reductase enzymes before it can be used in plant metabolism. The nitrogen fertilizer was dissolved in 500 ml of water and sprayed over an area of 2.25 m^2 around the tree. In the control treatment, trees were sprayed with pure water. In an additional control treatment, trees did not receive any fertilization or extra water (or any experimental larvae; see below).

The performance of *E. autumnata* larvae on living trees was used as a measure of birch resistance. Trees in the fertilization treatments and the control treatment were provided with 12 *E. autumnata* larvae. At the time of leaf flush (4 June 2005), a mesh bag with an identical mix of newly hatched moth larvae was attached around a branch of the tree. Each bag contained 1 larva from each of 12 moth broods. At the end of the larval period, the bags were checked daily and larvae were collected when they were preparing to pupate. Larvae were placed individually into plastic vials containing moist *Sphagnum* moss for pupation. After 7 d, pupae were weighed and sexed. Larval development rate was defined as the reciprocal of the duration of the larval period (in days) (Kause et al., 1999b).

Birch Measurement Tree phenology was recorded twice (31 May and 4 June 2005), using a classification on a scale of 1 to 8 based on bud morphology and leaf elongation (Sulkinoja and Valanne, 1987). In late summer, after the birch trees had completed their growth, we measured the number of long and short shoots and the number of female flowers (catkins). Short-shoot leaves are even-aged and form the majority of the trees' foliage. Long-shoots are responsible for branch growth, and they form new leaves as the shoot grows (Macdonald and Mothersill, 1983). The numbers of shoots and female flowers were calculated for 4 big branches facing in different directions from the tree, and the proportion of the whole tree covered by these branches was estimated. The length of long shoots was estimated by measuring the lengths of 10 long shoots from different parts of the tree and calculating their mean value.

In late summer, the overall *M. betulinum* (rust) frequency was estimated by taking the mean of the approximate percentages of the leaf area of 10 long shoots infected by the pathogen. The percentage of natural herbivory damage was estimated visually as the ratio of leaves eaten by herbivores to the total original number of leaves on the tree; it therefore excludes miners, galls, etc. The natural herbivory percentage and the senescence index are averages based on the whole tree.

Leaf Samples and Chemical Analyses Samples of leaves (2–3 g) for the analysis of enzymatic activities and chemistry were collected in early summer when the leaves had completed their active growth. Short-shoot leaves were collected throughout the canopy. Leaves for chemical analysis were sealed in plastic bags and brought to the laboratory in a cooler. Leaves were immediately weighed and freeze-dried for 72 h. For each tree, dried leaves were reweighed, homogenized to a powder, and stored at −20 °C prior to chemical analysis. For the enzymatic assays, leaves were flash-frozen in liquid nitrogen and stored at −80 °C prior to analysis.

Protein-bound amino acids were derivatized with 9-fluorenylmethyl chlorformat (FMOC-Cl) and analyzed by high-performance liquid chromatography (HPLC; Bank et al., 1996). Derivatized amino acids were separated using a

slightly modified ternary gradient system as described by Bank et al. (1996). Phenolic compounds were analyzed using HPLC with photodiode-array detection (HPLC-DAD). Methods for extraction, analysis, and identification of phenolic compounds are described in Salminen et al. (1999, 2001). Enzyme activities of the birch leaves were analyzed as in Ruuhola and Yang (2006) and Ruuhola et al. (2007). Enzyme activities were measured in triplicate. Protein concentrations of the enzyme extracts were measured in triplicate as in Ruuhola and Yang (2006) using the Bio-Rad protein assay method based on the Bradford method (Bradford, 1976). Bovine serum album was used for the protein standard curves.

Statistical Analysis Data were analyzed in two different ways to estimate (1.) the components of variance, and (2.) the effect of microhabitat and family. In the first analysis, both the family and the site in the garden were random variables, in the second both variables were fixed. The site of the birch on the slope was used as a continuous variable.

$$Y = \text{half} - \text{sib family} + \text{site in tree line garden} + \text{fertilization treatment}(+\text{plate in case of oxidase activities}) + \text{error}.$$

The statistical analyses were performed with the SAS proc mixed procedure (SAS Institute, 1990). Degrees of freedom were calculated with a *Kenward-Roger* approximation. If necessary, we applied the logarithmic transformation to the birch traits. Twelve birch trees were removed from the analysis of amino acid content as outliers.

Nitrogen treatment was statistically significant only for catechin levels and long shoot length, and these traits were excluded from subsequent analyses.

We calculated heritabilities (h^2) as

$$h^2 = V_A/V_P = (4{*}\text{covariance due to half} - \text{sib family}) / (\textstyle\sum \text{all covariance components})$$

Coefficients of genetic variation (CV) was calculated for additive genetic variance (CV_A) and phenotypic variation (CV_P) for easier comparison of variances (Houle, 1992)

$$CV_A = [\text{sqrt}(4{*}\text{covariance due to half} - \text{sib family})]/\text{trait mean}$$
$$CV_P = [\text{sqrt}(\textstyle\sum \text{all covariance components})]/\text{trait mean}$$

We calculated the percentages of observed variation explained by the half sib family and the site of a birch tree in the garden in order to compare the effects of genotype and environment.

$$\text{The proportion of variation}(\%)\text{explained by the half sib family} = [\text{covariance due to half} - \text{sib family}/(\textstyle\sum \text{all covariance components})]{*}100\%$$
$$\text{The proportion of variation}(\%)\text{explained by the site of a birch tree in the garden} = [\text{covariance due to site in the garden}/(\textstyle\sum \text{all covariance components})]{*}100\%$$

Phenotypic correlations were calculated as Spearman's non-parametric correlations with the SAS proc corr procedure (SAS Institute, 1990). Before calculating the correlations, we calculated the least square mean values for birch trees with regard to the pupal mass and developmental rate of moth larvae. Least square mean values were obtained with variance analysis (proc glm), where larval sex was used as a fixed variable. We also analyzed male and female larvae separately. Since the results did not differ, however, we provide only those results for the complete data.

Results

The coefficient of additive genetic variance was high for many phenolic compounds, especially the galloylglucoses. Birch family explained approximately one quarter of the observed variation in phenolics (Table 1). The amino acids, on the other hand, generally had low coefficients of both additive genetic and phenotypic variances, and all birch trees were relatively similar regarding their amino acid levels (Table 2). Oxidative enzyme activities had relatively high phenotypic variances, but their additive genetic variances were not as high as those of phenolics (Table 1).

The chemical properties of the foliage varied with the site of the tree in the tree-line garden (= microhabitat), but this environmental factor explained only 1–4 % of the variation observed (Table 1). The effect was clearest in the phenolics (galloylglucoses, pedunculagin, casuarictin, and gallic acid) (Fig. 1a–e). In the case of the amino acids, site in the garden explained 2–4 % of variation observed in serine, glysine, methionine, and phenylalanine (Table 2, Fig. 1f).

The number of long shoots had a high genetic component, and 26 % of variation in long shoots was explained by genetics. Tree site in the garden explained 2–3 % of the observed variation in growth, measured as the number of

Table 1 Heritability (h^2), coefficients of additive genetic (CV_A) and phenotypic (CV_P) variation of levels in phenolic compounds and oxidative activities in *Betula pubescens* spp. *czerepanovii* (mountain birch) leaves

Birch trait	h^2	CV_A	CV_P	Family	Site	*N*	Mean	SD	log
Monogalloylglucose	1	0.71	0.67	28*	1	72	6.38	4.12	
Digalloylglucose	0.86	0.59	0.64	22*	1*	72	1.11	0.7	
Trigalloylglucose	0.92	1.54	1.61	23*	3*	71	1.69	1.09	log
Total galloylglucoses	1	0.67	0.6	31*	1*	72	9.18	5.33	
Pedunculagin	0.37	1.08	1.79	9*	3*	69	2.19	2.16	log
Pedunculagin derivates	0.15	0.32	0.82	4	0	72	9.9	7.93	
Casuarictin	0.61	0.85	1.09	15*	4*	72	1.76	0.99	log
Ellagitannin 1	1	1.15	0.91	40*	1*	72	2.43	1.47	log
Ellagitannin 2	0.32	0.3	0.53	8	0	72	0.65	0.47	log
Total ellagitannins	0.43	0.18	0.28	11*	0	72	16.94	9.87	log
Total hydrolysable tannins	0.83	0.53	0.58	21*	0	72	26.11	14.49	
Myricetin glycosides	0.08	0.12	0.43	2	0	72	1.68	0.7	
Quercetin glycosides	1	0.33	0.31	28*	0	72	9.86	2.98	
Kaempferol glycosides	0.74	0.29	0.34	19*	0	72	3.16	1.03	
Total flavonoid glycosides	0.97	0.27	0.27	24*	0	72	14.7	3.99	
Gallic acid	0.71	0.74	0.88	18*	4*	72	0.12	0.12	log
Chlorogenic acid	0.56	0.3	0.4	14*	0	72	8.33	3.17	
Coumaroylquinic acids	0.4	0.32	0.5	10*	0	72	0.64	0.45	log
Total phenolics	1	0.3	0.3	25*	0	72	51.14	14.67	
Acidic PPO (with catalase)	0.13	0.18	0.51	3	0	66	362	185	
Acidic PPO	0.06	0.12	0.47	2	1	69	645	305	
Alkaline PPO (with catalase)	0.06	0.16	0.64	2	3*	68	67.85	41.94	
Alkaline PPO	0		0.48	0	0	69	147	72.2	
Phenoloxidase activities									

Heritability values, additive genetic variances and phenotypic variances below 0 are marked as 0, heritability values over 1 are marked as 1. Columns "family" and "site" show the proportion of variation (%) explained by the half sib family and the site of a birch in the garden, respectively. An asterisk means that family or site explains a statistically significant amount of the variation. The mean and standard deviation (SD) of the birch trait (e.g. concentration of the compound mg/g dw) are also given. *N* = number of trees, log = logarithmic transformation of the trait value. Bold and underlined traits are the sums of the individual compounds

long and short shoots. There was clearly more phenotypic variation in the number of long shoots than in that of short shoots (Table 3, Fig. 2a–b).

The number of female flowers showed high additive genetic and phenotypic variation (Table 3).

The extent of natural herbivory and *M. betulinum* infection had a clear additive genetic component (Table 3). Natural herbivory damage varied with site in the garden (4 % of observed variation, Table 3, Fig. 2c) as did the levels of the pathogen, *M. betulinum* (2 %, Table 3, Fig. 2d).

The birch phenology index showed low additive genetic and phenotypic variance (Table 2). The senescence index in turn showed high phenotypic variance but no genetic variance (Table 3).

The resistance status of the trees was negatively correlated with the amino acid level of the foliage. This was always the case, whether we measured the intensity of natural herbivory (Fig. 3a), pathogen *M. betulinum* incidence (Fig. 3b) or the performance of the herbivore *E. autumnata* (Fig. 3c–d). Trees with longer long shoots had higher amino acid levels (r=0.3, P=0.006, N=72). The numbers of long shoots (r=0.4, P=0.001, N=60) and short ones (r=0.3, P=0.026, N=60) were correlated with *E. autumnata* pupal weight. The numbers of long shoots (r=0.6, P=0.002, N=26) and short ones (r=0.7, P<0.001, N=26) also correlated with the number of female flowers.

The younger the leaves, the higher the level of phenolics in almost all cases. In other words, the phenology index co-varied negatively with 10 out of 15 individual phenolics. Chlorogenic acid was an exception. Younger leaves had less chlorogenic acid than older ones. Younger leaves (low phenology index) also had more amino acids that older ones (r=-0.4, P=0.002, N=63).

Table 2 Heritability (h^2), coefficients of additive genetic (CV_A) and phenotypic (CV_P) variation of levels in amino acids in *Betula pubescens* spp. *czerepanovii* (mountain birch) leaves

Birch trait	h^2	CV_A	CV_P	Family	Site	*N*	Mean	SD	log
Histidine	0.01	0.01	0.1	0	1	60	3.22	0.31	
Arginine	0.22	0.05	0.1	6	0	60	8.78	0.88	
Serine	0.29	0.05	0.09	7	2*	60	7.36	0.67	
Aspartic acid	0.31	0.05	0.1	8	1	60	15.26	1.43	
Glutamic acid	0.28	0.05	0.1	7	1	60	19.11	1.79	
Threonine	0.17	0.04	0.1	4	1	60	7.38	0.7	
Glysine	0		0.1	0	4*	60	7.57	0.77	
Alanine	0.04	0.02	0.09	1	1	60	9.37	0.81	
Proline	0		0.08	0	1	60	7.81	0.64	
Methionine	0		0.32	0	2*	54	0.9	0.29	
Valine	0.1	0.03	0.09	2	1	60	7.92	0.67	
Phenylalanine	0		0.09	0	2*	60	8.03	0.71	
Isoleusine	0.06	0.02	0.08	1	1	60	6.4	0.53	
Leusine	0.07	0.02	0.09	2	1	60	14.71	1.26	
Lysine	0.23	0.04	0.09	6	1	60	11.93	1.07	
Tyrosine	0.26	0.06	0.12	7	0	60	5.37	0.6	
Amino acids	0.15	0.03	0.09	4	1	60	141	12.53	

Heritability values, additive genetic variances and phenotypic variances below 0 are marked as 0, heritability values over 1 are marked as 1. The columns "family" and "site" give the proportion of variation (%) explained by the half sib family and the site of a tree in the garden, respectively. An asterisk means that family or site explains a statistically significant amount of the variation. The mean and standard deviation (SD) of the birch trait (e.g. concentration of the compound mg/g dw) are also given. *N* = number of trees, log = logarithmic transformation of the trait value. Bold and underlined traits are the sums of the individual compounds.

Amino acids levels co-varied with each other (out of all 120 possible correlations between 16 individual amino acids, in all cases $r>0$ and in 112 $P<0.05$, $N=72$), as did phenolics (out of all 105 possible correlations between 15 individual phenolics, in 39 cases $P<0.05$ and in all those $r>0$, $N=72$). All hydrolyzable tannins (galloylglucoses and ellagitannins) showed a positive intercorrelation. In 25 cases (out of 28), the correlations were significant ($P<0.05$, $N=72$). All measures of oxidative activities showed positive, statistically significant correlations.

Discussion

Heritable differences among birch trees with regard to their herbivore resistance status are a prerequisite for the evolution of plant-herbivore interactions (an "arms race"). In the absence of variation, herbivores would easily be able to specialize in monotonic birch foliage (Adler and Karban, 1994; Roslin et al., 2006). The variation can be caused by environmental or genetic factors, or by some combination of these. We found that the topography of the garden (environmental variation) affects tree architecture: the trees were taller and tended to have more long and short shoots at the lower, moister end of the garden. Tree architecture has been shown to affect the herbivores foraging on them (Riihimäki et al., 2006). Natural herbivory damage was most abundant at the drier, sandier, and steeper high end of the garden, while both pathogenic rust and the main insect herbivore, *E. autumnata*, performed better at the lower, moister end. According to Senn et al. (1992), fast-growing birch trees seem to be most vulnerable to herbivores and pathogens. Our results with *E. autumnata* and rust are in accordance with the plant vigor hypothesis, according to which vigorous growth makes plants more vulnerable to herbivores (Price, 1991), but the level of natural herbivory damage does not follow this hypothesis.

While the environment affected some traits, genetic factors seem to be most important in giving rise to chemical variation in birch. This study, together with an independent crossing study (Haviola et al., 2006), provides clear evidence of a genetic basis for the wide variation observed in phenolics. The primary function of phenolic compounds has been suggested to lie in their anti- and pro-oxidative capacity, and in the protection of the plant against reactive oxygen species (Harborne and Williams, 2000; Close and McArthur, 2002; Salminen and Karonen, 2011). The levels of phenolic compounds, especially the galloylglucoses and proanthocyanidins (= condensed tannins), are negatively related to the growth of herbivores in birch foliage (Kause

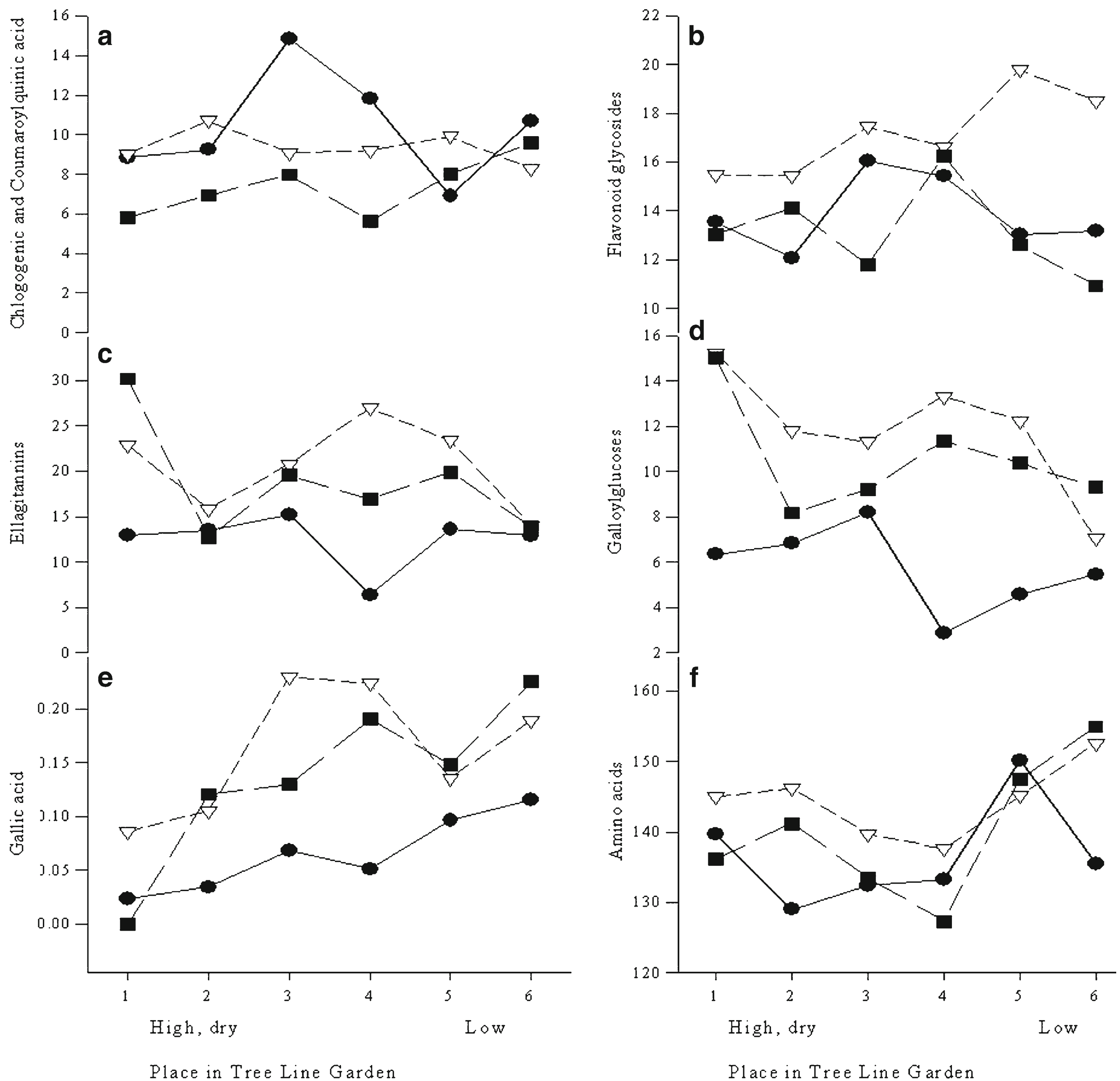

Fig. 1 Genetic and environmental differences in *Betula pubescens* spp. *czerepanovii* (mountain birch) half sib families trees in a tree-line garden. *Different lines* represent half sib families, the *symbols* representing averages. Sum of chlorogenic and Coumaroylquinic acid (**a**), total flavonoid glycosides (**b**), total ellagitannins (**c**), total galloyl-glucoses (**d**), gallic acid (**e**) total amino acids (**f**)

et al., 1999a; Haukioja et al., 2002). However, several studies conducted on relationships between individual phenolic compounds and herbivory have given contrasting results (Haviola et al., 2007; Ruuhola et al., 2007). A plant's suitability for herbivores also has been connected to plant oxidases, such as polyphenoloxidases (PPOs) and peroxidases (PODs) (Felton et al., 1989; Appel, 1993). Phenoloxidases transform phenolics into an active form, quinones, thus reducing the nutritive value of plant tissue for herbivores. Quinones precipitate amino acids and other important nutrients forming a brownish polymer, melanin. In addition, the oxidation of phenolics also may produce reactive oxygen species (ROS), which are harmful to plant-eating animals. Yang et al. (2007) found considerable variation in peroxidases (POD), polyphenoloxidases (PPO's), and catalase activity within individual trees, but less between trees. In our study, mainly environmental differences gave rise to variation in defensive enzymes. We did not find much

Table 3 Heritability (h^2), coefficients of additive genetic (CV_A) and phenotypic (CV_P) variation in *Betula pubescens* spp. *czerepanovii* (mountain birch) traits (growth parameters)

Birch trait	h^2	CV_A	CV_P	Family	Site	*N*	Mean	SD	log
Herbivory %	0.31	0.52	0.94	8	4*	72	1.74	1.69	
M. betulinum % (rust)	0.08	0.45	1.62	2	2*	72	0.63	1.01	
Long shoots per tree (log)	1	0.32	0.31	26*	3*	72	7.38	1.98	log
Short shoots per tree (log)	0.27	0.08	0.15	7	2*	72	3007	2631	log
Female flowers pre tree	1	1.65	1.61	26*	0	72	85.17	299.9	log+1
Phenology index (31 May)	1	0.05	0.04	41*	0	63	1.3	0.05	
Senescence index	0		0.94	0	0	72	4.13	3.7	

Heritability values, additive genetic variances and phenotypic variances below 0 are marked as 0, heritability values over 1 are marked as 1. The columns "family" and "site" give the proportion of variation (%) explained by the half sib family and the site of a tree in the garden, respectively. An asterisk means that family or site explains a statistically significant part of the variation. The mean and standard deviation (SD) of the birch trait are also given. *N* = number of trees, log = logarithmic transformation of the trait value

variation in the amino acids of individual birch trees, which is in line with earlier studies on variation in primary metabolites—sugars and amino acids (Suomela et al., 1995; Ruuhola et al., 2003; Riipi et al., 2004). Amino acids are important actors in plant-herbivore interactions, because they provide nutrition to herbivores (Berenbaum, 1995), and are positively associated with herbivore growth (Kause et al., 1999a).

Heritability estimates of secondary chemistry typically are high in plants, as shown in reviews by Berenbaum and Zangerl (1992) and Geber and Griffen (2003), and foliar chemistry has been found to respond to selection (reviewed in Geber and Griffen, 2003). In a closely related species, *Betula pendula*, resistance has a genetic basis. Keinänen et al. (1999) found that *B. pendula* clones showed substantial variation in the phenolic composition of the leaves. Studies with *B. pendula* clones by Laitinen et al. (2000) showed that shoot secondary chemistry is under strong genetic control. Mutikainen et al. (2000) observed significant differences in resistance among their *B. pendula* clones. Many studies of *B. pubescens* spp. *czerepanovii* have suggested genetic variation in leaf phenolics. Ruohomäki et al. (1996) showed that birch families differed in their foliage chemistry, and Suomela et al. (1995) found that most of the variation in phenolics occurred between trees, as compared to within-tree variation. Anttila et al. (2010) found that birch clones differed in their foliar chemistry. Despite the huge dynamic changes occurring in foliar chemistry during a season, individual birch trees show some consistence in phenolic composition (Riipi et al., 2004). Nevertheless, this and our earlier experiment (Haviola et al., 2006) are the only studies with *B. pubescens* spp. *czerepanovii* that have provided genetic estimates, although several studies have pointed to the importance of genetics. The high genetic variation found in *B. pubescens* spp. *czerepanovii* might be due partly to its hybrid origin; the number of *Betula nana* genes might vary within mountain birch populations (Elkington, 1968).

In addition to chemistry, we found indications of heritability in traits that affect birch architecture and the phenology index (measured as timing of bud burst). Even a low level of genetic variation in the advancement of phenology may be important for subarctic birch. In the northern setting of cold springs and short summers, optimal phenology is a compromise between cold tolerance and the length of the growth season. The leaf chemistry profile of the trees changes dramatically with the leaf phenology as the summer advances (Riipi et al., 2002). Phenology also is tied to herbivore performance: the main herbivores are able to eat off a tree only during certain stages of the phenology. If the phenology is genetically determined, trees sampled at the same time may have different chemical profiles simply because they differ in phenology (Carmona et al., 2011). Studying individual traits separately may not lead to ecologically relevant results. Phenology and tree architecture are good examples of this.

We found indications of genetic differences among birch families with regard to the level of natural herbivory damage and the incidence of the pathogen *M. betulinum*. Our results are consistent with earlier experiments in the same garden, in which birch trees were found to have heritable variation in frequencies of the endophytes *Melanconium* sp., *Fusicladium* sp., and *Venturia ditrich* (Elamo et al., 1999; Ahlholm et al., 2002), and in infection levels of the pathogen *M. betulinum* (Elamo et al., 2000). Ruohomäki et al. (1996), on the other hand, did not find birch families to differ as food for *E. autumnata*.

A classic assumption of quantitative genetics is that high values of additive genetic variances and heritability indicate that the trait in question has not been operated upon by natural selection thus far, since selection typically erases genetic variation (Fisher, 1930; Mousseau and Roff, 1987; Roff and Mousseau, 1987). A more recent view is that some

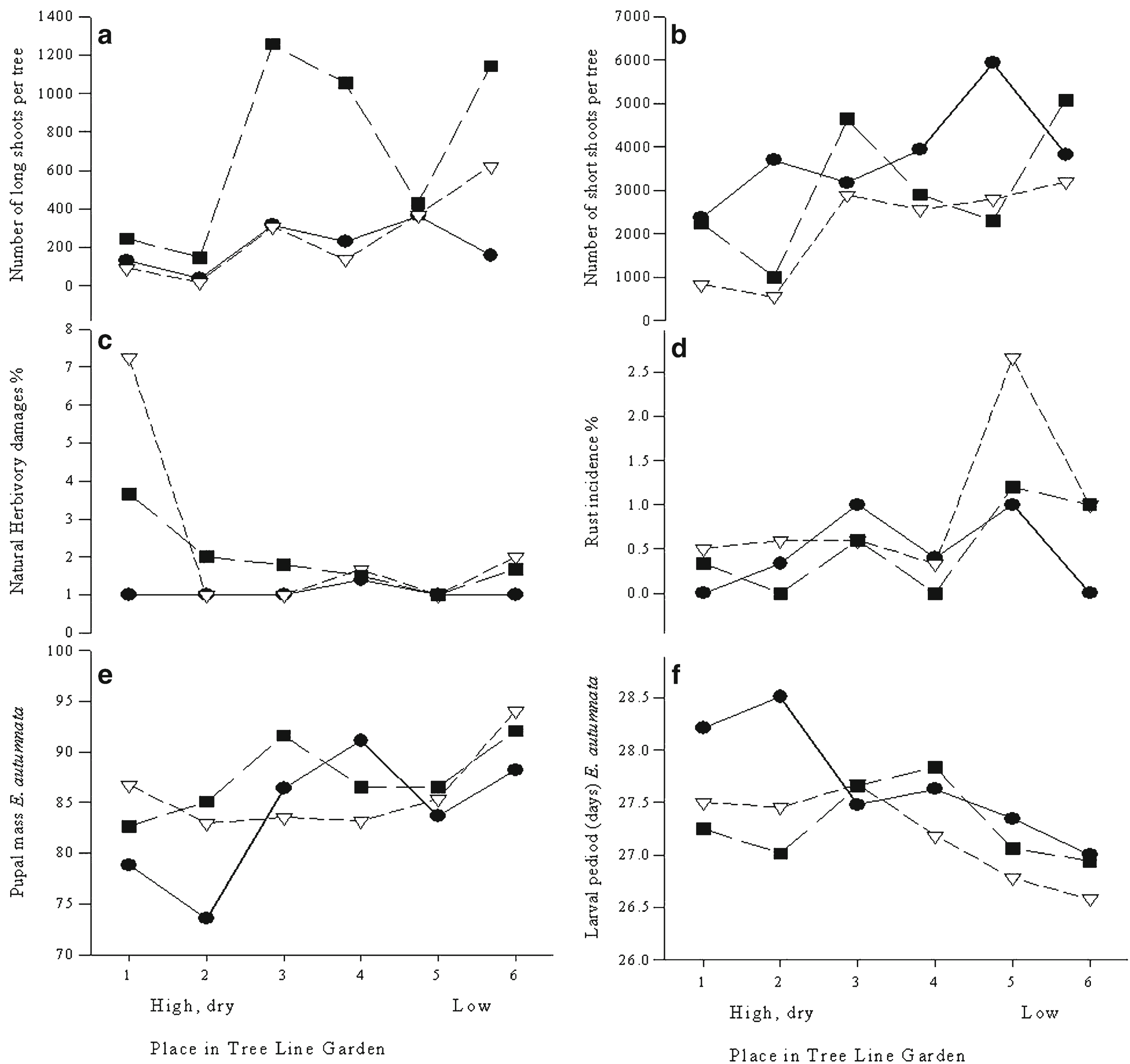

Fig. 2 Genetic differences and environmental differences in *Betula pubescens* spp. *czerepanovii* (mountain birch) half sib families trees in a tree-line garden. *Different lines* represent half sib families, the *symbols* representing averages. Number of long (**a**) and short (**b**) shoots in a tree, level of natural herbivory damage (**c**), *Melampsoridium betulinum* (rust) infection (**d**), pupal mass (**e**) and larval period (days) (**f**) of *Epirrita autumnata* (autumnal moth)

traits are affected by such a number of genes that they easily collect additive genetic variance, even when exposed to severe selection (Houle, 1992; Geber and Griffen, 2003). It is clear that herbivory resistance in birch is a multifaceted trait. We, therefore, consider that it is not unlikely for resistance to contain a great deal of genetic variation and heritability, even if it is a target of selection.

Amino acid levels play such a pivotal role in plant metabolism, and are so tightly regulated that newly arising mutations, which affect amino acid levels, would in all likelihood be detrimental to the plant. In could on the other hand be argued that there is a possibility for advantageous phenol-metabolism mutants to arise, since we already see huge differences in the phenolic composition of the foliage, both between and within plant species. Plants typically contain tens, if not hundreds, of secondary compounds that contribute to resistance (Agrawal, 2011). It is possible that phenolics have become the most important resistance components, but not because they have the strongest effect on herbivores; in fact, amino acids seem to be more important

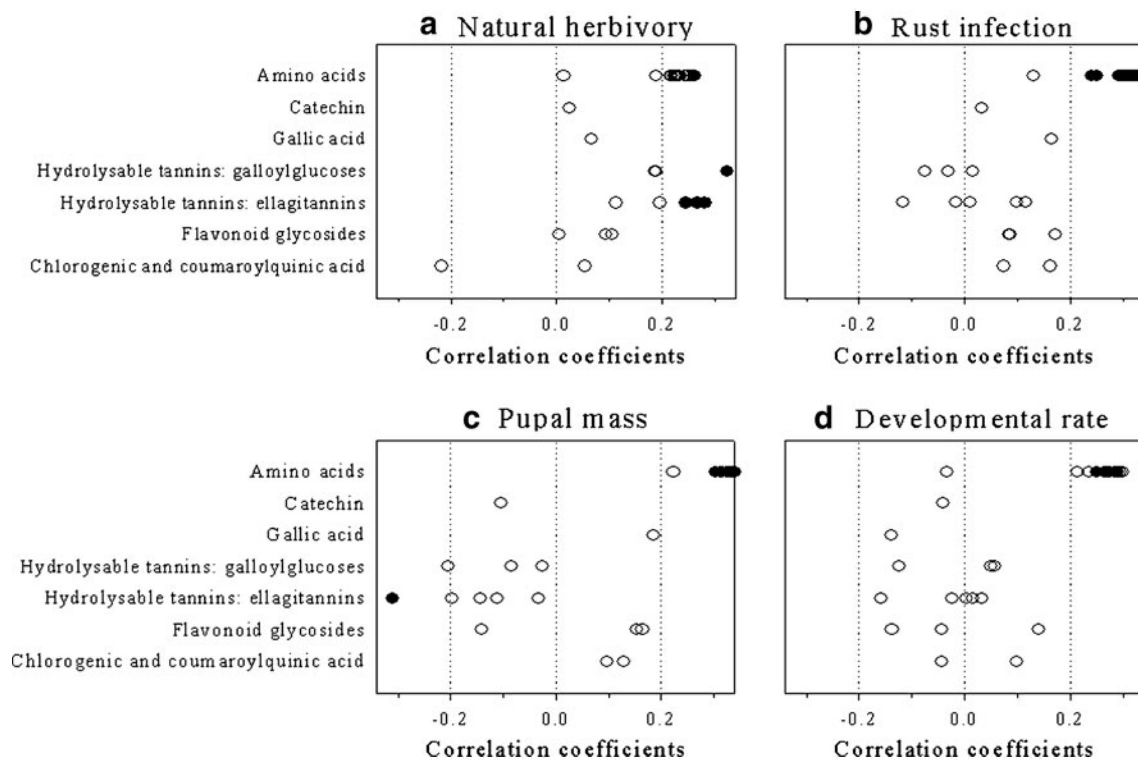

Fig. 3 Overview of phenotypic correlations between phenolic levels of *Betula pubescens* spp. *czerepanovii* (mountain birch) foliage and intensity of herbivory. Natural herbivory damage (%) (**a**), *Melampsoridium betulinum* (rust) infection (**b**) Pupal mass (**c**) and developmental rate (reciprocal of larval period, days) (**d**) of *Epirrita autumnata* (autumnal moth). The figure shows the Spearman correlation coefficients of individual amino acids and phenolics (catechin, gallic acid, hydrolyzable tannins (galloylglucoses and ellagitannins), flavonoid glycosides or chlorogenic acid and coumaroylquinic acid). *Filled circles* represent correlations significantly different from zero

to insects performance (Berenbaum, 1995). Rather, phenolics may have become the most relevant resistance compounds because the constraints on their evolution are the weakest (Carmona et al., 2011).

Genes that are part of shared biosynthetic pathways, such as those coding for phenolics, or genes that are involved in allocating resources to plant functions are likely to produce phenotypic and genetic correlations. Selection tending to increase trait values slows down the adaptation of negatively correlated traits, but enhances adaptation if the traits have positive genetic correlations (Lande and Arnold, 1983). We found positive phenotypic correlations between individual hydrolyzable tannins (galloylglucoses and ellagitannins). When a tree is under herbivore or pathogen attack, or is suffering from reactive oxygen species (Harborne and Williams, 2000; Close and McArthur, 2002; Salminen and Karonen, 2011), hydrolyzable tannin molecules may be needed by the plant as defensive compounds. Since all hydrolyzable tannins are produced via the same metabolic pathway, activation of the pathway may cause many hydrolyzable tannins to be induced at the same time; only some of them, however, may have a defensive role (Moilanen and Salminen, 2008).

Due to global warming, some changes are likely to occur in the interaction of temperature, solar radiation, and herbivore abundance in subarctic birch forests. Rising temperatures and the associated changes affect many aspects of the ecosystem, including herbivore performance, overwintering, and their natural enemies (Virtanen and Neuvonen, 1999). Additionally, variables that affect soil properties, such as decomposition, microbial activity, and the duration of snow cover might change (Aerts et al., 2006). Soil properties affect the trees' foliar chemistry, which in turn affects herbivory and thus the trees again indirectly. Changes in the combinations of phenolics and polyphenoloxidases may provide a route for birch to adapt to these challenges.

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Mosaic Eucalypt Trees Suggest Genetic Control at a Point That Influences Several Metabolic Pathways

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Abstract Mosaic trees contain more than one phenotype. The two *Eucalyptus* mosaic trees studied here (*E. melliodora* and *E. sideroxylon*) are predominantly susceptible to insect herbivory, with the leaves on a single large branch on each tree resisting herbivory. We used gas chromatography–mass spectrometry and high-pressure liquid chromatography to analyze the chemical profile of leaves of the mosaic trees, as well as leaves of adjacent non-mosaic conspecifics. We show that the leaves of the two phenotypes are distinctly different. Compared to the susceptible (S) leaves on the same tree, the resistant (R) leaves on the mosaic trees had low concentrations of sesquiterpenes (*E. melliodora*: 2 vs. 24 mg·g^{-1} dry matter; *E. sideroxylon*: 8 vs. 22 mg·g^{-1} dry matter), high concentrations of FPCs (*E. melliodora*: 5.4 vs. 0.3 mg·g^{-1} dry matter; *E. sideroxylon*: 9.8 vs. 0.2 mg·g^{-1} dry matter) but similar concentrations of nitrogen (*E. melliodora*: 15.4 vs. 16.8 mg·g^{-1} dry matter; *E. sideroxylon*: 13.1 vs. 14.0 mg·g^{-1} dry matter). The only difference between the two mosaic trees was in the levels of monoterpenes. The R leaves from the mosaic *E. melliodora* contained higher concentrations of monoterpenes compared to the S leaves (12 vs. 6 mg·g^{-1} dry matter). In contrast, the leaves from the *E. sideroxylon* mosaic contained much higher concentrations of monoterpenes with a reversed pattern (R: 26 vs. S: 45 mg·g^{-1} dry matter). There were qualitative differences too on the mosaic trees. The R leaves of both species contained much higher concentrations of the monoterpene, 1,8-cineole, whereas the S chemotype of *E. sideroxylon* only had high concentrations of phellandrenes. Furthermore, the chemical differences between leaves on the R and S branches of the mosaic trees resembled those between the leaves of R and S con-specific trees in the same population. We use these data and knowledge of secondary metabolite biosynthesis to propose that high-level transcriptional differences may control the profile of specialized metabolites in eucalypts.

Keywords Terpene · Formylated phloroglucinol compound (FPC) · Chemotype · Ecotype · Somatic mutation · Mosaicism · Herbivory · Insect outbreaks

Introduction

Long-lived organisms, such as forest trees, may accumulate somatic mutations throughout their lives that result in an adult supporting several genotypes. This is known as genetic mosaicism (Whitham and Slobodchikoff, 1981; Gill, 1986) – a hypothesis proposed to explain variable resistance to pests within a single plant. However, genetic variation probably will go unnoticed unless accompanied by phenotypic variation. There are excellent examples of such variation among horticultural species. For example, nectarines are a genetic variant of the peach (*Prunus persica*), and peach fruits have occurred on nectarine trees and *vice versa* since 1937 (McGregor, 1976). There are many other examples of intra-individual variation of plants that have profound influences on plant herbivore interactions (e.g., Herrera, 2009).

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Two phenotypically variable mosaic *Eucalyptus* trees were identified in open woodland and pastoral environments in central New Soth Wales, Australia (NSW): an *E. melliodora* (Edwards et al., 1990) and an *E. sideroxylon* (Edwards et al., 1993) (Fig. 1). The two species are members of the "box-ironbark group" of *Eucalyptus* in the sub-genus *Symphyomyrtus*, section *Adnataria*, sub-section *Terminales*, series *Melliodorae* (Brooker, 2000; Slee et al., 2006). Despite their close affinities, they tend to occupy different ecological niches with *E. melliodora* preferring acidic clay, whereas *E. sideroxylon* occurs on more alkaline sandy soils (Brooker, 2000). Other unusual phenotypes grow nearby. Approximately 100 m from the mosaic *E. melliodora* is another of that species with unusual bark on its trunk (Fig. 1c). Chemical mosaics also occur in *E. camaldulensis* (Edwards et al., 1993) and in *E. radiata* (Penfold and Morrison, 1937), which suggests that genetic mosaicism in eucalypts may be more common than appreciated.

Central NSW periodically experiences outbreaks of several insect species, which result in widespread defoliation of dominant trees (Edwards et al., 1990). Damage by several species of Christmas beetles (*Anoplognathus spp.*) revealed the two phenotypic mosaics that we studied because the beetles ate most leaves but left leaves on the resistant (R) branches virtually untouched (Edwards et al., 1990, 1993). Those workers showed that both the composition and concentration of several monoterpenes differ between the leaves of the R and susceptible (S) ecotypes (Edwards et al., 1990, 1993). They proposed that heavy insect feeding released a favorable mutation in the meristematic tissue (the resistant ecotype) that persisted to form an entire branch. Herbivorous insects frequently feed on meristematic tissue. They will either destroy the meristem and alter the growth form of the plant or consume part of the meristem whereby different cells form the developing branch (Marcotrigiano, 2000). One possible scenario is that exposure of damaged cells to

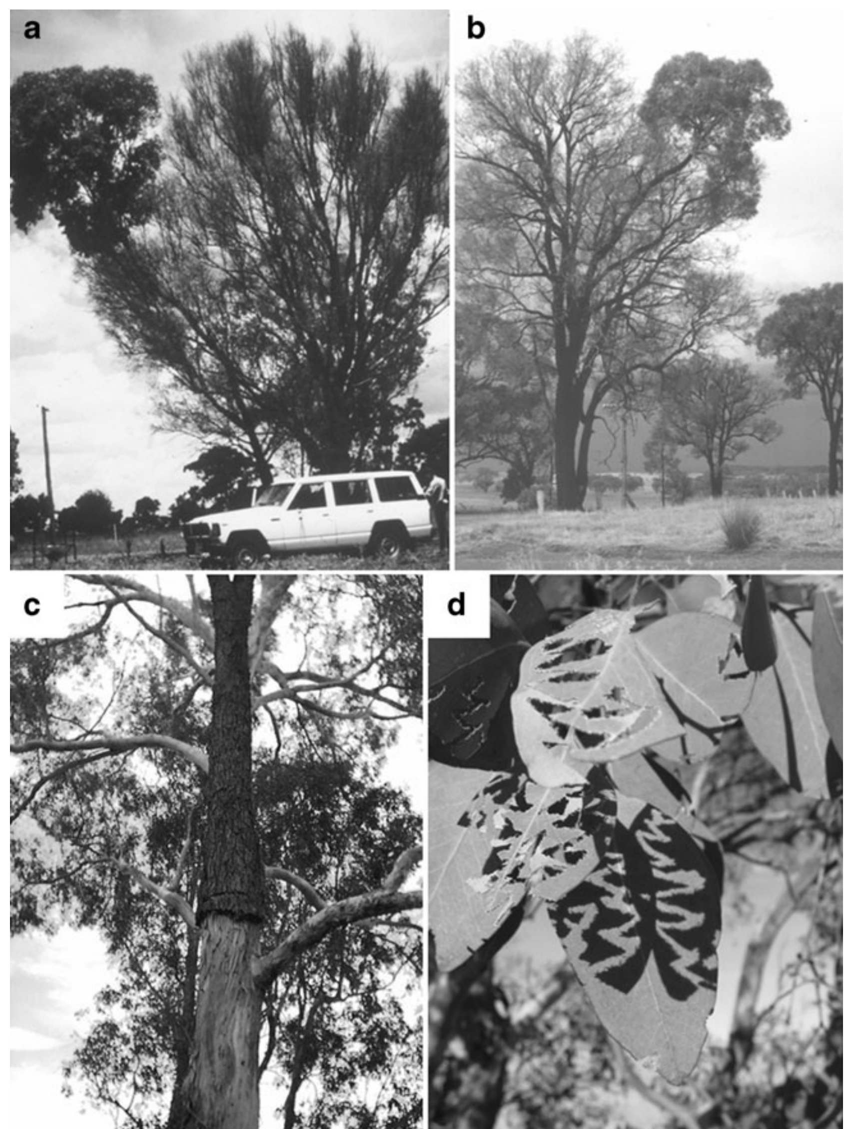

Fig. 1 Photographs of mosaic trees. **a** *Eucalyptus melliodora* and **b** *E. sideroxylon* supporting leaves of two ecotypes (Edwards et al., 1993; Edwards et al., 1990). **c** a bark-mosaic *E. melliodora* with typical box-bark on the lower trunk, which changes to an iron-bark before returning to a box-bark on the newest branches (left). **d** the typical pattern of leaf damage by Christmas beetles (*Anoplognathus spp*.)

reactive oxygen species causes mutations to the nuclear DNA (Halliwell, 1999). If the genotype of these cells differs from the rest of the plant, then a genetic mosaic will arise. A phenotypic mosaic, like the *Eucalyptus spp* described, may result if this genotypic difference also encodes a phenotypic change. Understanding the chemical changes in the mosaics will enable us to develop hypotheses regarding the specific type of mutation. For example, changes in monoterpenes, such as those found by Edwards et al. (1990, 1993), would suggest a mutation in the monoterpene biosynthetic pathway. In contrast, widespread changes in several chemical traits would suggest that the mutation is at a higher control point, such as a transcription factor.

In this study, we report the profiles of three biosynthetically distinct groups of foliar metabolites−the monoterpenes, sesquiterpenes, and the formylated phloroglucinol compounds (FPCs), from both phenotypes of the mosaic trees and from non-mosaic individuals in the same population.

In particular, we ask: (i) What similarities and differences are there in the chemical profiles of the two eucalypt species? (ii) Do the mosaic trees retain their chemical profiles over a period of several years? (iii) Do the chemical profiles of the leaves of R and S trees differ in all three groups of metabolites? (iv) Do the chemical profiles of leaves from non-mosaic individuals mirror the profiles of the leaves from the R and S branches of the mosaic trees?

By answering these questions, we propose a mechanism for the evolution and maintenance of multiple chemotypes in a single individual using the genetic mosaic hypothesis (GMH) framework.

Methods and Materials

Plant Material Foliage samples were collected at two sites in south-eastern NSW – Yeoval (32°45′00″S; 148°39′00″E), where there is a chemically mosaic *E. melliodora*, and at Cumnock (32°55′00″S; 148°46′01″E), where a mosaic *E. sideroxylon* grows (Edwards et al., 1990, 1993). In 2005 and 2009, we collected leaf samples (ca 60 g) using an elevated platform mounted on a truck. Each of the mosaic trees has one large R branch from which smaller R branches originate. Therefore, we collected a sample from 5 of these smaller R branches and 5 samples from random points in the S part of the canopy. In 2005, we also collected a sample of foliage from two neighboring trees of *E. melliodora* (M11, M12) and of *E. sideroxylon* (S14, S15). Edwards et al. (1990, 1993) had already identified these as R (M12, S15) and S (M11 and S14). The collection in 2009 was larger. Apart from sampling the mosaic trees again, we also collected 3 – 5 samples from each of 10 non-mosaic *E. melliodora* and from 11 non-mosaic *E. sideroxylon* growing within a 50 m radius of the mosaic tree. These included M11, M12, S14, S15, and several *E. melliodora* (M2-M8, M10) and *E. sideroxylon* (S2, S8, S16-S22) of unknown ecotype. Each leaf sample was sub-sampled. We immediately placed a weighed amount (ca 0.5 g) into a sealed vial containing 5 ml of 100 % ethanol (Southwell and Russell, 2002) with 0.254 g·l^{-1} tetradecane (Sigma-Aldrich, Sydney Australia) as an internal standard for terpene analysis. A second sub-sample of leaves was stored in a sealed polythene bag and kept frozen on a bed of CO_2 pellets. Approximately 1 g of these leaves was dried at 50 °C for 72 h to determine water content. Another sample (ca 20 g) was freeze-dried and ground to pass a 1 mm screen in a Tecator Cyclotec mill for analysis of formylated phloroglucinol compounds (FPCs) and nitrogen. We retained the remaining portion of the sample at −20 °C pending further work.

Analyses

Terpenes Terpenes were separated using gas chromatography on an Agilent 6890 GC and detected with an Agilent 5973 Mass Spectrometer. The Alltech AT-35 column (35 % phenyl, 65 % dimethylpolyoxylane) (Alltech, Wilmington, DE, USA) was 60 m long with an internal diameter of 0.25 mm and a stationary phase film thickness of 0.25 µm. The carrier gas was helium, and we injected 1 µl of the ethanol extract at 250 °C at a 1:25 split ratio. The temperature program for the 25 min elution was 100 °C for 5 min, ramping to 200 °C at 20 °C·min^{-1}, followed by a ramp to 250 °C at 5 °C·min^{-1}, where it remained for 4 min. We identified peaks by comparing mass spectra to reference spectra in the National Institute of Standards and Technology library (Agilent Technologies, Deerfield, IL, USA) and verified major peaks with authentic standards. One sample from each individual was reanalyzed on the final day of chromatography to confirm that retention times remained stable. The area under each peak was measured manually with the help of MSD Chemstation Data Analysis (Agilent Technologies, Deerfield, IL, USA) and converted to a relative concentration by comparison with the internal standard.

Formylated phloroglucinol compounds Formylated phloroglucinol compounds (FPC) were extracted by sonicating 50 mg of freeze-dried, ground leaf with a known mass (ca 4.0 g) of solvent (7 % water in acetonitrile containing 0.1 % trifluoroacetic acid and 0.300 g·l^{-1} of the internal standard, 2-ethylphenol) for 5 min (Wallis and Foley, 2005). The resulting mixture was filtered (0.2 µm) directly into an auto sampler vial, and then we injected 20 µl onto a Wakosil 250×4 mm GL 3C18RS (SGE, Ringwood, Victoria) column maintained at 37 °C with a flow rate of 0.75 ml·min^{-1}on a Waters Alliance Model HPLC. We eluted the FPCs under gradient conditions with 0.1 % trifluoroacetic acid in water

(A) and 0.1 % trifluoroacetic acid in acetonitrile (B) as follows: 40 % A/60 % B for 5 min, linear gradient to 90 % B/10 % A at 60 min, held for 10 min and returned to starting conditions over 10 min. We measured the peak response at 275 nm and quantified the FPCs present using authentic standards purified in the laboratory (Eschler and Foley, 1999). The coefficient of variation between duplicate measurements was typically less than 4 %.

Total nitrogen We determined the total nitrogen concentration on duplicate samples (200±10 mg) of freeze-dried ground leaf using a Leco CNS-2000 combustion analyzer. Duplicates that differed by more than 2 % were repeated. We re-analyzed 8 random samples to ensure repeatability (Wallis et al., 2011).

Terminology – Ecotypes and Chemotypes In 1990 and 1993, Edwards et al. made comprehensive observations of herbivory, focusing on Christmas beetle (*Anoplognathus spp*) damage. From this we can define the S ecotype as leaves that suffer heavy herbivory, while leaves of the R ecotype suffer little or no herbivory. This can occur for two reasons. First, all ecotypes have the same suite of chemicals, but differences in concentrations convey resistance or susceptibility. Alternatively, the R and S ecotypes may contain different suites of chemicals. Either way, it is likely the ecotypes will have a different chemical profile or chemotype, defined as consistent and discontinuous differences in chemical composition that occur in the same species (Keszei et al., 2008; Kleine and Müller, 2011).

Statistical Analyses We used GenStat 12th Edition Lawes Agricultural Trust (Rothamsted Experimental Station) for all analyses. We first used principal component analysis (PCA) to identify and describe the chemical variation within and between trees, using the monoterpene and sesquiterpene data expressed as a proportion of each pool, respectively (Butcher et al., 1994; Ramanoelina et al., 1994; Trilles et al., 2006; Padovan et al., 2010). Due to the limited data from 2005, which precluded other analyses, we also used the PCA to examine whether samples from the two collections grouped together, suggesting chemical stability over time. We then used the linear mixed model with restricted maximum likelihood (REML) to measure variation in monoterpenes, cineole, sesquiterpenes, FPCs, and nitrogen between the two species, trees within species, branches within trees, and ecotypes. The fixed model was "ecotype" plus "branch within tree within species", and the random model "branch within tree". Finally, we compared the chemistry of the leaves from R and S branches on the mosaic trees with that of leaves from R and S neighboring trees after first restricting the data to either "R" or "S" ecotypes. In this case, the fixed model was "mosaic" and the random model again "branch within tree".

Results

Chemical Profile of Leaves of Two Ecotypes We categorized four groups of monoterpenes (Groups A-D) and three groups of sesquiterpenes (Groups E-G) (Table 1) based on the carbocation precursor described in the terpene synthase reaction mechanisms proposed by Keszei et al. (2008). The chemical profile of the leaves of the R ecotype contains a high proportion of group C monoterpenes, a high concentration of FPCs, and a low concentration of sesquiterpenes, while that of the S ecotype, contains mainly group A compounds, a high concentration of sesquiterpenes and a low concentration of FPCs (Fig. 2).

Principal component analysis, using proportional data, indicated that monoterpenes were the best predictor of ecotype because, for both *E. melliodora* and *E. sideroxylon*, there was a clear distinction between the R and S ecotypes on the x-axis (Figs. 3a, b). This axis explained 94 and 80 % of the variation for *E. melliodora* and *E. sideroxylon*, respectively. It depended on 1,8-cineole in the negative direction and both β-phellandrene and *p*-cymene in the positive direction. The species differed a little on the y-axis, both by the amount of variation explained (3 % for *E. melliodora* and 18 % for *E. sideroxylon*) and by the compounds explaining the variation. For *E. melliodora*, β-pinene in the negative direction and α-pinene in the positive direction explained the variation. In contrast, α-pinene in the negative direction and 1,8-cineole and β-phellandrene in the positive direction explained the variation in *E. sideroxylon*.

The PCA using sesquiterpenes did not separate the ecotypes even though the first principal component in *E. sideroxylon* explained 50 % of the variation. Instead, despite the same compounds influencing the variation in both species, these compounds (bicyclogermacrene, aromadendrene, spathulenol, and β-caryophyllene) tend to occur in the leaves of all trees and often are the most abundant sesquiterpenes. Thus, the resultant PCA (not shown) indicated a continuous rather than discrete distribution of sesquiterpenes.

The PCA identified the non-mosaic con-specifics growing near the mosaic trees as predominantly R-ecotypes. We identified no further S ecotypes among the eight unknown *E. melliodora* but two S ecotypes (S8 and S16) among the nine *E. sideroxylon*. Therefore, in the following comparisons of R and S ecotypes we had a mosaic tree from each species (M1 and S1) – that is, with both R and S ecotypes, 9 resistant and 1 susceptible *E. melliodora* and 8 resistant and 3 susceptible *E. sideroxylon*. This gave 12 samples for *E. melliodora* and 13 for *E. sideroxylon*.

Quantitative Variation (Table 2) Ecotype explained most of the variation in the concentrations of 1,8-cineole, sesquiterpenes and FPCs (all $P<0.001$), monoterpenes ($P=0.003$)

Table 1 Compounds in the four groups of monoterpenes (Groups A-D) and three groups of sesquiterpenes (Groups E-G)

Monoterpenes				Sesquiterpenes		
Group A	Group B	Group C	Group D	Group E	Group F	Group G
α-thujene	α-pinene	1,8-cineole	β-myrcene	elixene	β-caryophyllene	copaene
sabinene	camphene	limonene	trans-β-ocimene	isoledene	α-caryophyllene	β-cubebene
α-terpinene	β-pinene	cis-β-terpineol	β-linalool	β-guaiene	caryophyllene oxide	cubebene
α-phellandrene	fenchol	α-terpineol		β-elemene		β-cadinene
β-phellandrene	trans-pinocarveol	thymol		α-gurjuene		calamenene
p-cymene	borneol	*p*-cymen-7-ol		aromadendrene		trans-nerolidol
γ-terpinene	pinocarvone	α-terpinyl acetate		alloaromadendrene		
terpinolene	myrtenol			viridiflorene		
terpinen-4-ol	cryptone			viridiflorol		
	myrtenal			bicyclogermacrene		
	trans-3(10)-caren-2-ol			epiglobulol		
	verbenone			globulol		
				spathulenol		

a Compounds were grouped based on the carbocation precursors described in the terpene synthase reaction mechanisms proposed by Keszei et al. (2008)

and nitrogen (P=0.009), while "tree within species" explained significant variation for monoterpenes (P=0.042) and sesquiterpenes (P=0.019). "Branch within tree" did not contribute significant variation.

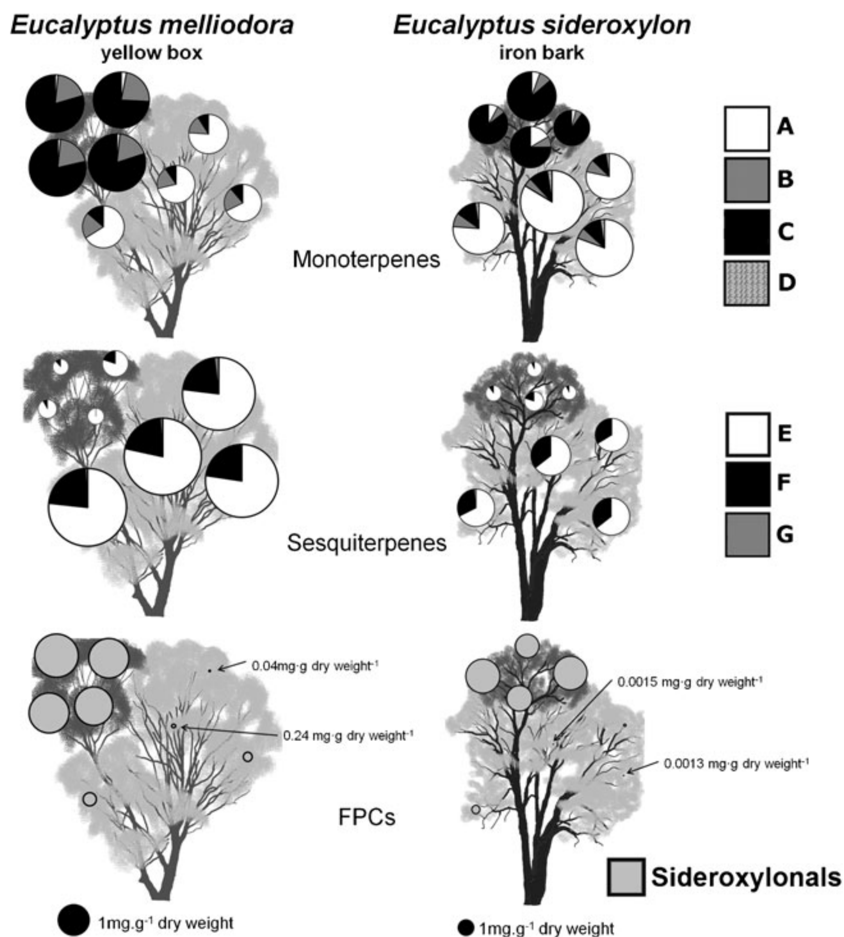

Fig. 2 A graphical representation of the chemical variation in monoterpenes, sesquiterpenes and formulated phloroglucinol compounds (FPCs) in the two mosaic trees (*Eucalyptus melliodora* on the left and *E. sideroxylon* on the right). The pie charts represent the relative proportion of specific groups of compounds and the size of the pie chart indicates total concentration in that sample. The pie charts for *E. melliodora* correspond to the larger scale on the left, whereas those for *E. sideroxylon* use the smaller scale on the right to clarify the pattern. Arrows indicate very low concentrations

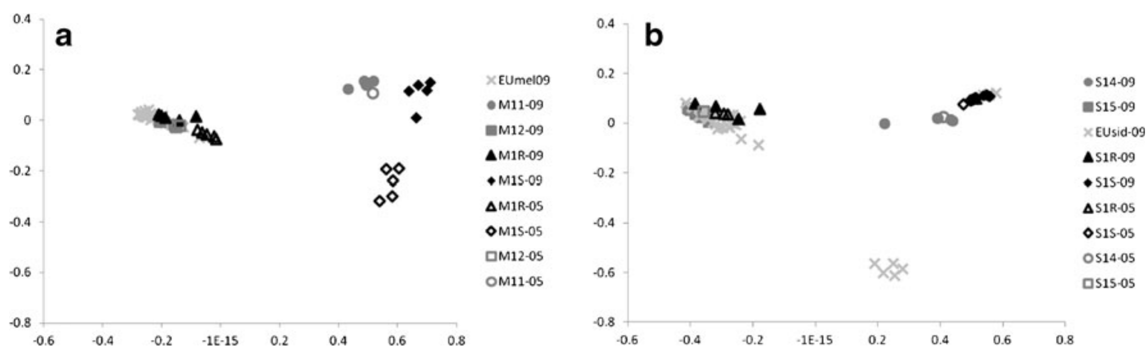

Fig. 3 Graphical representation of the principal component analysis of foliar terpenes. **a** *Eucalyptus melliodora* monoterpenes. **b** *E. sideroxylon* monoterpenes. Filled symbols represent data obtained from the 2009 collection (labelled -09), and open symbols represent data obtained from the 2005 collection (labelled -05). The resistant branches of the mosaic trees are labelled M1R and S1R (*triangle*) and the susceptible branches are labelled M1S and S1S (*diamond*). There are two non-mosaics from each species that we collected from in both years – the resistant M12 and S15 (*square*) and the susceptible M11 and S14 (*circle*). The trees of unknown chemotype from the larger 2009 collection are labelled EUmel09 and EUsid09 (*cross*)

Testing the Experimental Hypotheses

(i) What similarities and differences are there in the chemical profiles of the two eucalypt species (Table 2, Fig. 3)? The chemical profiles of the leaves of *E. melliodora* and *E. sideroxylon* are similar. We separated the same terpenes−29 monoterpenes and 39 sesquiterpenes, from the leaves of both species. A REML analysis indicated that the leaves of the two species had similar concentrations of total monoterpenes and of 1,8-cinole, the most abundant monoterpene in the resistant trees and of FPCs. The species did differ, however, in two ways both associated with susceptible trees. First, susceptible *E. sideroxylon* but not susceptible *E. melliodora* had very high concentrations of phellandrenes. Secondly, leaves of *E. sideroxylon* had higher concentrations of sesquiterpenes due to extremely high concentrations in the leaves of one tree – S8.

(ii) Do the mosaic trees retain their chemical profiles over a period of several years? The PCA results shown in Fig. 3, indicate that the leaves collected in 2005 group with those collected in 2009 for the resistant and susceptible branches of both *E. melliodora* and *E. sideroxylon*, suggesting that the mosaic ecotypes are stable over time. Similarly, leaves from the non-mosaic trees collected in both years (*E. melliodora* M11 and M12; *E. sideroxylon* S14 and S15) also group together indicating little change over time.

(iii) Do the chemical profiles of the leaves of R and S trees differ in all three groups of metabolites (Table 2)? Trees identified as resistant or susceptible differed in all three chemical profiles. Leaves of the resistant trees have higher concentrations of monoterpenes (P= 0.003) and FPCs (P<0.001) but lower concentrations of sesquiterpenes (P<0.001). Also, the leaves of susceptible trees contained more nitrogen, but this was caused by a species*ecotype interaction. There were higher concentrations of nitrogen in susceptible *E. melliodora* compared to resistant trees (S= 17.1 $mg{\cdot}g^{-1}$ DM; R=14.2 $mg{\cdot}g^{-1}$ DM) but little difference in *E. sideroxylon* (S=14.3 $mg{\cdot}g^{-1}$ DM; R=14.0 $mg{\cdot}g^{-1}$ DM).

(iv) Do the chemical profiles of leaves from non-mosaic individuals mirror the profiles of the leaves from the R and S branches of the mosaic trees (Table 2, Fig. 3)? We examined this in two ways – a REML analysis and visually, using a PCA with proportional monoterpene data. Because there was no difference between species for monoterpenes and FPCs, we removed the species identifier to give two mosaic trees in the analysis. We then compared the resistant and susceptible branches of the mosaic trees with the corresponding ecotypes of adjacent trees. The monoterpene (P=0.23), sesquiterpene (P=0.49), nitrogen (P=0.85) and FPC (P=0.08) concentrations of the leaves from resistant branches of the mosaic trees were similar to the concentrations in leaves from neighboring resistant trees. This is clear in the PCA (Fig. 3) that shows the species separately. The REML analysis also indicated that leaves from the susceptible branches on the mosaic trees and from neighboring susceptible trees contain similar concentrations of monoterpenes (P=0.45), sesquiterpenes (P= 0.45), nitrogen (P=0.93) and FPCs (P=0.34), but the PCA indicates far more variability among susceptible ecotypes. For *E. sideroxylon*, the PCA of monoterpenes indicate that the susceptible leaves from the mosaic tree group with the leaves from two other susceptible trees (S14 and S16). Lying well away and in a tight group are the leaves sampled from the five branches of another susceptible tree (S8). It is this tree, rather than the mosaic tree, that appears chemically different. Compared to other susceptible *E. sideroxylon*, the leaves of

Table 2 Concentration of metabolites (mg•g^{-1} DM; mean and standard deviation of three-five branches) in individuals of *Eucalyptus melliodora* and *E. sideroxylon* along with mean values and standard deviations for N individuals of a species

Tree	Ecotype[a]	Monoterpenes	Cineole	Sesquiterpenes	Formylated Phloroglucinol Compounds	Nitrogen
E. melliodora						
M1R[b] (*N*=5)	R	12.2 (1.18)	9.2 (0.6)	1.7 (1.3)	5.4 (0.3)	15.4 (0.6)
M1S[b] (*N*=5)	S	6.3 (1.55)	0.5 (0.1)	24.2 (4.6)	0.26 (0.3)	16.8 (0.9)
M2 (*N*=5)	R	23.4 (3.85)	19.2 (3.2)	0.9 (0.9)	11.8 (1.6)	13.0 (0.1)
M3 (*N*=5)	R	16.7 (1.61)	13.5 (1.6)	0.5 (0.5)	6.2 (0.9)	14.9 (0.3)
M4 (*N*=5)	R	19.6 (2.37)	16.6 (1.7)	2.3 (1.4)	19.7 (8.5)	14.6 (2.7)
M5 (*N*=5)	R	20.0 (3.77)	17.3 (3.4)	0.9 (0.3)	13.7 (0.4)	13.1 (0.4)
M6 (*N*=3)	R	27.2 (1.11)	21.0 (0.6)	2.1 (0.5)	17.8 (4.2)	14.2 (0.8)
M7 (*N*=3)	R	26.6 (8.63)	19.1 (4.3)	0.03 (0.04)	11.9 (0.8)	14.1 (1.3)
M8 (*N*=3)	R	22.8 (3.45)	19.1 (2.2)	0.7 (0.1)	14.7 (1.8)	13.8 (0)
M10 (*N*=5)	R	21.9 (7.64)	17.6 (5.1)	1.1 (0.7)	10.5 (1.6)	13.6 (0.6)
M11 (*N*=5)	S	12.5 (2.80)	2.4 (0.3)	3.5 (1.2)	0.44 (0.2)	17.4 (1.6)
M12 (*N*=5)	R	24.5 (10.34)	18.9 (8.0)	8.7 (3.6)	13.4 (2.9)	15.6 (1.2)
Mean (*N*=10)	R	21.5 (4.6)	17.1 (3.4)	1.9 (2.5)	12.5 (4.5)	14.2 (0.9)
(*N*=2)	S	9.4 (4.4)	1.5 (1.3)	13.9 (14.6)	0.4 (0.1)	17.1 (0.4)
E. sideroxylon						
S1R[b] (*N*=5)	R	25.7 (8.1)	17.9 (3.8)	7.9 (8.2)	9.8 (3.3)	13.1 (1.1)
S1S[b] (*N*=5)	S	44.8 (13.1)	2.0 (0.6)	21.6 (4.0)	0.2 (0.4)	14.0 (0.5)
S2 (*N*=5)	R	22.0 (3.1)	18.5 (2.6)	2.1 (0.8)	14.5 (4.2)	12.9 (0.7)
S8 (*N*=5)	S	3.64 (1.1)	0.7 (0.3)	67.6 (15.8)	0.7 (0.1)	15.2 (1.0)
S14 (*N*=5)	S	22.6 (4.0)	4.0 (1.8)	20.9 (4.5)	1.3 (0.9)	13.7 (1.0)
S15 (*N*=5)	R	27.8 (9.2)	23.4 (7.0)	2.5 (1.3)	15.8 (4.3)	14.1 (0.5)
S16 (*N*=5)	S	16.7 (4.4)	0.8 (0.3)	9.2 (2.6)	0.4 (0.3)	14.3 (0.4)
S17 (*N*=5)	R	21.4 (6.7)	17.5 (5.2)	2.1 (1.1)	10.9 (1.4)	14.8 (0.8)
S18 (*N*=5)	R	20.2 (4.9)	15.0 (2.4)	1.0 (0.5)	8.7 (1.0)	14.6 (0.6)
S19 (*N*=4)	R	25.1 (4.0)	19.1 (3.1)	3.0 (0.4)	11.7 (0.4)	14.3 (0.4)
S20 (*N*=3)	R	24.8 (8.5)	18.9 (5.7)	2.3 (0.9)	12.6 (4.7)	13.9 (1.6)
S21 (*N*=3)	R	19.3 (5.4)	15.9 (3.7)	1.6 (0.8)	14.8 (4.3)	14.1 (0.8)
S22 (*N*=3)	R	7.6 (1.2)	6.2 (0.5)	0.86 (0.4)	3.8 (0.2)	13.8 (0.7)
Mean (*N*=9)	R	21.5 (5.9)	16.9 (4.7)	2.6 (2.1)	11.4 (3.7)	14.0 (0.6)
(*N*=4)	S	21.6 (16.6)	1.9 (1.5)	29.8 (25.8)	0.7 (0.5)	14.3 (0.7)
P values						
Species		ns	ns	<0.001	ns	ns
Tree		0.042	ns	0.019	ns	ns
Branch		ns	ns	ns	ns	ns
Ecotype		0.003	<0.001	<0.001	<0.001	0.009

a An "R" denotes the leaves of the resistant ecotype and an "S" the leaves of the susceptible ecotype

b The mosaic branches are those from M1R and S1R (resistant ecotype) and M1S and S1S (susceptible ecotype). All other samples refer to an individual tree

We used a Restricted maximum likelihood (REML) to evaluate the differences in concentrations between the leaves of the resistant and susceptible ecotypes

this tree contain low concentrations of monoterpenes, with no detectable phellandrenes, extremely high concentrations of sesquiterpenes, and the highest nitrogen concentration. In *E. melliodora,* the PCA indicates that the monoterpene profile of the mosaic's susceptible leaves group with those of the other susceptible *E. melliodora* (M11). If we superimpose Fig. 3a and b, the resistant ecotypes from the two species form a single group but the susceptible ecotypes do not. Instead, the data from the susceptible ecotype of the mosaic *E.*

melliodora and from M11 form a separate group that lies between most of the susceptible *E. sideroxylon* and S8, the *E. sideroxylon* outlier mentioned previously.

Discussion

The key finding of this work is the identification of both qualitative and quantitative differences in the foliar metabolites of the resistant and susceptible branches of mosaic *Eucalyptus* trees that go far beyond the original findings of Edwards et al. (1990, 1993). Their thesis, for both *E. melliodora* and for *E. sideroxylon*, was that monoterpenes, particularly 1,8-cineole, explained the ecological difference they observed: leaves from the resistant ecotype had much higher concentrations of 1,8-cineole compared to the leaves of the susceptible individuals. In addition to this finding, we also show that there are other qualitative and quantitative differences in monoterpenes and sesquiterpenes and higher concentrations of FPCs. In other words, what appeared as a relatively simple chemical difference between ecotypes to Edwards et al. (1990, 1993) is in fact a consistent but much more complex difference. It answers our third hypothesis by showing that leaves from resistant and susceptible branches differ substantially in the three groups of chemicals we studied.

Perhaps most important, however, was demonstrating that the chemotypes of the leaves of the resistant and susceptible branches of the mosaic trees do not differ chemically from the corresponding chemotypes of adjacent non-mosaic con-specifics. This answered our fourth hypothesis. Therefore, there is nothing novel about the chemical profiles of the resistant and susceptible parts of mosaic trees – they both occur in the surrounding population. If there is an outstanding tree, chemically, then it is not one of the mosaics but instead one of the non-mosaic susceptible *E. sideroxylon* we identified during this study. The leaves of tree S8 differed chemically from all other susceptible foliage, particularly in its extremely high concentrations of sesquiterpenes and very low concentration of phellandrenes. This suggests that while there appears just one way to be resistant – high concentrations of monoterpenes and FPCs and low concentrations of sesquiterpenes, there is more than one way to be susceptible. Because monoterpenes, sesquiterpenes, and FPCs come from different but related metabolic pathways (Külheim et al., 2011), we argue that the most likely cause of the resistant branches is a mutation at a higher point – one that influences several pathways. If so, these mosaic trees provide a unique opportunity to investigate the genetic basis of chemical variation in eucalypts.

Our first hypothesis considered the chemical similarities of *E. melliodora* and *E. sideroxylon*, which although closely related, show substantial morphological differences. For example, *E. melliodora* lacks the heavy kino-impregnated bark of *E. sideroxylon*, has smaller flowers, and tends to flower in summer rather than winter. In contrast, the chemical profiles of the leaves of the two species were mostly similar and were indicative of the ecotype regardless of whether they came from a resistant or susceptible tree or a specific branch on a mosaic tree. This suggests that common factors select for chemical traits, and that Australia's unpredictable environment with sporadic outbreaks of herbivores, such as Christmas beetles, enable susceptible trees to persist. We cannot explain the main difference we observed between the species – a high concentration of phellandrenes in susceptible *E. sideroxylon* (except S8), but not in susceptible *E. melliodora*.

Our remaining hypothesis questioned whether the mosaic trees retain their chemical profiles over periods of years. This is important because much of the discussion about chemical changes in plants concerns induced defenses, whereby plants elicit defenses in response to attack by herbivores or pathogens (Degenhardt and Gershenzon, 2000; Arimura et al., 2004). Furthermore, there is plentiful information about chemical differences within plants due to ontogenetic effects, such as the common sight of juvenile and adult leaves of differing chemical composition on a single eucalypt (Moore et al., 2004). Our finding that we could not discriminate between the chemical profiles of leaves collected from the resistant and susceptible branches in 2005 and those collected from the same branches in 2009 indicates that the chemical profiles of these trees are constitutive. Likewise, we could not detect changes in the chemical profiles of leaves from neighboring trees collected at these times thus providing further evidence for trees having chemical signatures. This conservation combined with the significant variation in leaf chemistry due to "ecotype" and to "tree within species" suggests the influence of genetic rather than environmental factors. Both environmental and genetic factors contribute to variation in defensive chemicals, but most research has considered variation in resource availability most important (Bryant et al., 1983). This may be true over larger areas, such as landscapes, but all branches of a mosaic tree presumably experience a similar environment and have access to the same resources. Furthermore, the similarity of the leaf chemistry in nearby trees provides convincing evidence that environmental factors have only a small influence, and that genetic factors likely determine the patterns we describe for terpenes and FPCs in mosaic trees. The narrow-sense heritability of foliar terpenes and FPCs in a closely related species, *E. tricarpa*, is about 0.6, indicating a strong genetic component to this trait (Andrew et al., 2007). Furthermore, Andrew et al. (2010) showed that there was only a small genotype by environment effect influencing the concentration of FPCs in the leaves of *E. tricarpa*.

The fact that herbivores rarely attack the resistant branches of the mosaic trees and that the resistant trees themselves suffer little herbivory could be due to several factors. For instance, there is substantial evidence that nitrogen is a key determinant of insect herbivory in eucalypt forests (Morrow and Fox, 1980). Even the present study suggested nitrogen playing a role. While the total nitrogen concentrations did not differ between the leaves of the *E. sideroxylon* ecotypes (R=14.0 mg·g^{-1} DM; S=14.3 mg·g^{-1} DM), the leaves of the susceptible *E. melliodora* contained more nitrogen (17.0 mg·g^{-1} DM) than did the leaves of the resistant ecotype (14.2 mg·g^{-1} DM) (Table 2). Although deserving of further study, especially identifying more susceptible trees, we believe this difference in nitrogen is relatively unimportant compared to the changes in terpenes and FPCs. Further evidence comes from Matsuki et al. (2011), who showed that Christmas beetles, the organisms responsible for the defoliation of these susceptible eucalypts, ate less with increasing concentrations of sideroxylonals and 1,8-cineole. Although nutritional quality is almost certainly important, insects appear to make their initial selection of which leaves to eat based on variation in specialized metabolites. A similar conclusion comes from Gypsy moths, whose feeding and development depended more on the concentration of terpenes and phenols than it did on the nitrogen concentrations of Douglas Fir leaves (Joseph et al., 1993).

Our understanding of the chemical differences of resistant and susceptible leaves on mosaic and non-mosaic trees along with substantial knowledge of the terpene biosynthetic pathway enables us to propose hypotheses to explain the origins of the resistant branches on the mosaic trees. In lieu of RNA sequencing experiments and measurements of enzyme concentrations, we propose that the significant qualitative variation in the profile of foliar monoterpenes between the branches of the mosaic trees points to a factor regulating the terpene synthases. However, our results show that resistance or susceptibility depends on FPCs as well as terpenes. From what we know of terpene and FPC biosynthesis (Külheim et al., 2011), we propose that a simple somatic mutation invoked chemical changes involving several biosynthetic pathways. Support for this thesis comes from Henery et al. (2007), who showed that quantitative trait loci (QTLs) associated with monoterpenes, sesquiterpenes, and FPCs co-locate to the same location on the linkage groups. They claim that this could be a regulatory site governing the transcription of genes from both pathways. Thus, we expect a change in a single regulatory gene, most likely a transcriptional regulator that affects genes in several pathways possibly through this 'regulatory region'.

Can the Genetic Mosaic Hypothesis (GMH) explain these results? The GMH was proposed to explain intra-individual variation in susceptibility of clonal plants to pests (Whitham and Slobodchikoff, 1981) and extended to explain how long-lived host plants co-evolve with short-lived pests (Gill, 1986). Mosaic *Eucalyptus* support these ideas and provide an excellent model to study them partly because the biosynthesis of the compounds responsible for the two ecotypes is well-understood and the products have high heritability (Pichersky et al., 2006; Andrew et al., 2007). Eucalypts must defend themselves against various mammalian and insect herbivores, as well as fungi, bacteria and viruses (Edwards et al., 1993; Lawler et al., 1999; Keane et al., 2000; Moore et al., 2005; Crous et al., 2007; Matsuki et al., 2011). Compared to eucalypts, these are all relatively short-lived organisms yet there appears to be a co-evolutionary arms race between them. The mosaic eucalypts explain why this is possible. If a mutation occurs that allows part of the plant to escape predation pressures, then that part gains a selective advantage.

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Volatiles Emission Patterns in Poplar Clones Varying in Response to Ozone

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Abstract The volatiles emitted from young and old leaves of two poplar clones (*Populus deltoides x maximowiczii*, Eridano, and *P. x euramericana*, I-214) were sampled after exposure to ozone (80 ppb, 5 hd^{-1}, for 10 consecutive days) by solid phase microextraction and characterized by GC-MS. Only mature leaves of the ozone-sensitive Eridano clone developed necrosis in response to ozone exposure, and their membrane integrity was significantly affected by ozone (+86 and +18 % of levels of thiobarbituric acid reactive substances in mature and young leaves). The head-space of the poplar clones studied here contained mono- and sesquiterpenes, both hydrocarbons and oxygenated ones in Eridano, and only hydrocarbons in the clone I-214. Furthermore, some non-terpenes, such as C_9-C_{15} straight-chain aldehydes and C_{12}-C_{16} saturated and unsaturated aliphatic hydrocarbons, were detected. Other common non-terpene volatiles were oxygenated aliphatic compounds, mainly C_6-alcohols and their acetates. Ozone exposure induced a strong change in volatile profiles, depending on clones and leaf age. Regardless of leaf age, in clone I-214, quantities of oxygenated monoterpenes tended to increase after ozone exposure, however, "O_3 x leaf age" was not significant. In clone Eridano, increases were observed in emissions of hydrocarbons and oxygenated sesquiterpenes in response to ozone treatment. (*Z*)-3-Hexen-1-ol and (*Z*)-3-hexenol acetate were present in traces in the headspace of untreated Eridano mature leaves, but quantities slightly increased after ozone treatment. Quantities of non-terpene oxygenated compounds dropped in the headspace of young leaves of both clones (−24 and −44 % in Eridano and I-214) and also in mature ones of I-214 (−50 %) after ozone exposure. Similarly, quantities of non-terpene hydrocarbons in the emissions from mature leaves of both clones (−58 and −49 %, respectively) decreased, while these compounds increased in young leaves of Eridano (+83 %). We suggest that the resistance of the poplar clone I-214 to O_3 is achieved by: i) monoterpenes constitutively present in young leaves and ii) increase of monoterpene content induced by O_3 in mature leaves.

Keywords Green leaf volatiles · Oxidative stress · *Populus deltoides x maximowiczii* · *Populus x euramericana* · Terpenes · Volatile organic compounds

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Introduction

Plant volatile organic compounds (BVOCs) play key roles in large-scale atmospheric processes and serve the plants as important signals and defense molecules. Biotic and abiotic stresses can induce emissions of an array of organic compounds in any plant species (Niinemets, 2010). The main emphasis in quantitative BVOC studies has been on constitutive emissions of isoprene and specific monoterpene species that are present only in certain plant species. Vickers et al. (2009) proposed a 'single biochemical mechanism for multiple physiological stressors' model, whereby the protective effect by volatile isoprenoids against abiotic stress is exerted through direct or indirect improvement in resistance to damage by reactive oxygen species (ROS).

Trees of the genus *Populus* are important emitters of VOCs (Kesselmeier and Staudt, 1999). The history of research on the emission of volatile isoprenoids is closely linked to poplar, making this genus the best studied tree system for analysis of biosynthesis, regulation, and ecophysiological functions of volatile isoprenoids (Schnitzler et al., 2010). Studies of the direct effects of increasing ozone levels on the emission of this deciduous plant have been carried out previously (Calfapietra et al., 2009).

In the present study, we aimed to deepen the knowledge on the hybrid and clone specificity of poplar volatile emissions in response to ozone exposure. Furthermore, we investigated the impact of leaf age on volatile emissions of poplar that had been exposed to ozone. We analyzed volatile profiles emitted *in vivo* by the systems *Populus deltoides x maximowiczii*, Eridano clone (sensitive to O_3), and *Populus x euramericana*, I-214 clone (resistant) by means of solid phase microextraction (SPME) coupled with gas chromatography-mass spectrometry (GC-MS). These poplar clones are a suitable O_3-responsive model, well-characterized in terms of phenomenological response to ozone (i.e., visible injury) (Nali et al., 1998), photosynthetic activity (Guidi et al., 2001; Ranieri et al., 2001), and antioxidant response (Biagioni et al., 1997; Rizzo et al., 2007).

Emissions of mono- and sesquiterpenes from poplar are estimated to be much lower than those of isoprene; however, levels of mono- and sesquiterpenes show potential to increase under stress conditions (Schnitzler et al., 2010). As previously observed by Beauchamp et al. (2005), O_3 is a good 'model' agent of plant stress for several reasons: (i) exposure can be conducted under well-defined conditions; (ii) experiments may be easily repeated mimicking the same conditions; (iii) doses of O_3 can be varied over a wide range, allowing investigation of the plants' responses in relation to different degrees of stress. Furthermore, O_3 is used as elicitor for VOC emission because the plant's response to this pollutant is similar to a hypersensitive response (HR), and O_3 exposure has been suggested as a tool for testing programmed cell death (PCD) in plants (Rao et al., 2000).

Methods and Materials

Plant Material and Ozone Exposure Growth conditions of rooted cuttings of two poplar hybrid clones (*P. deltoides x maximowiczii*, Eridano, and *P. x euramericana*, I-214) were as described in Meroni et al. (2008). After the appearance and complete expansion of the 6th leaf, exposures to O_3 were performed according to Pellegrini et al. (2011), in the form of a square wave, 80 ppb from 9.00 to 14.00 (solar time) for 10 consecutive days. Analyses were performed at the end of O_3 fumigation on young expanding (5th and 6th leaf, YL) and fully mature (2nd and 3th leaf, ML) leaves.

Phenomenological and Biochemical Markers of Ozone Stress Mature foliar symptoms were evaluated manually at the end of the exposure period, on the basis of the percentage of necrotic area on the adaxial surface by overlaying a transparent plastic grid (4-mm) and counting the percentage of intersections covering injured areas with respect to healthy ones.

A TBARS (thiobarbituric acid reactive substances) assay was carried out according to Pellegrini et al. (2011). The test quantifies oxidative stress by measuring the peroxidative damage to membrane lipids that generates free radicals and results in the production of MDA (malondialdehyde, an indicator of lipid peroxidation).

Emission of Volatiles SPME sampling and GC and GC/EIMS analyses were performed as previously described by Flamini and Cioni (2010). Briefly, Supelco SPME devices coated with polydimethylsiloxane (PDMS, 100 μm) were used for sampling the headspace of intact leaves collected when rooted cuttings were about 30 cm tall. They were cut a few millimeters below the petiole, whose end portions were wrapped in aluminium foil to minimize water loss. They were inserted into a 50 ml glass septum vial and allowed to equilibrate for 30 min. Then, the fiber was exposed to the headspace for 50 min at room temperature. Once sampling was finished, the fiber was withdrawn into the needle and transferred to the injection port of a GC-FID or GC-MS system.

GC-EIMS separations were performed with a Varian CP 3800 gas chromatograph equipped with a DB-5 capillary column (30 m×0.25 mm; coating thickness=0.25 μm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions were as follows: injector and transfer line temperature at 250 and 240 °C, respectively; oven temperature was programmed from 60 to 240 °C at 3 °C min^{-1}; carrier gas, helium at 1 ml min^{-1}; splitless injection. Identification of the constituents was based on the comparison of the retention times with those of authentic samples, comparing their linear retention indices relative to the series of *n*-hydrocarbons, and on computer matching against commercial (NIST 98 and ADAMS) and home-made libraries of mass spectra built from pure substances and components of known essential oils and MS literature data (Adams, 2007).

Prior to volatile sampling, the saplings were transported to the laboratory. All analyses were performed at the same time of the day in order to avoid any possible influence of circadian rhythms and/or of external stimuli, such as daylight. Intact leaves (area: 8–10 cm^2) were collected when rooted cuttings were about 30 cm tall, cut a few millimeters below the petiole, and wrapped in aluminium foil to minimize water loss. Sampling conditions were under artificial fluorescent illumination and controlled environment temperature. Blank analyses of the glass vials were run before

starting each set of sampling. Results were expressed as mean percentages obtained by FID-peak area normalization.

Statistical Analysis The organization of the experiment is reported as follows: we had 8 types of samples (Eridano ML -O_3, Eridano ML + O_3, Eridano YL -O_3, and Eridano YL + O_3; I-214 ML -O_3, I-214 ML + O_3, I-214 YL -O_3, and I-214 YL + O_3) and a minimum of 3 replicates (plants) for each type. An experiment was repeated 3 times. Values are presented as means (±SE). Following performance of the *Shapiro-Wilk W test* (Zar, 1984), data were analyzed using two-way analysis of variance (ANOVA) and *LSD post tests*. If the interaction term is significant, this can involve an amplified effect (synergism) or a reduced effect (antagonism). The statistical test alone does not reveal this. Thus, plots of the means against each factor were used, according to Dunne (2010): there is interaction when all lines are non-parallel (slopes are statistically different); there is antagonism when lines cross; and there is synergism in the opposite case (Underwood, 1997). Where appropriate, the means were compared with paired *Student's t-test*. Percentages were normalized by arcsine transformation prior to perform the tests for data comparison. Finally, the dataset was subjected to multidimensional scaling (MDS), a multivariate analysis that treats the dissimilarity matrix as a distance matrix and provides a visual representation of the pattern of proximities (i.e., similarities or distances) among a set of objects. MDS plots the objects on a map where those similar to each other are placed near each other, and those that are perceived different from each other are placed far away (Chatfield and Collins, 1980). Cluster analysis, using squared Euclidean distance as a measurement of distance and the Ward algorithm, were applied to verify the results obtained by MDS. Analyses were performed with NCSS 2000 Statistical Analysis System Software.

Results

Markers of Ozone Stress At the end of the O_3 fumigation, mature leaves (ML) of the sensitive clone Eridano developed severe minute (Ø 1–2 mm) roundish dark-brown necrosis spots localized in the interveinal area of the adaxial surface. The injured area was about 27 % of the total leaf surface (range 23–31 %). Only slight marginal lesions were present on the young apical leaves (YL). No damage was observed in unfumigated controls of either clone or in treated plants of resistant material from clone I-214.

In Eridano, membrane integrity was significantly affected by O_3 (Table 1). According to the two-way ANOVA test, the main effects "O_3" and "leaf age" and their interaction effect were statistically significant. Interactions were more than additive (i.e., synergistic). In treated plants, an evident increase of peroxidation was observed both in ML and YL (+86 and +18 % of TBARS levels, respectively, in comparison with controls). In clone I-214, no significant differences were observed between the separate four groups (ML and YL, treated and untreated) after LSD tests, although an overall O_3 effect was found (F=7.31; $P\leq0.05$).

Emission of Volatiles Profiles of detached and living leaves showed similar patterns (Table S1, Supplemental data). Isoprene was not detected in our experimental conditions because the volatile fraction isolated from cut materials may not reflect the real composition of the emitted mixture *in vivo* (Zini et al., 2001); furthermore, the isoprene emission responses of poplar are known to be very low at a photosynthetic photon flux density of 530 μmol m^{-2} s^{-1} (in fumigation chamber) or under artificial fluorescent illumination (in the laboratory) and at leaf temperature of 20 °C (both in fumigation chamber and in the laboratory) (Sharkey and Singsaas, 1995; Tani et al., 2011).

Levels of constitutively released poplar volatiles are shown in Fig. 1 (clone Eridano) and Fig. 2 (clone I-214). In untreated plants (controls) of Eridano, 18 compounds were identified, and in I-214 we identified 17 compounds. The volatiles were mono- and sesquiterpenes, both hydrocarbons and oxygenated terpenes in Eridano, and hydrocarbons only in I-214; in addition, some non-terpene derivatives were detected, such as C_9-C_{15} straight-chain aldehydes and C_{12}-C_{16} saturated and unsaturated aliphatic hydrocarbons. Other common non-terpene volatiles were oxygenated aliphatic compounds, mainly C_6-alcohols and their acetates.

When considering constitutive levels of volatile emissions from both ML and YL of Eridano, traces of 4 compounds, such as (*Z*)-3-hexen-1-ol, 6-methyl-5-hepten-2-one, (*Z*)-3-hexenyl acetate, and (*E*-*E*)-α-farnesyl acetate were detected in addition to those shown in Fig. 1. Among terpene-derivatives, sesquiterpene hydrocarbons and oxygenated monoterpenes and sesquiterpenes were identified. However, oxygenated sesquiterpenes were represented by a single compound, (*E*-*E*)-α-farnesyl acetate. In comparison with ML, YL showed lower emission rates of non-terpene hydrocarbons and oxygenated sesquiterpenes content (−57 % in both classes). For example, emission rates of *n*-tetradecane were considerably lower in YL (−66 %) than ML. In contrast, emission rates of oxygenated monoterpenes were higher in YL (+57 %) than ML, in particular because of the higher percentage of (*E*)-geranyl-acetone (+60 %).

As in clone Eridano, in both untreated ML and YL of clone I-214, traces of (*Z*)-3-hexen-1-ol, 6-methyl-5-hepten-2-one, and (*Z*)-3-hexenyl acetate were detected. In addition, *neo*-menthol and pentadecanal were found. In the headspace of untreated YL of clone I-214, traces of *n*-dodecane, *n*-tridecane, *n*-tetradecane, and *n*-pentadecane also were present, as well as β-caryophyllene in the case of in ML (Fig. 2).

Table 1 TBARS (thiobarbituric acid reactive substances, nmol g^{-1} FW) determination in mature (ML) and young (YL) leaves of *Populus deltoides* x *maximowczii*, clone Eridano, and *Populus* x *euroamericana*, clone I-214, exposed to ozone (80 ppb for 10 consecutive days, 5 hd^{-1}) (+O_3) or to ozone—free air (−O_3)

Clone	ML		YL		Source of variation		
	−O_3	+O_3	−O_3	+O_3	Ozone	Leaf age	Ozone x Leaf age
Eridano	1.34±0.169 a	2.59±0.179 c	2.20±0.239 b	2.54±0.255 c	54.19***	13.90**	17.48**
I-214	1.85±0.185	1.58±0.314	2.37±0.462	1.61±0.500	7.31*	2.02^{ns}	1.61^{ns}

Values are shown as means ± SE. The F value for each source of variation is given. For each type of sample, N=9. In each row, different letters indicate significant differences for: *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$; *ns* $P > 0.05$

As reported for Eridano, in ML of I-214, non-terpene hydrocarbons (5 compounds), non-terpene oxygenated volatiles (8 compounds), terpene-derivatives, oxygenated monoterpenes, and sesquiterpene hydrocarbons also were identified. In comparison with ML, untreated YL of clone I-214 showed lower emission rates of non-terpene hydrocarbons (−96 %); all compounds of this class were detected in traces only in untreated YL of this clone. However, emission rates of oxygenated monoterpenes (+41 %), sesquiterpene hydrocarbons (+44 %), and non-terpene oxygenated compounds (+67 %) were higher in untreated YL of I-214 than in ML. Noteworthy, β-caryophyllene content, present at trace levels

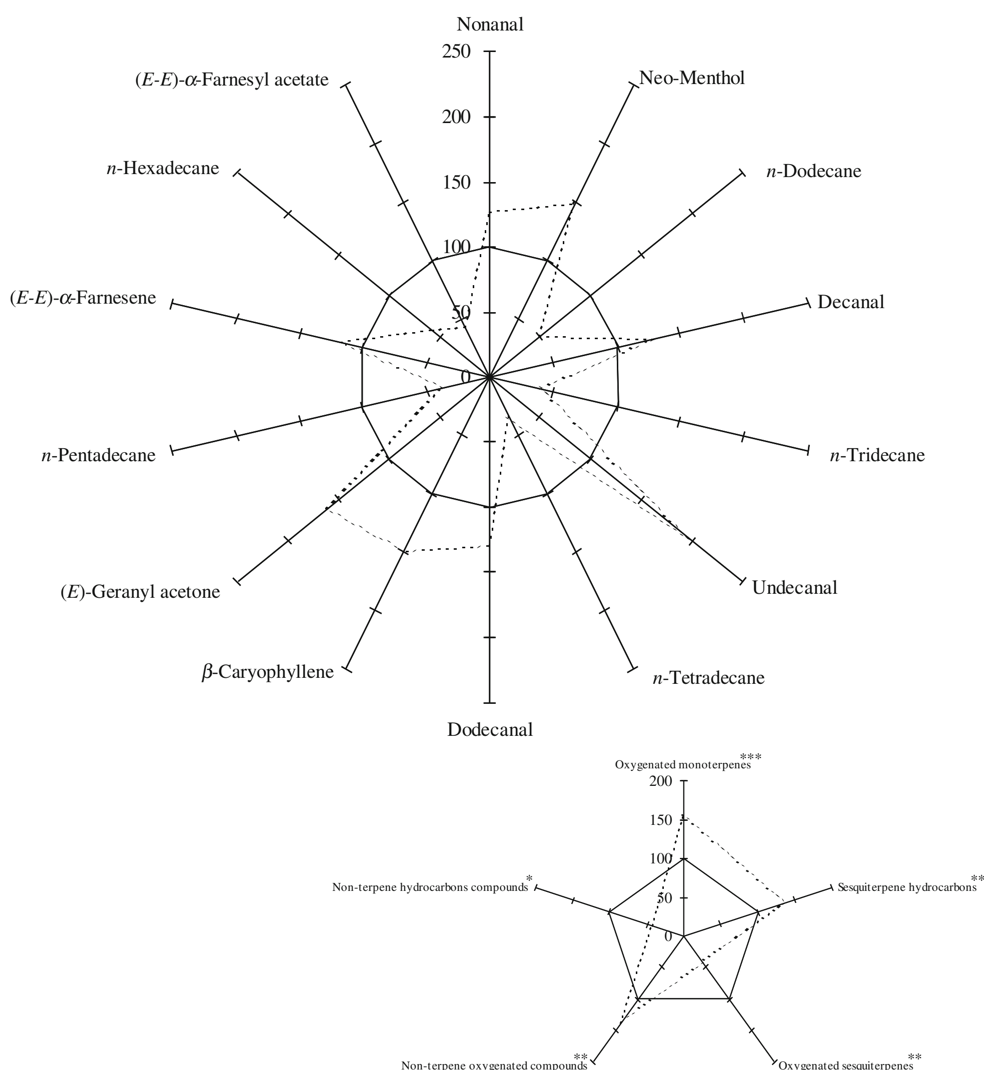

Fig. 1 Polar graphs for the visual comparison of constitutive levels of volatile emission patterns (referred to single compounds: 14-rays profile; referred to classes of compounds: 5-rays profile) in mature and young leaves of *Populus deltoides* x *maximowczii*, clone Eridano (ozone sensitive clone). Data are expressed in relative terms: the *solid line* refers to mature leaves in comparison to dotted profile, which represents the young leaves. For each type of sample, N=9. *Asterisks* related to classes of compounds show that the differences between mature and young leaves are significant for: *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$. Absolute values (%) of volatile compounds emitted by mature leaves are: nonanal, 4.9; neo-menthol, 3.8; *n*-dodecane, 6.4; decanal, 9.6; *n*-tridecane, 6.3; undecanal, 3.7; *n*-tetradecane, 7.3; dodecanal, 4.8; β-caryophyllene, 7.1; (*E*)-geranyl acetone, 10.1; *n*-pentadecane, 5.5; (*E-E*)-α-farnesene, 5.1; *n*-hexadecane, 5.5; (*E-E*)-α-farnesyl-acetate, 5.4

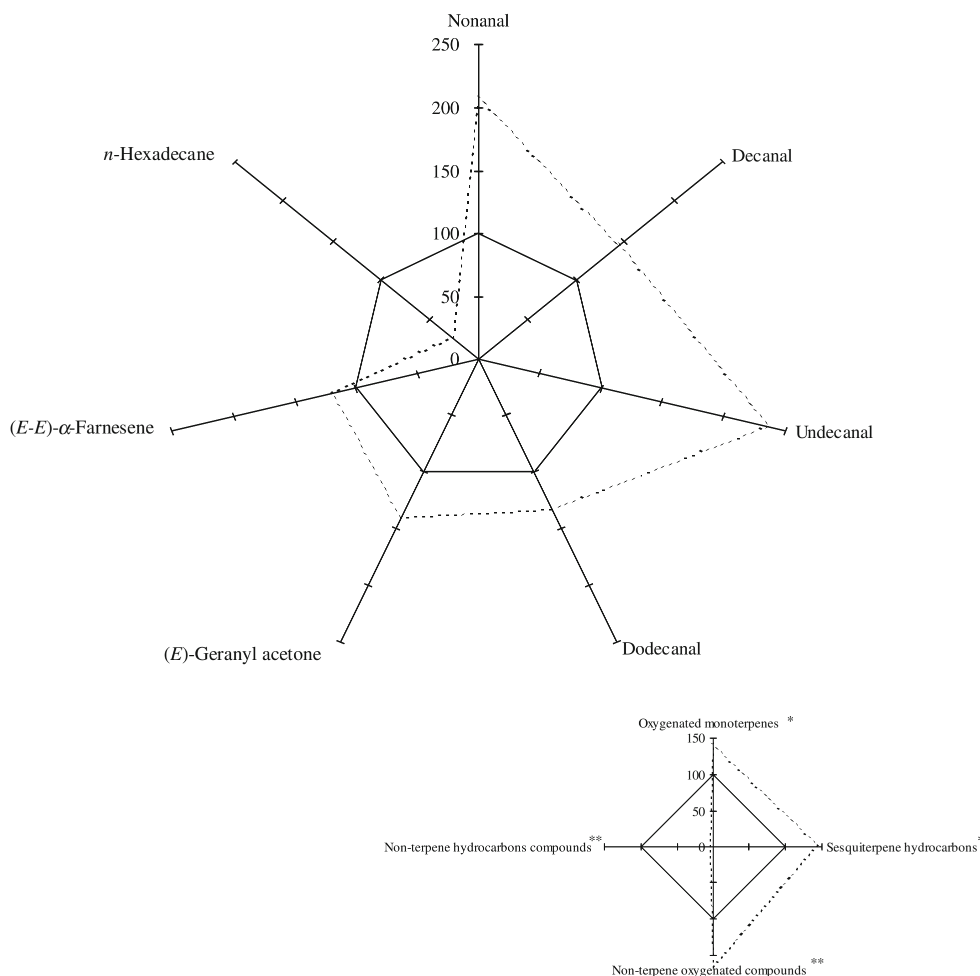

Fig. 2 Polar graphs for the visual comparison of constitutive levels of volatile emission patterns (referred to single compounds: 7-rays profile; referred to classes of compounds: 4-rays profile) in mature and young leaves of *Populus* x *euroamericana*, clone I-214 (ozone resistant clone). Data are expressed in relative terms: the *solid line* refers to mature leaves in comparison to dotted profile, which represents the young leaves. For each type of sample, $N=9$. *Asterisks* related to classes of compounds show that the differences between mature and young leaves are significant for: ** $P \leq 0.01$; * $P \leq 0.05$. Absolute values (%) of volatile compounds emitted by mature leaves are: nonanal, 6.8; decanal, 17.4; undecanal, 2.7; dodecanal, 2.4; (*E*)-geranyl acetone, 10.7; (*E-E*)-α-farnesene, 19.9; *n*-hexadecane, 4.9

in ML, reached 4.9 % in YL. However, in the case of non-terpene hydrocarbons, during the senescence of leaves in I-214, not simply an increase of the various constituent levels in ML was noted, but significant changes of composition were detected.

Results of the analyses of ozone-induced volatile emissions are shown in Table 2. In clone Eridano, the interaction between "O_3" and "leaf age" was significant for all chemical classes (i.e., oxygenated and hydrocarbons sesquiterpenes and non-terpene compounds), with the exception of oxygenated monoterpenes. In this case, simple effects of both factors were significant. Interactions were synergistic both for oxygenated sesquiterpenes and non-terpene oxygenated compounds, and antagonistic for both sesquiterpenes and non-terpenes hydrocarbons. In response to O_3 exposure, both ML and YL leaves of Eridano showed a trend of increasing emission of oxygenated sesquiterpenes (about 3- and 2-fold in ML and YL, respectively) and hydrocarbons sesquiterpenes (about 2- and 1-fold). A rise also was observed in the non-terpene hydrocarbons content of YL (about 2-fold). Concerning single compounds (Table 3), in ML the main ozone-induced increases were detected for (*E-E*)-α-farnesene and (*E-E*)-α-farnesyl-acetate (3-fold in both cases). Furthermore, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenol acetate which were present at trace levels in ML controls, reached higher emission rates in fumigated leaves. The emission of non-terpene oxygenated compounds and hydrocarbons decreased after ozone exposure in both Eridano YL (−24 %) and ML (−58 %), respectively. In the former class, quantities of undecanal decreased most after ozone exposure. In the latter class, *n*-tetradecane levels decreased most in response to ozone exposure.

In clone I-214, the interaction between "O_3" and "leaf age" was significant in all classes (i.e., oxygenated monoterpenes, oxygenated non-terpene compounds, and hydrocarbons), with the exception of sesquiterpenes hydrocarbons. In this case, simple effects of both factors were significant. Interactions were antagonistic for the variable oxygenated monoterpenes and synergistic in both non-terpene oxygenated compounds and hydrocarbons. O_3 exposure caused an increase of

Table 2 Classes of compounds (% of total peak area) emitted by mature (ML) and young (YL) leaves of *Populus deltoides* x *maximowczii*, clone Eridano, and *Populus* x *euroamericana*, clone I-214, exposed to ozone (80 ppb for 10 consecutive days, 5 hd^{-1}) (+O_3) or to ozone—free air (−O_3). Comparison of percentages is valid only for between-sample comparisons

Classes of compounds	ML		YL		Source of variation		
	−O_3	+O_3	−O_3	+O_3	O_3	Leaf age	Leaf age x O_3
Eridano							
Oxygenated monoterpenes	13.9±0.14	18.5±0.57	21.7±0.14	25.7±0.50	241.66***	758.75***	1.40ns
Sesquiterpene hydrocarbons	12.2±0.07 a	25.9±0.78 d	16.6±0.42 b	19.7±0.21 c	672.01***	7.34ns	271.67***
Oxygenated sesquiterpenes	5.4±0.35 b	17.0±1.13 c	2.3±0.21 a	5.1±0.33 b	279.33***	303.01***	104.78***
Oxygenated non-terpenes	23.0±0.85 a	24.3±0.56 a	32.1±0.49 b	24.4±0.28 a	59.08**	122.67***	122.67***
Non-terpene hydrocarbons	31.0±1.17 c	12.9±1.13 a	13.2±2.19 a	24.1±0.71 b	10.68*	8.18*	174.42***
I-214							
Oxygenated monoterpenes	10.7±0.07 a	29.0±0.07 d	15.1±0.78 b	22.5±0.42 c	1668.08***	10.57*	296.16***
Sesquiterpene hydrocarbons	19.9±0.28	39.4±0.78	28.7±1.56	46.0±0.24	681.97***	120.08***	2.45ns
Oxygenated non-terpenes	29.2±0.57 b	14.7±1.20 a	48.8±1.34 c	27.4±0.07 b	719.01***	578.25***	25.49**
Non-terpene hydrocarbons	33.4±1.84 c	17.0±3.96 b	1.3±0.28 a	1.5±0.05 a	27.93**	234.21***	28.96***

Legend: oxygenated monoterpenes = neo-menthol + (*E*)-geranyl acetone; sesquiterpene hydrocarbons = β-caryophyllene + (*E-E*) α-farnesene; oxygenated sesquiterpenes = (*E-E*) farnesyl-acetate; non-terpene oxygenated compounds = nonanal + decanal + undecanal + dodecanal + pentadecanal + (*Z*)-3 hexen-1-ol + (*Z*)-3-hexenol acetate + 6-Methyl-5-hepten-2-one; non-terpene hydrocarbons compounds = *n*- dodecane + *n*-tridecane + *n*- tetradecane + *n*- pentadecane + *n*- hexadecane. Values are shown as means ± SE. The F value for each source of variation is given. For each type of sample, $N=9$. In each row, different letters indicate significant differences for: *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$; *ns* $P > 0.05$

oxygenated monoterpenes (about 3- and 1.5-fold in ML and YL, respectively) related to *(E)*-geranyl acetone. Quantities of (*E-E*)-α-farnesene in ML were twice as high in ozone treated leaves as in controls. In response to ozone treatment, the emission rates of non-terpene oxygenated compounds and hydrocarbons dropped in ML and YL (−50 and −44 %, respectively) in the former, and only in ML (−49 %) in the latter. Among non-terpene oxygenated compounds, quantities of

Table 3 Percentage of increase/reduction of emission rates of volatile compounds released from mature (ML) and young (YL) leaves of *Populus deltoides* x *maximowczii*, clone Eridano, and *Populus* x *euroamericana*, clone I-214 after exposure to ozone (80 ppb for 10 consecutive days, 5 hd^{-1}) in comparison to ozone—free air

Constituents	l.r.i.	Eridano		I-214	
		ML	YL	ML	YL
(*Z*)-3- Hexen-1-ol	314	*4.5*	Tr	tr	tr
6-Methyl-5-hepten-2-one	465	tr	Tr	tr	tr
(*Z*)-3-Hexenyl acetate	517	*9.2*	Tr	tr	tr
Nonanal	652	−59.2	−17.7	−48.5	−39.0
Neo-Menthol	933	+13.2	+26.8	tr	tr
n-Dodecane	1000	−56.3	+67.7	−64.6	tr
Decanal	1085	−47.9	−10.5	−56.9	−48.2
n-Tridecane	1147	−54.0	+72.0	−44.8	tr
Undecanal	1189	−54.1	−56.2	−14.8	−40.6
n-Tetradecane	1200	−68.5	+108.0	−61.1	tr
Dodecanal	1220	−58.3	−19.4	−37.5	−37.5
β-Caryophyllene	1249	+25.4	+9.4	tr	+57.1
(*E*)-Geranyl acetone	1371	+40.6	+14.8	+171.0	+49.0
n-Pentadecane	1500	−60.0	+160.0	0.0	tr
(*E-E*)-α-Farnesene	1599	+233.3	+33.3	+96.5	+60.3
n-Hexadecane	1619	−50.9	+38.7	−46.9	+15.4
Pentadecanal	1925	tr	Tr	tr	tr
(*E-E*)-α-Farnesyl acetate	1959	+214.8	+121.7	–	–

Legend: *l.r.i.* linear retention indices (DB-5 column); *tr* detected content is <0.1 %. Data reported in italics are referred to absolute value (%) (when the compound is present in controls at trace level); -=not detected

decanal decreased most after ozone exposure. Among hydrocarbons, quantities of *n*-dodecane were most reduced in response to ozone treatment (Table 3).

O_3 treatment induced a strong change in volatile profiles, depending on clones and leaf age, as highlighted by MDS (Fig. 3). The fraction of total variance explained by these two dimensions was 74.8 %. In the whole data population, the degree of homogeneity observed within the two clones strongly differed, with the I-214 data being more homogeneous than those of Eridano, as suggested by the lower scattering of data. The distribution of points over the space defined by dimensions 1 and 2 identified 4 groups: (i) I-214 group, having in common the absence of oxygenated sesquiterpenes, with 2 subgroups: control ML (with lower levels of terpenes) and control and treated YL and treated ML, dominated by a higher content of terpenes; (ii) Eridano ML control; (iii) Eridano YL control; (iv) ML and YL treated Eridano, characterized by equal quantities of several classes of compounds. These results have been confirmed by cluster analysis (*data not shown*).

Discussion

We found that both Eridano and I-214 poplar clones constitutively emitted considerable amounts of monoterpenes and sesquiterpenes from leaves that did not experience any kind of stress. Since it is known that monoterpenes are by far

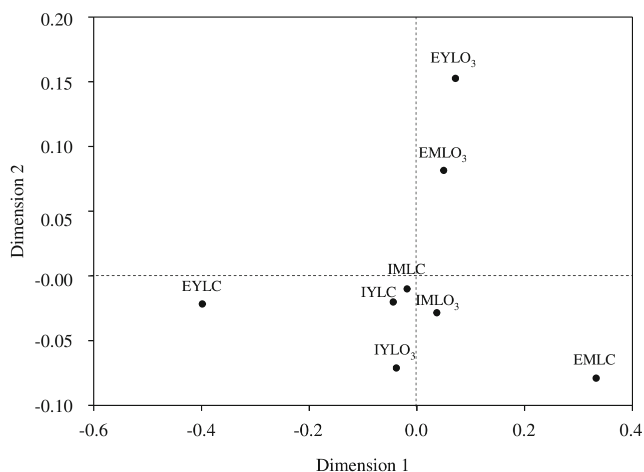

Fig. 3 Multidimensional scaling of volatiles emitted by mature and young leaves of *Populus deltoides* x *maximowczii*, clone Eridano, and *Populus* x *euroamericana*, clone I-214, exposed to ozone (80 ppb for 10 consecutive days, 5 hd^{-1}). Controls are maintained in filtered air. For each type of sample, *N*=9. Legend: EMLC = untreated mature leaves of Eridano; EYLC = untreated young leaves of Eridano; $EMLO_3$ = treated mature leaves of Eridano; $EYLO_3$ = treated young leaves of Eridano; IMLC = untreated mature leaves of I-214; IYLC = untreated young leaves of I-214; $IMLO_3$ = treated mature leaves of I-214; $IYLO_3$ = treated young leaves of I-214

more reactive than isoprene (Atkinson, 1997) and may more efficiently scavenge O_3 (Fares et al., 2008), their strong antioxidant activity (Loreto et al., 2004) might contribute to protecting leaves from oxidative stress.

Monoterpenes are well-known to be emitted from plants in response to stressful conditions and to act as defensive compounds against biotic (Röse et al., 1996) and abiotic stresses (Loreto and Schnitzler, 2010). In agreement with other studies (Hartikainen et al., 2009), we have found that the emission of monoterpenes was induced by O_3 in ML that were emitting low amounts of the same monoterpenes before being fumigated. Our results are in agreement with the findings by Llusià et al. (2002) who reported increased concentrations of individual monoterpenes emitted from *Ceratonia siliqua*, *Olea europaea,* and *Quercus ilex rotundifolia* fumigated with O_3. Heiden et al. (1999) also compared the release of monoterpenes from young pine trees exposed to elevated O_3 concentration and observed a 3-fold increase of these compounds in treated plants.

Like isoprene and monoterpenes, many volatile plant sesquiterpenes rapidly react with ROS, and their emission is stimulated by abiotic stresses (Vickers et al., 2009). Sesquiterpenes released from stressed foliage appear to be much more reactive in the atmosphere than isoprene and monoterpenes, and they are involved in secondary aerosol formation (Joutsensaari et al., 2005; Van Reken et al., 2006). Both poplar clones studied here emitted sesquiterpenes after O_3 exposure, but the ozone-induced increase was less pronounced in the resistant I-214 clone. These results are in agreement with those obtained by Heiden et al. (1999) in Bel-W3 (O_3-sensitive) and Bel-B (O_3-resistant) tobacco varieties exposed to O_3. In contrast, Blande et al. (2007) reported that sesquiterpenes comprise a greater percentage of the emissions of tolerant than sensitive aspen clones under elevated O_3 levels. Referring to single compounds, a great amount of *(E-E)*-α-farnesene was released from both aspen clones in response to O_3 exposure, regardless of leaf age. This volatile is induced in several plants by herbivore-feeding damage (Turlings, 1994; Röse et al., 1996). Among the most common sesquiterpenes, emission of β-caryophyllene has been observed in many plant taxa, and overall in the *Salicaceae* (Duhl et al., 2007). It either can be emitted constitutively or be induced by abiotic or biotic environmental factors. In agreement with the study of ozone-treated pine by Heiden et al. (1999), the emission rate of this compound increased in the poplar clones studied here after fumigation, particularly in YL of I-214. Several reports have indicated that sesquiterpene emissions from poplar leaves are induced by several stresses and may actively influence communication with insects (Blande et al., 2007; Frost et al., 2007). Stresses may elicit general responses, such as production of ROS, which are important signalling molecules and serve to initiate defense responses (Apel and Hirt, 2004).

Green Leaf Volatiles (GLVs), which owe their name to the distinctive scent produced by crushed or injured leaves, are C_6 aldehydes and alcohols and their derivatives, often collectively called LOX-products (Loreto and Schnitzler, 2010). Plants start to form GLVs after disruption of their tissues and after suffering biotic or abiotic stress (Matsui, 2006). Physiologically, these compounds have antibiotic properties that inhibit the invasion of damaged tissues (Croft et al., 1993) and have signaling functions within plants to induce or prime defense (Frost et al., 2007). Recent findings have shown that abiotic stresses, such as O_3 and high temperatures, affect these emissions, indicating membrane degradation (Hartikainen et al., 2009). In our study, a marked difference was found in the response of the two poplar clones: a GLV emission was observed only in Eridano, when symptoms appeared in treated ML, suggesting that concentration and duration of exposure to O_3 were sufficient to cause breakdown of cell membranes (as evidenced by increased TBARS content) and implying a relation between GLVs emission and leaf injury. Heiden et al. (1999) reported an increase of GLV emissions in ozone-treated leaves of an O_3-sensitive (cv. Bel-W3) tobacco plant at the end of an acute treatment. In contrast, in the tolerant cv. Bel B that did not show visible damage in response to ozone treatment, no emission of C_6 aldehydes and alcohols was observed. Vuorinen et al. (2004) showed a relationship between visible effects of ozone treatment and emission of (*Z*)-3-hexenyl acetate and homoterpenes (but not monoterpenes) in lima bean plants exposed to O_3. Beauchamp et al. (2005) demonstrated that exposure of tobacco to high O_3 concentrations caused emissions of C_6 volatiles in older leaves of Bel-W3 showing necrotic spots. Peñuelas et al. (1999) reported that high O_3 concentrations stimulated emission of LOX products in tomato plants showing leaf injury. Heiden et al. (2003) demonstrated that corn plants exposed to acute O_3 treatment have different emission patterns of C_6 compounds in comparison to controls. Thus, there is a positive relationship between the development of symptoms and the emission of LOX products as part of the signal cascade that results in programmed cell death (PCD) triggered by O_3, in agreement with Pinto et al. (2010).

In addition to the oxygenated volatiles produced by LOX activity, we detected C_9-C_{15} compounds in the headspace of ML and YL of both poplar clones studied. The emissions of these volatiles (except undecanal) decreased significantly in response to O_3 in all leaves of the resistant clone I-214, and in the YL of the sensitive Eridano clone. Similar results were obtained by Vuorinen et al. (2005) with two clones of silver birch exposed to CO_2 and O_3, singly or in combination.

This study shows that in the poplar clones Eridano and I-214 a realistic O_3 exposure stimulates emission of volatiles as a function of clone and leaf age. Several results suggest that the differences in profiles of the volatile emissions are linked with different sensitivity to the pollutant: *i)* monoterpenes were abundant in the headspace of YL of both clones and increased following O_3 exposure, especially in resistant material; *ii)* only small amounts of sesquiterpenes were emitted constitutively, but their levels increased after ozone treatment; *iii)* GLV emissions were observed only in ML of Eridano, which showed leaf injury and breakdown of membranes after ozone treatment, initiating a PCD pathway (similar to HR during plant-pathogen interactions) that is associated with O_3-sensitivity. For these reasons, we hypothesize that the resistance to O_3 of the poplar clone I-214 is achieved by: i) monoterpenes constitutively present in YL and ii) increase of monoterpene emissions from ML induced by O_3. Future studies need to test this hypothesis using a range of poplar cultivars/clones differing in O_3 sensitivity.

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BOA Detoxification of Four Summer Weeds during Germination and Seedling Growth

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Abstract A recent greenhouse study revealed a significant reduction of germination and growth of redroot pigweed (*Amaranthus retroflexus*) and common purslane (*Portulaca oleracea*) by rye mulch, whereas velvetleaf (*Abutilon theophrasti*) and common lambsquarters (*Chenopodium album*) were not suppressed. Since BOA detoxification by metabolic alteration may influence the relation between the benzoxazinoid content of the soil mulch and weed suppression, we tested the dynamics in BOA detoxification in different plant organs of three and 10-day-old seedlings of four warm season weeds incubated with five BOA concentrations (4, 20, 40, 80, and 200 μmol g^{-1} fresh weight). In addition, germination and length of 3-day-old seedlings were measured after exposure to 0, 0.3, 1.5, 3, 6, and 15 μmol BOA. Finally, we tested the influence of the MDR translocator inhibitors verapamil, nifedipine, and the GST inhibitor ethycrynic acid on BOA accumulation and detoxification activity. Due to BOA-detoxification, all weeds were able to grow in environments with low BOA contents. At higher contents, *Abutilon theophrasti* and *Chenopodium album* had a better chance to survive because of highly active mechanisms that avoided the uptake of BOA (*A. theophrasti*) and of efficient detoxification activities in youngest seedlings (*C. album*). The interpretation of all of the data gave the following sequence of increasing sensitivity: *A. theophrasti* <<< *C. album* << *P. oleracea* ≤ *A. retroflexus*. The results were in agreement with recent findings of the suppression of these weeds by rye mulches and their benzoxazinoid contents. Our studies demonstrate for the first time that the detoxification of BOA influences the survival of certain weeds in environments enriched with this allelochemical. Therefore, detoxification processes affect the potential for weed suppression by soil allelochemicals in sustainable weed management.

Keywords Allelopathy · Benzoxazolinones · Bioherbicides · BOA detoxification · Summer weeds

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Introduction

Benzoxazinone-β-D-glucosides are characteristic secondary compounds of several Poaceae and some dicotyledoneous species (Frey et al., 1997, 2009; Sicker et al., 2000; Gierl and Frey, 2001; Sicker and Schulz, 2002). The synthesis, investigated in maize, starts with the conversion of indole-3-glycerol phosphate to indole. Subsequently, four cytochrome P450 dependent monooxygenases (*BX2-BX5*) convert indole to benzoxazinone by incorporation of oxygen. DIBOA-glucoside is synthesized by glucosylation of DIBOA (2,4-dihydroxy-1,4(2*H*)-benzoxazin-3-one) at the 2-position. The resulting glucoside is the precursor of DIMBOA-glucoside (Glawischnig et al., 1999; von Rad et al., 2001; Jonczyk et al., 2008; Schullehner et al., 2008). The compounds are stored in the vacuole until the tissue is damaged and hydrolysis of the sugar moiety by β-glucosidases takes place. The highly bioactive aglycones can be released into the soil also by root exudation or by plant residue degradation (Barnes and Putnam, 1987).

After deglucosylation, the aglucons undergo a ring contraction yielding benzoxazolinones. For this reason, benzoxazinones have a short lifetime whereas benzoxazolinones persist in the soil for several weeks. These compounds are thought to present one of the toxic principles of rye mulch, although the amount of benzoxazinoids varies highly with plant age, organ, and cultivar. Rye cultivars differ in the total benzoxazinoid amounts from 250 to 1800 μg g^{-1} dry tissue in young plants to about 100 or less μg g^{-1} in old plants (Burgos et al., 1999; Reberg-Horton et al., 2005; Rice et al., 2005; Zasada et al., 2007). Nitrogen fertilization significantly influences the benzoxazinoid content (Gavazzi et al., 2010).

Benzoxazolinones have been proposed for the use as bioherbicides for weed control in sustainable agricultural systems. Therefore, rye mulches have been tested for weed control in several studies (Putnam and DeFrank, 1983; Shilling et al., 1985; Tabaglio and Gavazzi, 2006). Cultivars used by Tabaglio et al. (2008) as mulches differed in the benzoxazinoid contents from 177 to 545 μg g^{-1} (dry weight).

The phytotoxic properties of benzoxazinoids have been investigated mainly with crops such as *Brassica rapa, Lactuca sativa, Raphanus sativus, Allium cepa, Solanum lycopersicon, Lepidium sativum, Phaseolus vulgaris*, and *Ph. aureus* (Macias et al., 2005; Batish et al., 2006; Sanchez-Moreiras and Reigosa, 2005; Singh et al., 2005 and many others), whereas weeds have been less-considered in studies (Belz and Hurle, 2004; Macias et al., 2006; Hussain et al., 2008; Hussain and Reigosa, 2011). According to Macias et al. (2005, 2006), seedlings of dicotyledonous crops such as tomato and the monocotyledonous weeds *Lolium rigidum* and *Avena fatua* showed no difference in their sensitivity to 5.5 μmol BOA (benzoxazolin-2(3*H*)-one. The growth of all species was inhibited 60 to75% .

Plants react to benzoxalinone (BOA) differently, dependent on species and dosage. These reactions can be dramatic as shown for *Arabidopsis thaliana*. In seedlings exposed to a sub-lethal dose of the compound, more than 150 genes were induced or up-regulated, 22 % of them seemingly components of a complex, coordinately regulated network of defense responses (Baerson et al., 2005). Quite a number of the up-regulated genes encoded for proteins belonging to the functional group of detoxifying enzymes.

In a recent study, we found a significant reduction of redroot pigweed (*Amaranthus retroflexus* L.) and common purslane (*Portulaca oleracea* L.), whereas common lambsquarters (*Chenopodium album* L.) and velvetleaf (*Abutilon theophrasti* Medicus) were moderately or not suppressed, respectively (Gavazzi et al., 2010). One possibility to explain the different reactions of the four weeds could be differences in the detoxification activities or in other, currently not considered strategies to cope with rye allelochemicals. Metabolic reduction of BOA content might be correlated with a lower susceptibility of a plant species to BOA. At present, there is no study available that demonstrates that in agricultural systems detoxification of allelochemicals can influence the efficiency of compounds for weed suppression.

BOA uptake into cells is an active, energy dependent process. Almost all investigated higher plant species detoxify absorbed BOA via 6-hydroxylation and subsequent O-glucosylation. The *P. oleracea* variety "Gelber", a garden cultivar of *P. oleracea*, is one known species that produces also BOA-5-O-glucoside (Hofmann et al., 2006). Monocots and some dicots synthesize, mainly or at least to a considerable portion, glucoside carbamate. In contrast to BOA-6-O-glucoside, glucoside carbamate is not toxic up to concentrations of 1 mM. The compound is also resistant to common β-glucosidases. Thus, detoxification via glucoside carbamate production represents the better detoxification pathway (Wieland et al., 1998; Schulz and Wieland, 1999; Sicker et al., 2000, 2001, 2004; Sicker and Schulz, 2002; Schulz et al., 2006). Glucoside carbamate can be modified subsequently by malonylation or by the addition of a second glucose molecule yielding gentiobioside carbamate (Hofmann et al., 2006). These modifications enhance the mobility of the compounds in an aqueous environment such as the cytosol. BOA-6-O-glucoside is the major detoxification product when maize or other seedlings are incubated with 6-methoxybenzoxybenzoxazolin-2(*3H*)-one (MBOA). Methoxyglucoside carbamate has been found hitherto only as a minor compound in maize when the incubation was extended to more than 48 hr. Known detoxification products are illustrated in Fig. 1. The BOA-detoxification process realized in maize roots starts with the production of BOA-6-O-glucoside. However, after 3–6 hr, glucoside carbamate accumulation is initiated. About 10 hr after the incubation starts, this compound becomes the major detoxification product, whereas BOA-6-O-glucoside accumulation stops. The accumulation of gentiobioside carbamate and malonylglucoside carbamate occurs 18–20 hr after start of the incubation. Thus, BOA detoxification is a highly dynamic process.

As detoxification of BOA is widespread among plants, it is particularly important to elucidate the detoxification capacities of problematic weeds such as *A. theophrasti*, *A. retroflexus, C. album,* and *P. oleracea*. BOA detoxification in *C. album* roots and shoots was formerly investigated with seeds from plants grown in organically managed wheat fields in Germany (Schulz and Wieland, 1999). For elucidation of the different reactions to rye mulch, we tested the warm season weeds *A. theophrasti*, *A. retroflexus, C. album,* and *P. oleracea* for their dynamics in BOA detoxification dependent on plant organ and BOA concentration, and also their germination and growth of seedlings in the presence of different BOA concentrations. From these studies of weeds

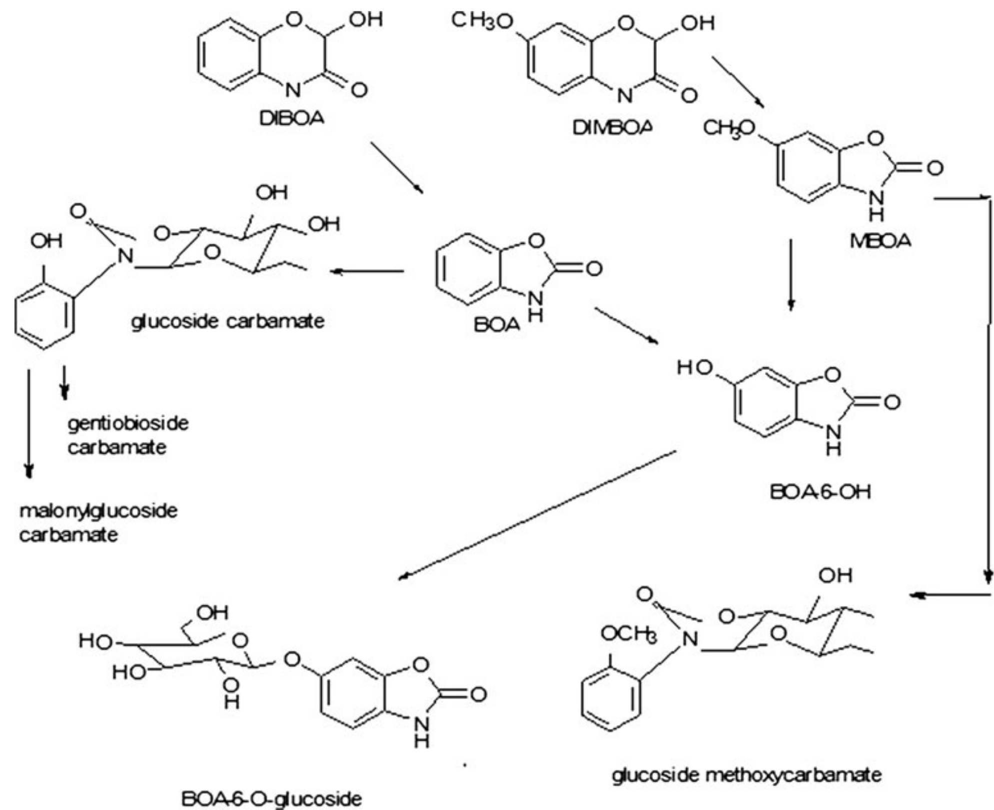

Fig. 1 Stable BOA/MBOA detoxification products found in higher plants (see text for explanation)

in environments enriched with this allelochemical, a relevant agronomic tool may be provided for integrated weed control in sustainable and organic agriculture.

Methods and Materials

Plant Material, BOA Incubation, and Determination of BOA Detoxification Products

Seeds of *Abutilon theophrasti* Medicus (velvetleaf), *Amaranthus retroflexus* L. (redroot pigweed), and *Chenopodium album* L. (common lambsquarters) were purchased from Herbiseed (Twyford, UK). *Portulaca oleracea* L. (common purslane) seeds were collected from fields in Piacenza, Italy. Seeds were aeroponically grown on cheesecloth at 30°C under greenhouse conditions (Schulz and Wieland, 1999). After 10 d, the seedlings were harvested and incubated for 24 hr with (0.1 mM) 4, (0.5 mM) 20, (1 mM) 40, (2 mM) 80, (5 mM) 200 μmol BOA g^{-1} fresh weight (FW), as described in Schulz and Wieland (1999). Benzoxazolinone was purchased from Fluka (Germany). After incubations, seedlings were washed x 3 with water, carefully dried, and separated into roots and shoots. Plant material was extracted immediately with 70 % methanol (3 ml g^{-1} FW) by mortaring with sea sand. Homogenates were centrifuged for 15 min at 14,000 g, and supernatants were used for HPLC analysis. HPLC was performed as described in Schulz and Wieland (1999) and Gavazzi et al. (2010). All experiments were repeated independently at least three times and by different investigators. Reference substances were: synthetic BOA-6-OH and glucoside carbamate; BOA-6-O-glucoside isolated from BOA incubated oat (Wieland et al., 1998, 1999); malonylglucoside carbamate and gentiobioside carbamate isolated from BOA incubated maize (Sicker et al., 2001; Hofmann et al., 2006).

In addition, we selected the 3 μmol application to test whether detoxification activity is different in 3-d-old seedlings compared to 10-d-old seedlings. Experiments were performed as described above, but whole seedlings were extracted with 70 % methanol (3 ml g^{-1} FW), and extracts analyzed by HPLC.

Moreover, the effect of three inhibitors on BOA uptake and accumulation of detoxification products was tested on the four weeds. Ten-day-old seedlings were incubated as described above with (0.5 mM) 20 μmol, and (2 mM) 80 μmol g^{-1} FW BOA for 24 hr supplemented with of 4 μmol g^{-1} FW verapamil (MDR/P6P inhibitor, Ca^{2+} channel blocker), 20 μmol g^{-1} FW ethacrynic acid (glutathione transferase inhibitor) and 20 μmol g^{-1} FW nifedipine (Ca^{2+} channel blocker). Extraction of whole seedlings and analyses were done as described. All inhibitors were purchased from Sigma (Germany).

Germination and Seedling Length

Germination rates in control conditions were 55, 45, 6, and 59 % for *A. theophrasti*, *A. retroflexus*, *C. album,* and *P.*

oleracea, respectively. The low germination percentage of *C. album* could be due to different seed geno- or morphotypes (Yao et al., 2010). Seeds of *A. theophrasti* (0.6 g=50 seeds, expected to germinate: 28 seeds), *A. retroflexus* (0.046 g=150 seeds, expected to germinate: 67 seeds), *C. album* (0.1 g=345 seeds, expected to germinate: 21 seeds), and *P. oleracea* (0.05 g=454 seeds, expected to germinate: 268 seeds) were placed on filter paper in Petri dishes (8 cm) and kept in the dark at 30°C. Prior to the study, the BOA concentrations used for the germination tests and seedling growth experiments had been evaluated for each species to cover the BOA concentrations where the species exhibits no inhibition or pronounced inhibition. The concentration range where seedling growth of the different species could be compared was between 0.3 and 15 μmol/Petri dish. Concentrations were calculated in nmol $seed^{-1}$ and μmol g^{-1} seed. Table 1 gives an overview of the concentrations used for the seeds of the different species. The BOA solutions with lower concentrations than 15 μmol/3 ml were prepared by dilutions of the 5 mM BOA stock solution.

Petri dishes were filled with 3 ml of water containing 1 ml ethanol/100 ml (controls) or with 3 ml (0.3, 1.5, 3, 6, 15 μmol) of the appropriate BOA solution and incubated at 30°C in the dark. After 24 hr, 1 ml water was added to compensate water uptake of the seeds. After an additional 48 hr, the seedlings, now 3-d-old, were counted, and the seedling length was measured. Germination in the presence of BOA was compared with controls. Determinations of germination percentage and seedling length were repeated at least three times.

Statistical Data Analysis

Analysis of variance was performed for statistical analysis of all data. The Tukey's test ($P \leq 0.05$) was used for mean separation. In tables, data are presented as mean±standard deviation. Regression analysis was performed between the concentrations of BOA and detoxification products in roots and shoots, and BOA concentration in solution. The statistical significance of each regression equation was calculated by PASW Software Package ver. 18.

Results

BOA Accumulation and Detoxification in 10-Day-old Seedlings

The phenotype of 10-d-old seedlings treated with BOA concentrations up to 80 μmol was not affected in comparison to controls. With 200 μmol BOA, numerous seedlings of all species exhibited wilting at the leaf tops. All four warm season species were able to detoxify BOA via BOA-6-O-glucoside and the glucoside carbamate pathway. Detoxification exhibited a species, dose, and plant organ dependency. With 4 μmol BOA g^{-1} FW, no BOA accumulated in shoots and roots of *C. album* and *A. retroflexus,* and traces of BOA-6-O-glucoside were found as the only detoxification product. *Portulaca oleracea* contained traces of BOA in shoots and roots, BOA-O-glucoside in the roots, and glucoside carbamate in the shoots. In *A. theophrasti*, BOA was not detectable in shoots and roots. Its shoots contained traces of an unknown detoxification product, which was not present when higher BOA concentrations were used for the incubation. Traces of detoxification products and BOA after exposure to 4 μmol BOA g^{-1} FW were not considered in the following presentations of BOA uptake, translocation, and characteristic patterns of detoxification products found in shoots and roots with higher BOA concentrations. The results obtained with 4 μmol BOA demonstrated that none of the species was considerably affected at very low BOA concentrations within 24 hr.

BOA Uptake in Roots

BOA concentrations in roots of the four summer weeds are shown in Table 2 and Fig. 2. Accumulation of BOA in roots increased with increasing BOA concentrations in solution for all species. BOA increased drastically in roots of

Table 1 BOA concentrations used for seed treatment of four weed species. *SW* Seed weight (mg)

BOA solution (mM) μmol per Petri dish	*Abutilon theophrasti* SW: 9–11 mg nmol per seed/μmol per g seeds	*Amaranthus retroflexus* SW: 0.3 mg nmol per seed/μmol per g seeds	*Chenopodium album* SW: 0.3 mg nmol per seed/μmol per g seeds	*Portulaca oleracea* SW: 0.1 mg nmol per seed/μmol per g seeds
(0.1 mM) 0.3	11 / 1	4 / 1.3	14 / 4.7	1 / 1
(0.5 mM) 1.5	53 / 5.3	22 / 7.3	70 / 23	6 / 6
(1 mM) 3	110 / 11	45 / 15	140 / 47	10 / 10
(2 mM) 6	200 / 20	90 / 30	280 / 93	20 / 20
(5 mM) 15	530 / 53	220 / 73	700 / 233	60 / 60

Table 2 BOA concentration and total amount of detoxification products in roots for the four weeds at four BOA concentration levels in incubation medium (mean±standard deviation; *AT Abutilon theophrasti*; *AR Amaranthus retroflexus*; *CA Chenopodium album*; *PO Portulaca oleracea*). F signif. shows the ANOVA results between weeds within the same BOA concentration level (*n.s.* not significant)

BOA concentration (μmol g^{-1} FW)	AT	AR	CA	PO	F signif.
	BOA in roots (nmol g^{-1} FW)				
20	396±95	335±77	303±41	411±153	0.01
40	555±140	752±145	702±298	891±392	n.s.
80	1166±348	1279±269	692±154	2632±497	0.001
200	2110±726	2651±420	5980±359	6109±665	0.001
	Total amount of detoxification products in roots (nmol g^{-1} FW)				
20	181±55	346±55	497±146	1184±195	0.001
40	258±96	512 ±118	1036±195	1860±451	0.001
80	142±81	293±88	143±36	1487±259	0.001
200	4±5	226±140	81±32	504±135	0.001

P. oleracea, C. album, and *A. retroflexus*, while in *A. theophrasti* accumulation was lower. The correlation between BOA concentration in solution and in root tissues was significant for all the species (Fig. 2). Regression coefficients were ≥0.92 for *A. retroflexus*, *C. album,* and *P. oleracea*, and decreased to 0.77 for *A. theophrasti*. Two patterns of uptake were evident: *A. theophrasti* and *A. retroflexus* were characterized by a low slope ranging from 9.6 to 12.5 nmol BOA g^{-1} FW / μmol BOA g^{-1} FW; *C. album,* and *P. oleracea* showed higher uptake with slope values approaching 32 nmol BOA g^{-1} FW / μmol BOA g^{-1} FW.

BOA Detoxification at Root Level

In *P. oleracea* roots, BOA-6-O-glucoside was the major detoxification product (Fig. 3). Glucoside carbamate was partly converted to malonylglucoside carbamate. Traces of gentiobioside carbamate also were detected after exposure to 40 and 80 μmol BOA g^{-1} FW. *Chenopodium album* extracts contained BOA-6-O-glucoside and glucoside carbamate as the two major soluble detoxification products and traces of malonylglucoside carbamate, which was not found in a former study with seeds from a field collection (Schulz and Wieland, 1999). These detoxification patterns were observed with 20 and 40 μmol BOA g^{-1} FW. Treatments with higher BOA concentrations resulted in a decrease of product accumulation. This switch took place at an accumulation more than 750 nmol free BOA in *C. album* and *A. retroflexus* roots, and more than 900 nmol in roots of *P. oleracea*. *Amaranthus retroflexus* produced BOA-6-O-glucoside and glucoside carbamate in amounts comparable to *C. album*, but already after exposure to 40 μmol BOA g^{-1} FW, the carbamate production was reduced.

BOA Translocation to Shoots

The concentrations of BOA in shoots of the four weeds are shown in Table 3. As reported for roots, the BOA concentrations increased with the BOA concentration used for the incubations in all weeds. Translocation into *A. theophrasti* shoots was low compared to the other weeds: differences increased with the BOA concentration in the solution,

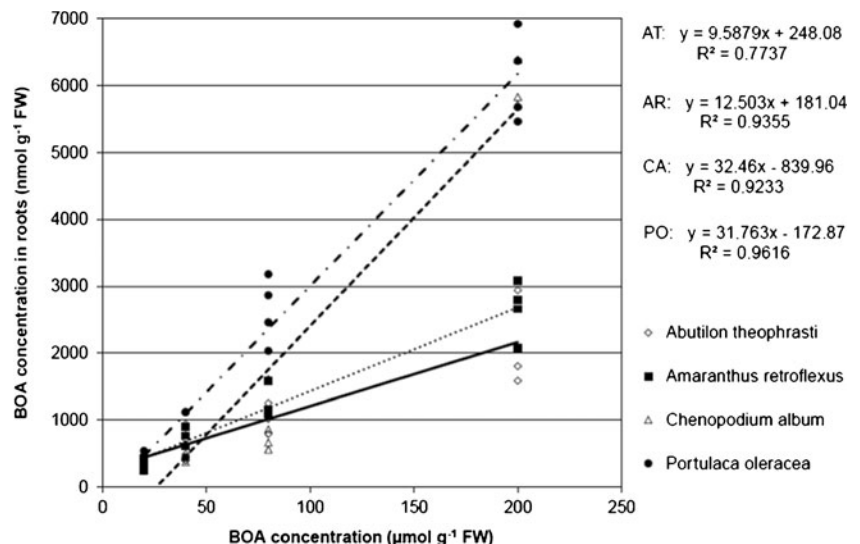

Fig. 2 Regression equations between BOA concentration in solution and free BOA concentration in roots of four weed seedlings. *AT Abutilon theophrasti*; *AR Amaranthus retroflexus*; *CA Chenopodium album*; *PO Portulaca oleracea*

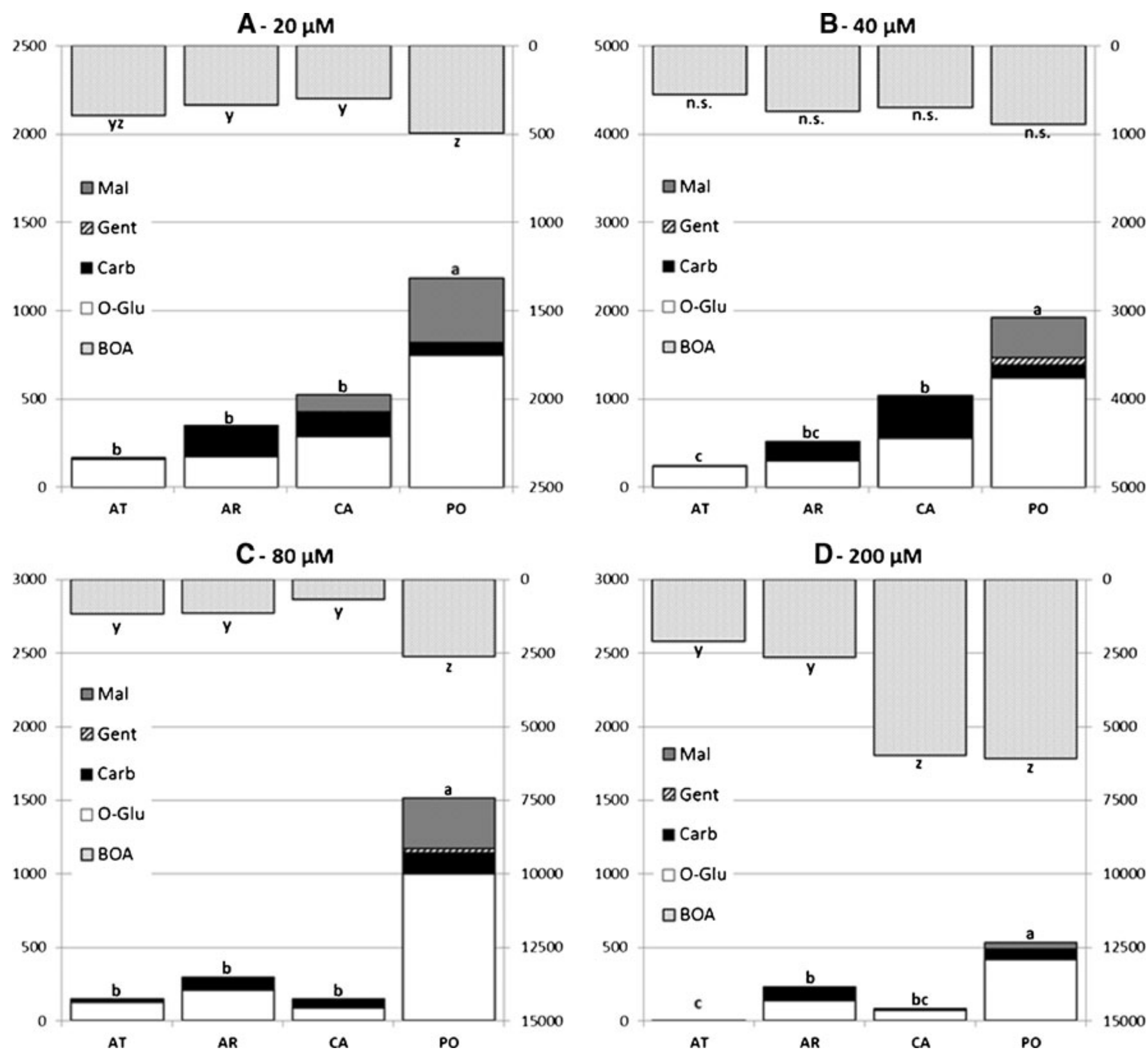

Fig. 3 Accumulation of detoxification products (below) and free BOA (above) in roots of four summer weeds exposed to 20 (**a**), 40 (**b**), 80 (**c**), and 200 (**d**) μmol BOA g^{-1} FW. All the values are expressed as nmol g^{-1} FW. Legend for the left Y axis: O-Glu, BOA-6-O-glucoside: □; Carb, Glucoside carbamate: ■; Gent, Gentiobioside carbamate: ▨; Mal, Malonylglucoside carbamate: ▩. Legend for the right Y axis: BOA, benzoxazolin-2(3*H*)-one: ⬚. *AT Abutilon theophrasti*; *AR Amaranthus retroflexus*; *CA Chenopodium album*; *PO Portulaca oleracea.* Letters on the bars indicate significant differences between means of total amount of detoxification products (**a**, **b**, **c**) and content of free BOA (**y**, **z**) at $P \leq 0.05$, according to Tukey's test

ranging from half to fifth (Table 3, Fig. 4). This could be one explanation of the allelopathic inefficacy in controlling this weed. *Chenopodium album* started to translocate BOA most effectively, resulting in an about 2 to 3 fold accumulation of the compound in the shoots compared to the roots when incubated with 20, 40, and 80 μmol BOA g^{-1} FW. Translocation broke down when seedlings were incubated with 200 μmol BOA. However, the amounts of free BOA were correlated with detoxification activity in the tissues.

Correlations between BOA concentration in solution and in shoot tissues are shown in Fig. 4. Regression coefficients were high as in roots for *A. retroflexus*, *C. album*, and *P. oleracea*, with the exception of the lower R^2 for *A. theophrasti* (0.54). The best function for *C. album* was represented by a logarithmic equation with R^2=0.83. In regard to BOA increase, *A. retroflexus* behaved differently in shoots with increments of about 31 nmol BOA g^{-1} FW / μmol BOA g^{-1} FW, compared to 12.5 found in roots.

BOA Detoxification at Shoot Level

At the 20 μmol g^{-1} FW level, *C. album* shoots exhibited the greatest portion of detoxification with glucoside carbamate as the major product and similar amounts of gentiobioside carbamate and BOA-6-O-glucoside, followed by *P. oleracea* and *A. retroflexus*, with BOA-6-O-glucoside and glucoside carbamate as the main products (Fig. 5). In *C. album*, the two glucosides decreased with BOA concentration ≥80 μmol. Gentiobioside carbamate was no longer detectable. *Portulaca oleracea* started to accumulate BOA-6-O-

Table 3 BOA concentration and total amount of detoxification products in shoots for four weeds at four BOA concentration levels in germinating solution (mean±standard deviation; *AT Abutilon theophrasti*; *AR Amaranthus retroflexus*; *CA Chenopodium album*; *PO Portulaca oleracea*). F signif. shows the ANOVA results between weeds within the same BOA concentration level (*n.s.* not significant)

BOA concentration (µmol g^{-1} FW)	AT	AR	CA	PO	F signif.
	BOA in shoots (nmol g^{-1} FW)				
20	190±111	322±69	460±194	596±178	n.s.
40	381±161	885±475	1481±483	901±194	0.01
80	566±392	2055±185	2131±138	2327±184	0.001
200	1105±585	5803±621	2655±660	5758±367	0.001
	Total amount of detoxification products in shoots (nmol g^{-1} FW)				
20	14±18	697±73	1053±262	443±147	0.001
40	24±47	1097±43	1495±108	442±97	0.001
80	28±23	1898±86	1044±204	1681±242	0.001
200	59±41	1466±42	671±175	1923±538	0.001

glucoside in addition to glucoside carbamate, when incubated with 80 µmol g^{-1} FW. In *A. retroflexus*, glucoside carbamate and BOA-6-O-glucoside accumulated in similar amounts. These latter detoxification products increased with increasing BOA concentration up to 80 µmol g^{-1} FW. Thus, the species *A. retroflexus, C. album*, and *P. oleracea* also performed BOA detoxification in the shoots. Significant break down of the detoxification processes at higher BOA doses as found in the roots of these species was not observed with shoots of *A. retroflexus* and *C. album*, whereas in *P. oleracea* the concentration of detoxification products progressively increased with BOA concentration. Shoots of *A. theophrasti* accumulated almost no detoxification products, which was in agreement with the low BOA uptake and translocation. This species again showed a different behavior.

Ratio of Detoxification in Roots and Shoots

The quotient [nmol of all detoxification products / nmol of absorbed free BOA] was used to measure species sensitivity towards BOA accumulation. Values lower than 1 indicate a low detoxification activity or high BOA uptake. Accumulation of BOA detoxification products in roots and shoots of *C. album* was higher than accumulation of free BOA up to incubations with 40 µmol BOA, which indicated a high detoxification activity (Table 4).

This ratio was higher in *P. oleracea* roots and *A. retroflexus* shoots up to incubations with 40 µmol BOA. However, in roots of *A. retroflexus* and shoots of *P. oleracea,* the accumulation of free BOA was higher than that of the detoxification products with all concentrations of BOA used, with the exception of 20 µmol BOA. Thus, regarding the whole plant, *C. album* was less sensitive up to 40 µmol g^{-1} FW compared with *A. retroflexus* or *P. oleracea*. Shoots of *A. theophrasti* were less sensitive to BOA, since the absorption into the cells was low up to the 80 µmol BOA (Table 3). In summary, *A. theophrasti* 10-d-old seedlings were not affected by BOA incubations with 20, 40, and 80 µmol g^{-1} FW, *C. album* was protected by a high detoxification activity in shoots and roots up to 40 µmol g^{-1} FW. *Amaranthus retroflexus* and *P. oleracea* had more susceptible roots and shoots, respectively. A dose of 200 µmol

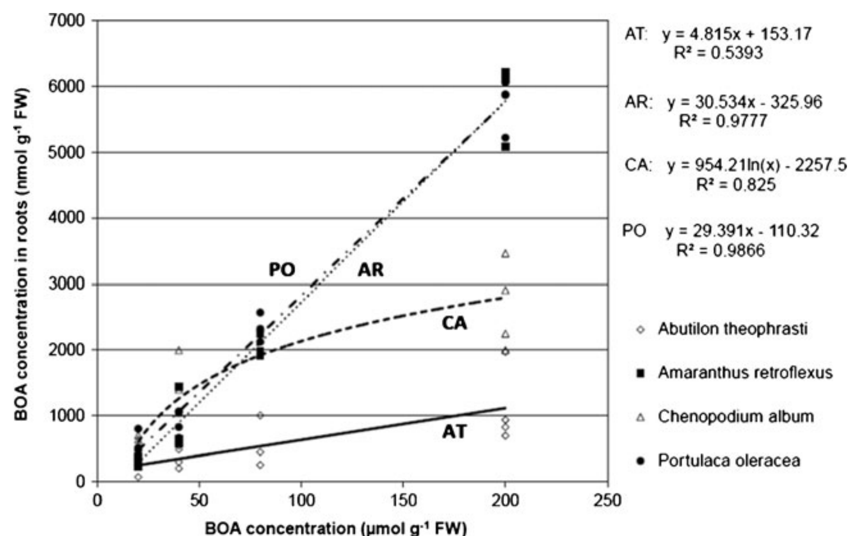

Fig. 4 Regression equations between BOA concentration in solution and free BOA concentration in shoots of four weed seedlings. *AT Abutilon theophrasti*; *AR Amaranthus retroflexus*; *CA Chenopodium album*; *PO Portulaca oleracea*

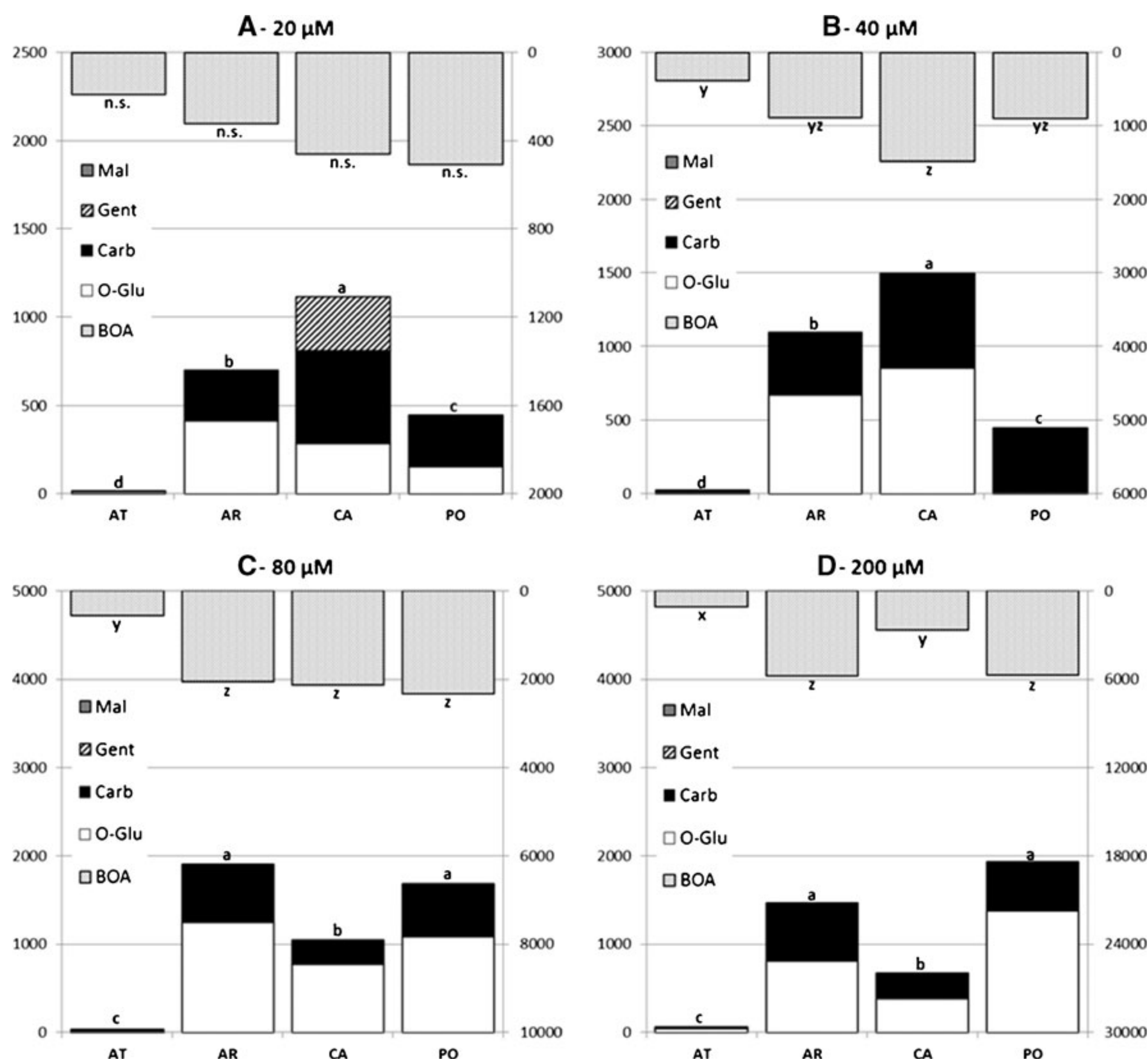

Fig. 5 Accumulation of detoxification products (below) and free BOA (above) in shoots of four summer weeds exposed to 20 (**a**), 40 (**b**), 80 (**c**), and 200 (**d**) μmol BOA g^{-1} FW. All the values are expressed as nmol g^{-1} FW. Legend for the left Y axis O-Glu, BOA-6-O-glucoside: □; Carb, Glucoside carbamate: ■; Gent, Gentiobioside carbamate: ▨; Mal, Malonylglucoside carbamate: ⬚. Legend for the right Y axis: *BOA* benzoxazolin-2(3*H*)-one: ▩. *AT Abutilon theophrasti*; *AR Amaranthus retroflexus*; *CA Chenopodium album*; *PO Portulaca oleracea.* Letters on the bars indicate significant differences between means of total amount of detoxification products (**a**, **b**, **c**, **d**) and content of free BOA (**x**, **y**, **z**) at $P \leq 0.05$, according to Tukey's test

BOA g^{-1} FW led to a breakdown of the detoxification process in all species.

Pattern of Detoxification Product Accumulation in Whole Seedlings

When accumulation of detoxification products was examined on the whole plant level, *Portulaca oleracea* showed the strongest accumulation up to 80 μmol (Fig. 6). The pattern of *C. album* showed the highest significative accumulation with 40 μmol BOA, with decrease at the succeeding increase in concentration. A similar pattern was recognized with *A. theophrasti* but at lower levels of detoxification products (ca. 6 fold less) and statistical significance ($P \leq 0.05$). The accumulation trend of detoxification products in *A. retroflexus* resembled that of *P. oleracea* without any significant differences between levels of BOA in the germination solution.

Germination and Growth of Seedlings at Three and Ten Days

Studies of germination and growth of seedlings after different applications again showed a strong species dependency (Table 5). Whereas the germination of *C. album* and *A. theophrasti* was not significantly affected by BOA up to 15 μmol, *P. oleracea* germination rate started to decrease with 3 μmol to 79 % and with 15 μmol to 71 %.

Table 4 Quotient of nmol of all detoxification products / nmol free BOA±standard deviation calculated at four concentrations of external BOA in roots and shoots of four weeds

Species	Roots				Shoots			
	Concentration free BOA (μmol BOA g^{-1} FW)				Concentration free BOA (μmol BOA g^{-1} FW)			
	20	40	80	200	20	40	80	200
Abutilon theophrasti	0.52±0.12	0.52±0.31	0.11±0.04	0.00±0.00	0.13±0.13	0.14±0.27	0.03±0.03	0.05±0.03
Amaranthus retroflexus	1.10±0.40	0.71±0.29	0.28±0.15	0.08±0.05	2.27±0.72	1.44±0.57	0.93±0.12	0.25±0.03
Chenopodium album	1.48±0.28	1.67±0.75	0.22±0.01	0.01±0.01	2.76±1.45	1.09±0.37	0.49±0.07	0.26±0.12
Portulaca oleracea	2.34±0.79	2.66±1.98	0.57±0.10	0.08±0.01	1.14±0.98	0.49±0.04	0.73±0.11	0.33±0.08

Germination of *A. retroflexus* declined rapidly with logarithmic pattern from 78 to 8 % at 0.3 and 15 μmol BOA, respectively. Again, *A. theophrasti* and *C. album* were less sensitive than *P. oleracea* and *A. retroflexus*. According to the germination data, *A. retroflexus* was the most sensitive species.

Figure 7 shows the seedling lenght of the four weeds in the presence of six different concentrations of BOA in solution. Three regression coefficients were significant at different probability levels, ranging from 0.68 for *P. oleracea* to 0.93 for *A. retroflexus*. In *C. album*, the linear regression with R^2=0.65 was not significant. Seedling growth of *A. theophrasti* showed a first inhibition between 6 and 15 μmol (200 nmol and 530 nmol seed^{-1}). Again, *A. retroflexus* and *P. oleracea* were more sensitive than velvetleaf. In *A. retroflexus,* growth inhibition started between 0.3 and 1.5 μmol BOA (4 nmol and 22 nmol seed^{-1}) and in *P. oleracea* with 1.5 μmol (6 nmol seed^{-1}).

The accumulation of detoxification products in 3-d-old seedlings of the four species was determined after incubation with 3 μmol BOA per Petri dish. These experiments revealed that all weeds differed significantly in the accumulation of detoxification products (Fig. 8). The highest detoxification activity was detected in *C. album* with no accumulation of free BOA. *Portulaca oleracea* produced, compared to *C. album*, about half of the amount of BOA-6-O-glucoside, a similar amount of glucoside carbamate (data not shown), but accumulated considerable amounts of free BOA. *Amaranthus retroflexus* also accumulated free BOA (360 nmol g^{-1} FW) with a low detoxification activity (827 nmol g^{-1} FW of total detoxification products) when incubated with 3 μmol BOA. Lower concentrations, between 0.3 and 1.5 μmol BOA, led to less BOA-6-O-glucoside plus glucoside carbamate: 696 and 55 nmol g^{-1} FW at 1.5 and 0.3 μmol BOA, respectively (data not shown). The accumulation of free BOA was more inhibitory for *A. retroflexus* and *P. oleracea*. Thus, 3-d-old seedlings of *P. oleracea* and *A. retroflexus* had a less developed detoxification than found in 10-d-old seedlings (Table 6). In contrast, the detoxification activity of *C. album* was more active. Three-day-old seedlings of *A. theophrasti* showed similar effects to those observed. The quotient [nmol of all detoxification products / nmol of absorbed free BOA] in 3-d-old and 10-d-old seedlings incubated

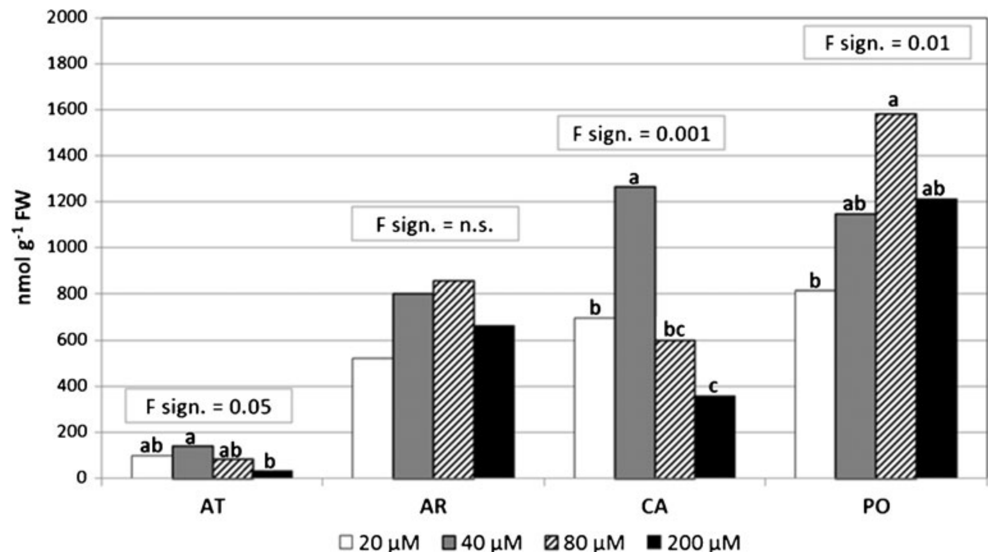

Fig. 6 Total detoxification products in the whole seedlings of four weed species exposed to 20, 40, 80, and 200 μmol BOA g^{-1} FW. *AT Abutilon theophrasti*; *AR Amaranthus retroflexus*; *CA Chenopodium album*; *PO Portulaca oleracea. F* signif. shows the ANOVA results between means within each weed (n.s., not significant). Letters on the bars indicate significant differences at $P \leq 0.05$, according to Tukey's test

Table 5 Mean germination values (%) and standard deviations of the four summer weeds in presence of 0.3, 3, 6 and 15 μmol BOA. Significant differences between the 0.3 and 15 μmol values (*Portulaca oleracea*) and between 0.3 μmol / 3 and 15 μmol (*Amaranthus retroflexus*) are indicated (*, **, ***: significant at 0.05, 0.01, 0.001 probability level). For *A.retroflexus,* only the difference between 6 and 15 μmol BOA is not significant as well as all differences of the other species which are not labeled

Species	BOA (μmol)				
	0 (control)	0.3	3	6	15
Abutilon theophrasti	100	104±6	113±13	84±5	88±5
Amaranthus retroflexus	100	78±4***	50±9.5**	16±13	8±6
Chenopodium album	100	87±18	104±10	104±15	74±26
Portulaca oleracea	100	104±18	79±7	80±5	71±4*

with 20 μmol BOA was similar for *A. retroflexus* and *P. oleracea*, but about 5 fold higher for *A. theophrasti* 3-day-old seedlings (Table 6). In three of four experiments, *C. album* did not accumulated BOA in 3-d-old seedlings, which indicated a very high detoxification activity in the early developmental stage of this species.

Effects of Inhibitors

Considering all data, the detoxification strategy of *Abutilon theophrasti* and perhaps *Chenopodium album* may include other features in addition to the synthesis of detoxification products. The low sensitivity of *A. theophrasti* towards BOA may be explained with highly active multidrug resistance transporters (MDRts) or multidrug and toxic compound extrusion (MATE) proteins that are responsible for the cellular efflux of BOA and its detoxification products. Thus, we tested the influence of the channel blockers verapamil, nifedipine, and the GST inhibitor ethacrynic acid on BOA accumulation and the pattern of detoxification products in 10-d-old seedlings of the four weeds. None of the inhibitors had an influence on BOA accumulation when *C. album* was incubated with 20 μmol BOA (Fig. 9). However, the accumulation of detoxification products was reduced in presence of all inhibitors in comparable proportions. With 80 μmol BOA, nifedipine and verapamil inhibited BOA

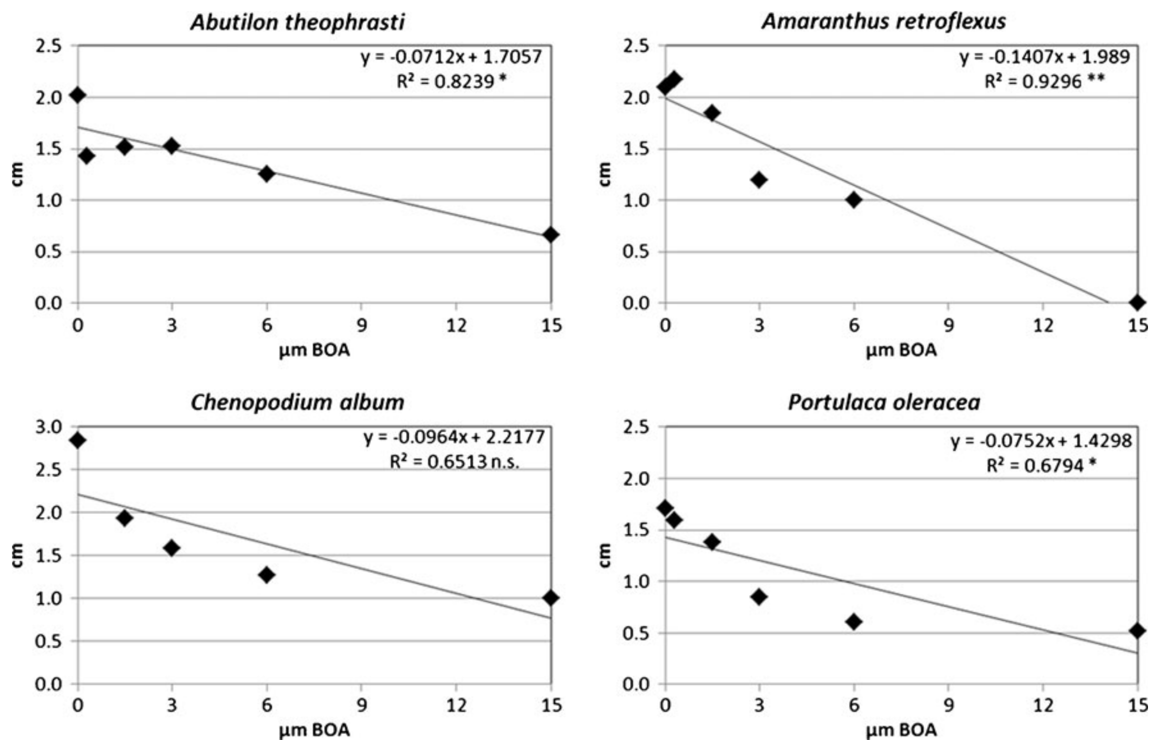

Fig. 7 Mean length of seedlings grown in presence of different concentrations of BOA, for the four weeds (*Abutilon theophrasti, Amaranthus retroflexus, Chenopodium album,* and *Portulaca oleracea*). The linear regressions, the determination coefficients and their statistical significance are shown (* = $P \leq 0.05$; ** = $P \leq 0.01$; *n.s.* not significant)

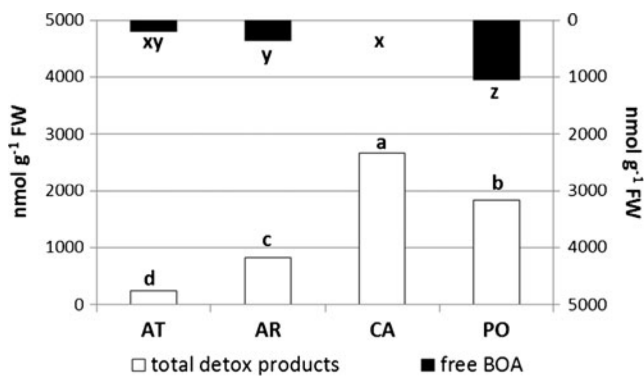

Fig. 8 Accumulation of detoxification products (below, left Y axis) and free BOA (above, right Y axis) in 3-day-old seedlings of four summer weeds incubated at 3 μM BOA. *AT Abutilon theophrasti*; *AR Amaranthus retroflexus*; *CA Chenopodium album*; *PO Portulaca oleracea.* Significance of F test is at $P \leq 0.001$ for both parameters. Letters on the bars indicate significant differences between means of total amount of detoxification products (**a**, **b**, **c**, **d**) and content of free BOA (**x**, **y**, **z**) at $P \leq 0.05$, according to Tukey's test

accumulation, whereas ethacrynic acid had no significant effect. The accumulation of detoxification products was not affected.

Interestingly, in *A. theophrasti,* ethacrynic acid and nifedipine led to a strong increase (2–3 folds) of BOA accumulation when the incubation was performed with 20 μmol BOA. Concerning accumulation of detoxification products, the presence of ethacrynic acid was without significant effect; verapamil and nifedipine were slightly inhibitory. With 80 μmol BOA, the inhibitors led to significant increase in BOA accumulation, while the effects on accumulation of detoxification products were similar to those found with the 20 μmol BOA incubation. In another experiment with 3-d-old *A. theophrasti* seedlings treated with ethacrynic acid in presence of 3 μmol BOA, an increase of detoxification products was observed, and the accumulation of BOA was not affected (data not shown). This may implicate age dependent alterations in the detoxification strategy of *A. theophrasti*.

In *P. oleracea*, nifedipine had an inhibitory effect on BOA accumulation, in contrast to ethacrynic acid when the incubation was performed with 20 μmol BOA. Verapamil had no significant effect. At 80 μmol BOA, accumulation of BOA was significantly inhibited with nifedipine and verapamil, whereas ethacrynic acid had no effect. All inhibitors caused a strong decrease of the accumulation of detoxification products. The inhibitors had no effect on accumulation of BOA in *A. retroflexus* at either BOA concentration, with the exception of verapamil at 80 μmol BOA. In this species, a dramatic breakdown of detoxification product accumulation was observed with both BOA concentration and with all inhibitors. The results of the inhibitor experiments were in agreement with all other results of this study.

Discussion

The four summer weeds were able to grow in the presence of low amounts of BOA, between 0.3 and 20 μmol g^{-1} FW, depending on the weed species. In fact, in previous experiments we showed that *A. theophrasti* was not influenced by rye allelochemicals (Tabaglio et al., 2008; Gavazzi et al., 2010). In 10-d-old seedlings, the detoxification activities of the three species *A. retroflexus*, *C. album,* and *P. oleracea* were rather high in comparison to *A. thaliana* ecotype Columbia 0 (Baerson et al., 2005). When exposed to 20 μmol BOA, whole seedlings of *C. album* produced 19.7 fold more glucoside carbamate/gentiobioside carbamate, *P. oleracea* and *A. retroflexus* 14.6 and 8.4 fold more glucoside carbamate/malonylglucoside carbamate than *A. thaliana* ecotype Columbia 0. The levels of BOA-6-O-glucoside were higher as well. The weeds seemingly have evolved alterations in defense gene qualities and copy number, as well as their expression, resulting in difference in levels and activities of enzymes that can detoxify BOA. When the amount of BOA is increased by rye mulch applications, *P. oleracea* and *A. retroflexus* are the first species injured because of the higher sensitivity of the germination process and seedling growth, and the lower detoxification activity in the shoots. The decrease of detoxification product accumulation indicates a breakdown of the detoxification pathways. From these results, it is obvious that a high detoxification activity is important to prevent the accumulation of free BOA in both roots and shoots. As long as most of the BOA absorbed is detoxified, the harmful effects are countered. However, absorption of high amounts of BOA leads to a breakdown of the detoxification mainly in the roots. This could be accompanied by membrane damage followed by an

Table 6 Comparison of the BOA detoxification activity in 3-day-old and 10-day-old seedlings when incubated with 3 μmol and 20 μmol BOA g^{-1} FW, respectively, for 24 hr. (DP, nmol of detoxification products; BOA, nmol of free BOA in seedlings; Quotient, detoxification products / BOA)

Species	3-day-old seedlings (3 μmol BOA)			10-day-old seedlings (20 μmol)		
	DP	BOA	Quotient	DP	BOA	Quotient
Abutilon theophrasti	261±29	167±15	1.6	178±43	583±136	0.3
Amaranthus retroflexus	575±89	359±157	1.6	1028±113	639±122	1.6
Chenopodium album	2661±137	0	–	1665±182	740±127	2.2
Portulaca oleracea	1110±87	653±64	1.7	1657±727	1014±311	1.6

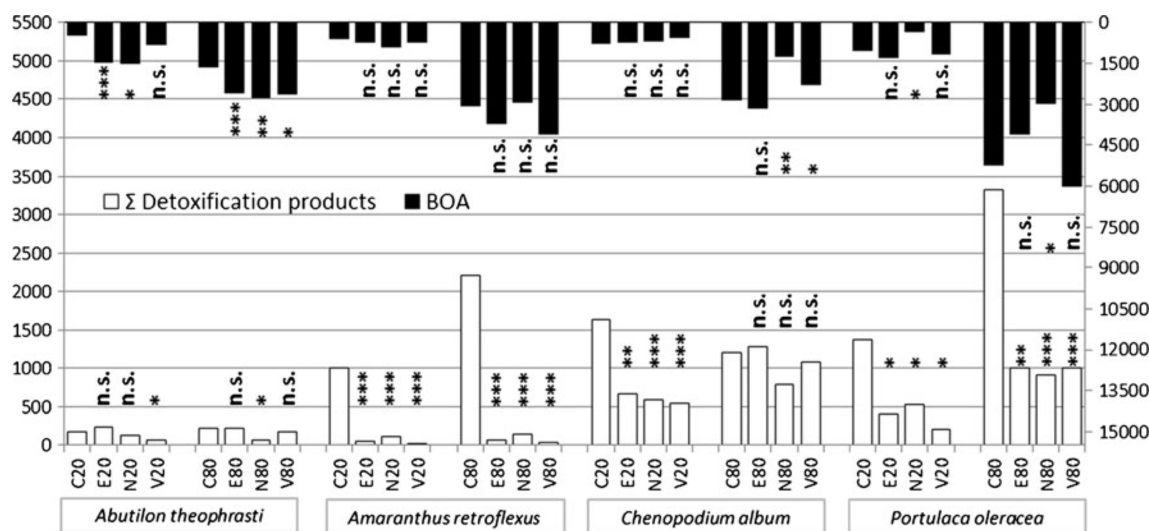

Fig. 9 Effect of three inhibitors (*E* ethacrynic acid; *N* nifedipine; *V* verapamil; *C* control) on detoxification product (left axis) and free BOA (right axis) contents in seedlings of four weeds treated with two levels of BOA (20 and 80 μmol BOA g^{-1} FW). All the values are expressed as nmol g^{-1} FW. (*n.s.* not significant; *, **, ***: significant at 0.05, 0.01, 0.001 probability level, respectively)

uncontrolled influx of BOA into the cells. This last observation could be due to a loss of membrane integrity, as BOA is reported to induce lipid peroxidation (Batish et al., 2006). Moreover, a high concentration of free BOA in shoots can inhibit photosynthesis (Sanchez-Moreiras et al., 2010). The interpretation of the detoxification data for 10-d-old seedlings results in the following sequence of increasing sensitivity:

A. theophrasti <<< *C. album* << *A. retroflexus* ≤ *P. oleracea*.

The results with 3-d-old seedlings mirror even stronger the results of the greenhouse studies (Tabaglio et al., 2008; Gavazzi et al., 2010). The early stage of seedling development is marked by a very high activity of cell division and growth rate. In this stage, the highest sensitivity of the seedlings to BOA has to be expected. Singh et al. (2005) described a stasis of the mitotic activity in onion root-tip cells after BOA treatment, abnormalities in cell shape and size, and effects on rhizogenesis. All effects resulted in a higher sensitivity of seedling growth. This correlates with a higher sensitivity of seedlings during the first days of development. The size of the seeds and the relationship between seed number and treatment solution volume is also important (Weidenhamer et al., 1987). It is known that inhibitory effects of allelochemicals on seed germination are greater in small seeds than in large seeds (Chase et al., 1991; Liebman and Sundberg, 2006). According to the germination and growth during the first three days, the sequence of sensitivity is as follows:

A. theophrasti ≤ *C. album* <<< *A. retroflexus* < *P. oleracea*.

Thus, very young seedlings of *P. oleracea* and *A. retroflexus* do not have a better detoxification activity than the 10-d-old seedlings, in contrast to *A. theophrasti* and especially *C. album*.

Although there are considerable differences in their sensitivity to higher BOA concentrations, *P. oleracea, A. retroflexus,* and *C. album* represent, nevertheless, a group of species with a considerable absorption and BOA detoxification capability, which *A. theophrasti* does not have. An efficient efflux can prevent a toxic accumulation of deleterious compounds in the cell (Simmons et al., 2003; Ito and Gray, 2006; Kuroda and Tsuchiya, 2009; Maron et al., 2010; Conte and Lloyed, 2011). Plant MDRts have been recognized during the last 15 years as important for the transport of a variety of compounds including glutathione conjugates of many xenobiotics (Awasthi et al., 1993; Loyola-Vargas et al., 2006). Transporters are, therefore, fundamental in herbicide resistance (Dixon et al., 2010), and also for the storage of detoxification products in the vacuole (Yuan et al., 2006). Plant MATE proteins have been characterized only recently (Moriyama et al., 2008). In BOA-incubated *Arabidopsis*, the genes of several ABC transporters of glutathione S-conjugate ABC transporter (AtMRP2), and other transporters including At2g04040, a MATE efflux protein, and At5g13750 (MFS antiporter) are up-regulated as well as a number of glutathione transferase encoding genes (Baerson et al., 2005).

The effects of inhibitors reveal a high differential species-specificity of the detoxification strategies, the involvement of suitable GSTs in *A. theophrasti* as well as the participation of translocators. The type of the translocators active in the extrusion may be variable depending on BOA concentration. The conjugates could be extruded by plasma membrane located translocators. However, we have not yet identified such BOA conjugates in the extracts. Clearly,

the highly efficient avoidance of BOA accumulation is the better strategy to escape deleterious effects due to BOA than only the production and storage of detoxification compounds. The translocator systems of *A. retroflexus* and *P. oleracea* seem not well adapted for BOA extrusion. The observed decrease in accumulation of detoxification products, heavily pronounced in *A. retroflexus* and *P. oleracea*, could be explained by a competitive interference of absorbed inhibitor molecules and BOA detoxifying enzymes. Absorption of some inhibitors might also interfere with BOA uptake, which would explain the reduced BOA accumulation observed in several experiments.

To summarize, the outstanding behavior and BOA tolerance of *A. theophrasti* is due to the avoidance of BOA accumulation in young and older seedlings. *Chenopodium album* is more active in metabolizing BOA in young seedlings than *P. oleracea* and *A. retroflexus*. In older seedlings of *C. album* and after exposure to higher BOA concentrations, extrusion of BOA is performed but is less efficient than in *A. theophrasti*. *Portulaca oleracea* and *A. retroflexus* have less efficient mechanisms to avoid BOA accumulation from soil. Thus, at high BOA concentrations both species are more susceptible to BOA than *A. theophrasti* and *C. album,* which possess highly complex detoxification machinery adapted to BOA concentration. From our studies, it has to be concluded that benzoxazolinone detoxification after uptake from soil enriched with this allelochemical is important to explain the differential behavior of weeds in field studies, which in turn is necessary to develop strategies for the use of benzoxazolinones from natural sources as bioherbicides.

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ERRATUM

Erratum to: BOA Detoxification of Four Summer Weeds during Germination and Seedling Growth

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The incorrect file for Fig. 1 of this paper was inadvertently inserted into this paper. Structures of DIBOA. DIMBOA, Glucoside carbamate, Glucoside methoxycarbamate, and BOA-6-OH were incorrect. No formulae were inserted for Gentiobioside- and malonylglucoside cabamate. The correct figure is presented here. The authors regret the error.

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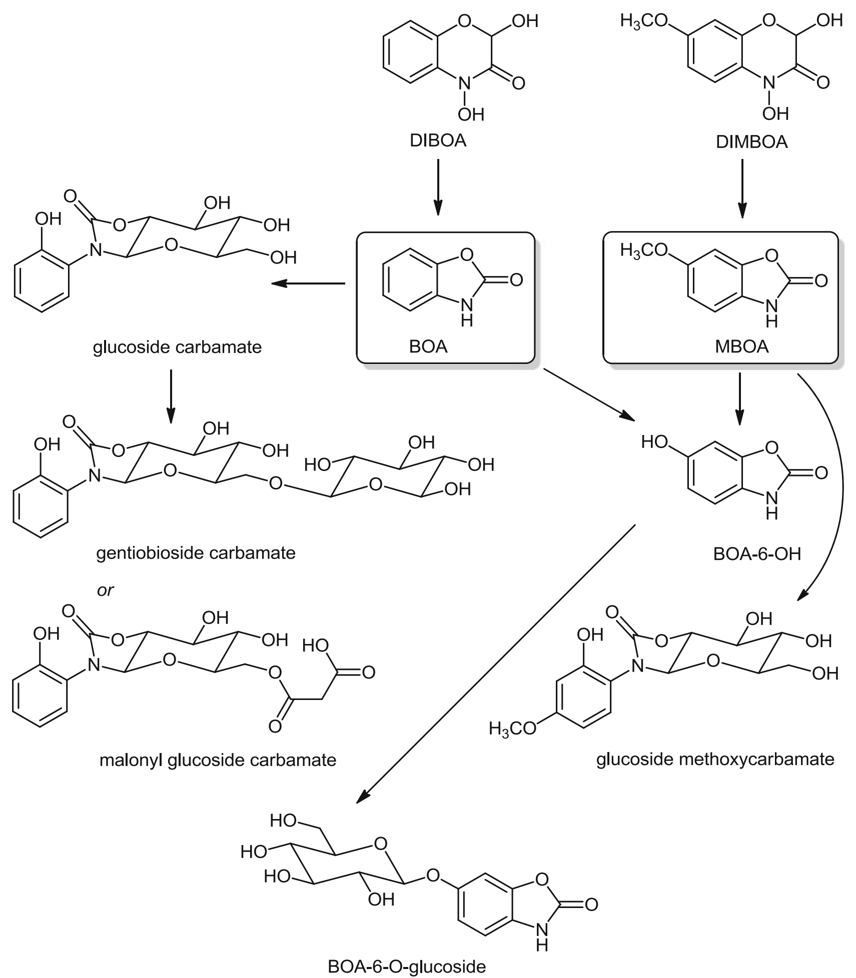

Fig. 1 Stable BOA/MBOA detoxification products found in higher plants (see text for explanation)