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Behavioral and electrophysiological responses of the emerald ash borer, *Agrilus planipennis*, to induced volatiles of Manchurian ash, *Fraxinus mandshurica*

Cesar Rodriguez-Saona,[1,*] Therese M. Poland,[2] James R. Miller,[1] Lukasz L. Stelinski,[1] Gary G. Grant,[3] Peter de Groot,[3] Linda Buchan[3], and Linda MacDonald[3]

[1]Department of Entomology, Michigan State University, East Lansing MI 48824, USA
[2]USDA Forest Service, North Central Research Station, East Lansing, MI 48823, USA
[3]Natural Resources Canada, Canadian Forest Service, Sault Ste. Marie, ON P6A 2E5, Canada

Summary. We investigated the volatile emissions of Manchurian ash seedlings, *Fraxinus mandshurica*, in response to feeding by the emerald ash borer, *Agrilus planipennis*, and to exogenous application of methyl jasmonate (MeJA). Feeding damage by adult *A. planipennis* and MeJA treatment increased volatile emissions compared to unexposed controls. Although the same compounds were emitted from plants damaged by beetles and treated with MeJA, quantitative differences were found in the amounts of emissions for individual compounds. Adult virgin female *A. planipennis* were similarly attracted to volatiles from plants damaged by beetles and those treated with MeJA in olfactometer bioassays; males did not respond significantly to the same volatiles. Coupled gas chromatographic-electroantennogram detection (GC-EAD) revealed at least 16 antennally-active compounds from *F. mandshurica*, including: hexanal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, 3-methyl-butylaldoxime, 2-methyl-butylaldoxime, (*Z*)-3-hexen-1-yl acetate, hexyl acetate, (*E*)-β-ocimene, linalool, 4,8-dimethyl-1,3,7-nonatriene, and *E*,*E*-α-farnesene. Electroantennogram (EAG) dose–response curves using synthetic compounds revealed that females had a stronger EAG response to linalool than males; and male responses were greater to: hexanal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, 3-methyl-butylaldoxime, 2-methyl-butylaldoxime, and hexyl acetate. These results suggest that females may use induced volatiles in long-range host finding, while their role for males is unclear. If attraction of females to these volatiles in an olfactometer is upheld by field experiments, host plant volatiles may find practical application in detection and monitoring of *A. planipennis* populations.

Key words. Host-plant finding – Fraxinus mandshurica – induced volatiles – methyl jasmonate – GC-EAD – EAG dose-response – olfactometer – attractants

[*]*Current address*: Blueberry & Cranberry Res. Center, Rutgers University, 125A Lake Oswego Rd., Chatsworth, NJ 08019, USA

Correspondence to: Cesar Rodriguez-Saona, e-mail: crodriguez@aesop.rutgers.edu

Introduction

Herbivorous insects of both sexes must find host plants for feeding and mating, and females must also find ovipositional sites (Miller & Strickler 1984; Bernays & Chapman 1994). Host finding might be more challenging for herbivores with narrow host ranges, because foraging specialists must find a single species of host plant surrounded by a range of non-hosts (Tahvanainen & Root 1972). To do so, specialist herbivores can use specific cues from host plants that are detectable and reliable. One such cue can be volatile chemicals (Bernays & Chapman 1994; Dicke 2000).

Some plants emit unique blends of constitutive volatiles that can attract herbivores (Visser 1986). Emissions of plant volatiles can be induced by biotic and abiotic environmental stresses, such as herbivore feeding damage, pathogen infection, fertilization rates, and light intensity (Takabayashi *et al*. 1994; Karban & Baldwin 1997; Gu & Dorn 2001; Gouinguené & Turlings 2002; Huang *et al*. 2003). Induced plant volatiles may direct herbivores to host plants for food (Inui *et al*. 2003), mating (Ruther *et al*. 2000), and oviposition (Stanjek *et al*. 1997).

Studies have demonstrated the critical role of induced plant volatiles in host and prey finding by parasitoids and predators (see reviews by Dicke & Vet 1999; Kessler & Baldwin 2002). Volatiles induced by feeding may increase the apparency of a plant and thus may affect host and mate finding by other herbivores (De Moraes *et al*. 2001; Kessler & Baldwin 2001; Heil 2004). For example, spider mites are generally attracted to conspecific-induced volatiles while these volatiles often repel moth oviposition (see reviews by Dicke & Vet 1999; Dicke 2000). Loughrin *et al*. (1996) also found that the Japanese beetle, *Popillia japonica* Newman, was attracted to plants damaged by conspecifics. While no practical application has yet been found for induced plant volatiles used in host finding by herbivorous insects, there is promise for the practical use of induced volatiles to attract natural enemies of agricultural insect pests (e.g., Thaler 1999; James 2003).

Jasmonic acid (JA) is a ubiquitous signaling molecule in plants; JA has a variable physiological functions including: induction of plant resistance to pests, senescence, and fruit ripening (He *et al*. 2002; Peña-Cortés *et al*. 2005). It is produced by the octadecanoid-signaling pathway and is a key elicitor of volatile production in plants (Hopke *et al*. 1994; Boland *et al*. 1998). Levels of JA often increase when plants are under stresses such as mechanical wounding and herbivory (Karban & Baldwin 1997). In fact, exogenous applications of JA, or its volatile derivative methyl jasmonate (MeJA), can mimic the volatile response of plants to herbivore feeding damage (e.g., Rodriguez-Saona *et al*. 2001; Gols *et al*. 2003).

The emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an invasive pest discovered in 2002 causing devastating mortality of ash trees, *Fraxinus* spp., in a small area in North America centered around the cities of Detroit Michigan, USA, and Windsor Ontario, Canada (Haack *et al*. 2002). Movement of firewood, nursery trees, and infested logs has resulted in rapid expansion of the infestation. Larvae of *A. planipennis* feed on the cambium, forming S-shaped galleries that disrupt the flow of nutrients in the phloem, often causing death of trees after 2 to 3 years of damage (Liu *et al*. 2003). There is no evidence that *A. planipennis* has an attractive long-range sex pheromone (Otis *et al*. 2005). However, host-plant volatiles might be essential cues for *A. planipennis* host and mate finding, as this insect has a narrow host range in its native Northeast Asia (China, Japan, Korea), where it feeds mainly on Chinese ash, *F. chinensis* Roxb., and Manchurian ash, *F. mandshurica* Rupr. (Yu 1992; Xu 2003). In North America, *A. planipennis* attacks almost exclusively green ash (*F. pennsylvanica* Marsh.), white ash (*F. americana* L.), and black ash (*F. nigra* Marsh.) (Liu *et al*. 2003). Compared to constitutive volatiles, induced volatiles from *Fraxinus* spp. may provide more detectable and reliable information to foraging *A. planipennis* for host and mate finding.

We investigated the role that induced volatile cues from ash may play in host finding by *A. planipennis* adults. Dunn *et al*. (1986) found that a related buprestid, *A. bilineatus* (Weber), was attracted to volatiles from stressed white oak, caused by removal of bark from a portion of the stem. Similarly, field data indicate that *A. planipennis* adults are attracted to girdled ash trees (Poland *et al*. 2005). Our objectives were to: (1) collect, identify, and compare the volatiles from ash *F. mandshurica* exposed to adult *A. planipennis* feeding, MeJA, and mechanical damage *vs* unexposed control trees; (2) quantify the behavioral responses of *A. planipennis* adults to induced volatiles from ash trees in the laboratory; and (3) quantify the antennal electrophysiological responses of *A. planipennis* adults to induced volatiles.

Materials and methods

Insects

Insects were collected from naturally infested bolts cut from trees in areas under quarantine for *A. planipennis* (Ann Arbor, Michigan; Hudson Mills and Island Lakes Metro Parks, Michigan; and Southwestern Ontario). Bolts (60 cm long, 20–30 cm diam) were brought to rearing facilities at Michigan State University and The Great Lakes Forestry Centre (GLFC; Sault Ste. Marie, Ontario) and held at 3–4 ºC. As beetles were needed, bolts were placed individually in horizontal emergence chambers at 25 ± 0.5 [SE] ºC, 40 % RH, and 16:8 h L:D. Emergent beetles were placed in sealed plastic rearing cups and supplied with water and fresh leaves from evergreen ash, *F. uhdei* (Wenzig) Lingelsh (Ponto Nursery, Vista, CA), a species of ash that does not occur in Asia or in infested areas in North America, at 24 ± 1.0 ºC, 65 % RH, and 16:8 h L:D. Evergreen ash was used because there is no artificial diet available to rear *A. planipennis*. At GLFC, the conditions for bolts and beetles were 21 ºC, 67 % RH, and 17:7 h L:D.

Plants

All experimental plants were Manchurian ash, a native host of *A. planipennis* (Yu 1992; Xu 2003; Liu *et al*. 2003). Bare-root seedlings (15-30 cm tall) were purchased from Lawyer Nursery Inc. (West Plains, MT), and maintained at 3–4 ºC until planted into 18 cm long × 18 cm wide × 30 cm tall pots with hi-porosity soil mix (Baccto; Michigan Peat Co., Houston, TX). Potted plants were fertilized weekly with a 20-20-20 N-P-K Scotts Peters Professional water-soluble fertilizer (Marysville, OH). Plants were grown in a greenhouse under natural lighting supplemented with 400 Watts high-pressure sodium lamps at 25 ± 5 ºC, until leaves had fully flushed and plants had reached 30 to 50 cm height (approx. 1-2 months after planting).

Treatments

Plants were assigned to one of four treatments: 1) insect damage, 2) MeJA treatment, 3) mechanical damage, or 4) control. Insect damaged plants were exposed to feeding by 10 virgin male or female *A. planipennis* (1- to 2-wk old) caged on foliage by placing 20 cm wide and 40 cm long mesh bags over the plants. Because of unexplained high mortality of beetles (64 ± 5 %); after 3 days, another 5-10 adults of the same sex were added for 2 additional days. Plants were kept at 24 ± 1.0 ºC, 65 % RH, and 16:8 h L:D. For a subset of 14 plants, the amount of leaf material consumed by *A. planipennis* was visually measured as the percentage of leaf area removed after 5 days of feeding (area removed per leaf = 17.1 ± 1.6 %). For the *MeJA* treatment, plants were sprayed to saturation (50–70 mL) with a 0.03 % MeJA (95 % purity; Sigma-Aldrich, St. Louis, MO) in a 0.1 % Tween-20 solution (1.4 mM MeJA solution). In preliminary tests, this dose was non-toxic, and induced volatiles from ash similar to those induced by herbivore feeding. Plants were treated at 18:00 h, the day before subsequent tests, and kept at room temperature for 15 h. For the *mechanical damage*, approximately 20 % of total leaf area of each tree was removed with scissors to mimic insect damage treatment. For *controls*, plants were either sprayed with 50 mL of a 0.1 % Tween-20 solution or bagged as described above, but without insects.

Collection of plant volatiles

Volatiles from plants in all treatments were collected using a push-pull system (Rodriguez-Saona *et al*. 2001). The aerial portion of ash seedlings (stem, branches, and leaves), including beetles in the insect damage treatment, was placed inside a 4 L, 30.5 cm tall and 15.2 cm diam, glass cylinder (Analytical Research Systems, Inc., Gainesville, FL). A guillotine support base (Analytical Research Systems, Inc.) closed the cylinder loosely around the stem of the plant. Air generated from a portable 2.0 HP, 20-gallon tank (Westward air compressor; Grainger; Northbrook, IL) was purified by drawing it through activated charcoal, entered the top of the glass cylinder, and flowed over the plant at a rate of 2 L/min. Volatiles were collected in Super-Q adsorbent traps (Alltech, Deerfield, IL), positioned 5 cm from the base of the cylinder, by pulling 50 % of the air at a rate of 1 L/min with the aid of a Gast ¼ HP vacuum pump (Grainger, Northbrook, IL). The remainder of the air was vented out the bottom of the system through an opening around the stem of the plant that was loosely closed

with cotton to prevent abrasion. Volatile samples were collected at 25 ± 0.5 ºC, 40 % RH, and 16:8 h L:D. The volatile collection apparatus was illuminated from above with 40 Watts fluorescent Chroma-50 lights (1875 lux; General Electric; Fairfield, CT) placed above each cylinder.

Volatiles from at least four plants per treatment were collected at two time intervals, for 24 h: during the day from 09:00-18:00 h, and at night from 18:00-09:00 h. Empty chambers sampled to test for contamination yielded no detectable volatiles. After volatiles were collected, all leaves from tested plants were excised, oven dried at 60 ºC, and weighed.

Gas chromatography and mass spectrometry (GC and GC-MS)

Volatiles were extracted from the Super-Q traps by rinsing with 150 µL of methylene chloride. An internal standard (400 ng of n-octane [Sigma-Aldrich] and 600 ng of nonyl acetate [Sigma-Aldrich] in 5 µl methylene chloride) was added to each extract. A 1-µL aliquot of each sample was injected onto a Hewlett-Packard (HP) 6890 Series GC, equipped with a flame ionization detector (FID) and a 15 m × 0.25 mm i.d., 0.25 µm film DB1 column (J&W Scientific, Folsom, CA). Injections were made in the splitless mode (40 °C for 1 min, increasing at 12 °C/min to 180 °C, and holding at 220 °C for 5 min). The carrier gas (helium) constant flow velocity was 22 cm/sec (0.7 ml/min).

Selected samples were analyzed by GC-MS with a HP 5890 Series II GC, equipped with a HP 5989A mass spectrometer, a DB1 column (25 m × 0.2 mm i.d., 0.33 µm film; J&W Scientific), and temperature program as described above. Compounds were identified by comparing spectral data with those from commercially available standards and spectra from Wiley 275.L and Nist98.L libraries, and confirmed by retention times of authentic standards, if available. Quantifications, in ng per g of dry leaf material, of individual components were based on comparison of peak areas from GC-FID with that of the n-octane internal standard.

Behavioral assays

We used a 2-arm olfactometer modified from a previously described 4-arm olfactometer (O'Neal *et al*. 2004) to test responses of walking *A. planipennis* to plant volatiles. The olfactometer consisted of a plastic central chamber (8 cm tall, 18 cm diam) connected to two opposing chambers by clear Tygon tubing (20 cm long, 2 cm diam; Fisher Scientific, Pittsburgh, PA). The plexiglas satellite chambers were sufficiently large (12.7 cm long × 12.7 cm wide x 73.7 cm tall) to hold the foliage, stem, and branches of a potted ash seedling. Three 4-by-1 cm clean cotton cylinders saturated with water provided humidity inside satellite chambers containing no plants. New cotton cylinders were used for each bioassay. To eliminate any visual cues from the satellite chambers, the central chamber was wrapped with black plastic. Air was drawn at 3 L/min from the satellite chambers through the central chamber by a Gast 1/8 HP vacuum pump (Grainger) connected to the top of the central chamber. All bioassays were conducted at 25 ± 0.5 ºC, 40 % RH, and 16:8 h L:D. Daytime light at 1400 lux was provided by a 40 Watts fluorescent light bulb placed 50 cm above each chamber.

In preliminary experiments, only 1 and 3 of 40 mixed sex beetles tested in groups of five moved to the central chamber when plants or moist cotton wicks, respectively, were provided in the satellite chambers. Thus, the movement of beetles was mainly unidirectional and upwind as observed by O'Neal *et al*. (2004).

Three dual-choice experiments were tested: 1) clean air *vs* control plants; 2) clean air *vs* plants damaged by beetles; and 3) clean air *vs* MeJA-treated plants. Beetles in the insect damage treatment remained on plants. Treatments were randomly assigned to each satellite chamber. Four plants were tested per day in four separate olfactometers, and new stimuli were used for each group of 5 to 10 virgin male or female beetles released in the central chamber. Bioassays began at 09:00 h and ran for 24 h. Approximately 1-wk old adult *A. planipennis* were used because females need to feed for several days prior to mating (Lyons *et al*. 2004), and might not exhibit orientation towards plant volatiles within the first few days after emergence. A positive response was scored when a beetle was found in a satellite chamber or in the connecting tubes (< 5 % of beetles were found in tubes). Insects were used only once and at least 70 males and females were tested per treatment combination. Beetles were starved for 15 h prior to testing. All connecting tubes and chambers were cleaned with 70 % ethanol and dried overnight after each bioassay.

We conducted additional experiments to determine: 1) the response of 80 beetles tested in groups of 10 to clean air from both satellite chambers; 2) the response of 40 beetles to 20 µl of MeJA released from Whatman No. 1 filter papers treated 15 h before bioassays *vs* clean air; 3) the response of 40 males to females *vs* clean air; and 4) the response of 40 females to males *vs* clean air.

Gas chromatography-electroantennogram detection (GC-EAD)

Aeration extracts were analyzed by GC-EAD to identify compounds from *F. mandshurica* that elicited an electrophysiological response from the antennae of 10-30 day-old male and female *A. planipennis*. An antenna was excised from the head and the distal tip cut-off with a scalpel. The cut ends of the antenna were inserted into small droplets of electrode gel (Signa Gel, Parker Labs., NJ) on a plexiglas platform. Glass electrodes filled with 0.9 % $NaCl_2$ were inserted into the gel. Gold wires inside each glass electrode were connected to a Syntech portable INR-2 amplifier (Hilversum, The Netherlands) connected to a personal computer containing a Syntech data acquisition interface board (Type ID AC-02) and Syntech GC-EAD software (version 2.2).

Two-uL of an extract from plants damaged by *A. planipennis* or treated with MeJA were injected into a Varian 3400 GC fitted with a nonpolar HP-1 capillary column (25m × 0.20 mm id, 0.33 µm film thickness) with helium as the carrier gas (temperature program to rapidly screen extracts started at 60 ºC for 1 min, then increasing at 35 ºC/min to 245 ºC, and held for 10 min). A second, slower GC program, adopted later to better separate components started at 40 ºC for 1 min, then increasing at 8 ºC/min to 160 ºC for 10 min then increasing to 190 ºC at 25 ºC/min and held for 5 min. One half of the column effluent went to the flame ionization detector (FID) of the GC and the other half went through a Syntech heated (215 ºC) transfer line and into a humidified airstream (about 300 mL/min) directed at the excised antenna.

Chemical sources

Hexanal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, hexyl acetate, (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, linalool, indole, and (*Z*)-jasmone were purchased from Sigma-Aldrich and were ≥ 98 % pure (confirmed by our GC analysis). A mixture of farnesene isomers (containing 66 % *E*,*E*-α-farnesene) was obtained from Phero Tech Inc. (British Columbia, Canada). A mixture of *Z* and *E* isomers of 2-methyl-butylaldoxime, and 3-methyl-butylaldoxime, was provided by Dr. Roman Kaiser (Givaudan Schweiz AG, Dübendorf, Switzerland).

EAG dose-response characterizations

EAG dose-response profiles for the responses of antennae from 10-14 day-old post-emergence *A. planipennis* of both sexes to induced plant volatiles were developed following the protocol of Stelinski *et al*. (2003). The EAG system consisted of a data acquisition interface board (Type IDAC-02) and universal single ended probe (Type PRS-1) from Syntech (Hilversum, The Netherlands). The recording and indifferent electrodes were silver-coated wire in pulled glass micropipettes (10 µL micro-hematocrit capillary tubes) containing 0.5 M KCl. Beetles were restrained on a wax-filled, 3.5 cm diameter Petri dish by placing clay (10 × 3 mm) over their thorax and abdomen. The terminal segment of an antenna was removed with fine scissors, and the recording electrode was placed over the severed end. The reference electrode was inserted into the back of the head through the cervical membrane.

Volatile stimuli were delivered through a glass Y-tube (each arm 2 cm in length, base 1 cm long, and 0.5 cm diam) positioned

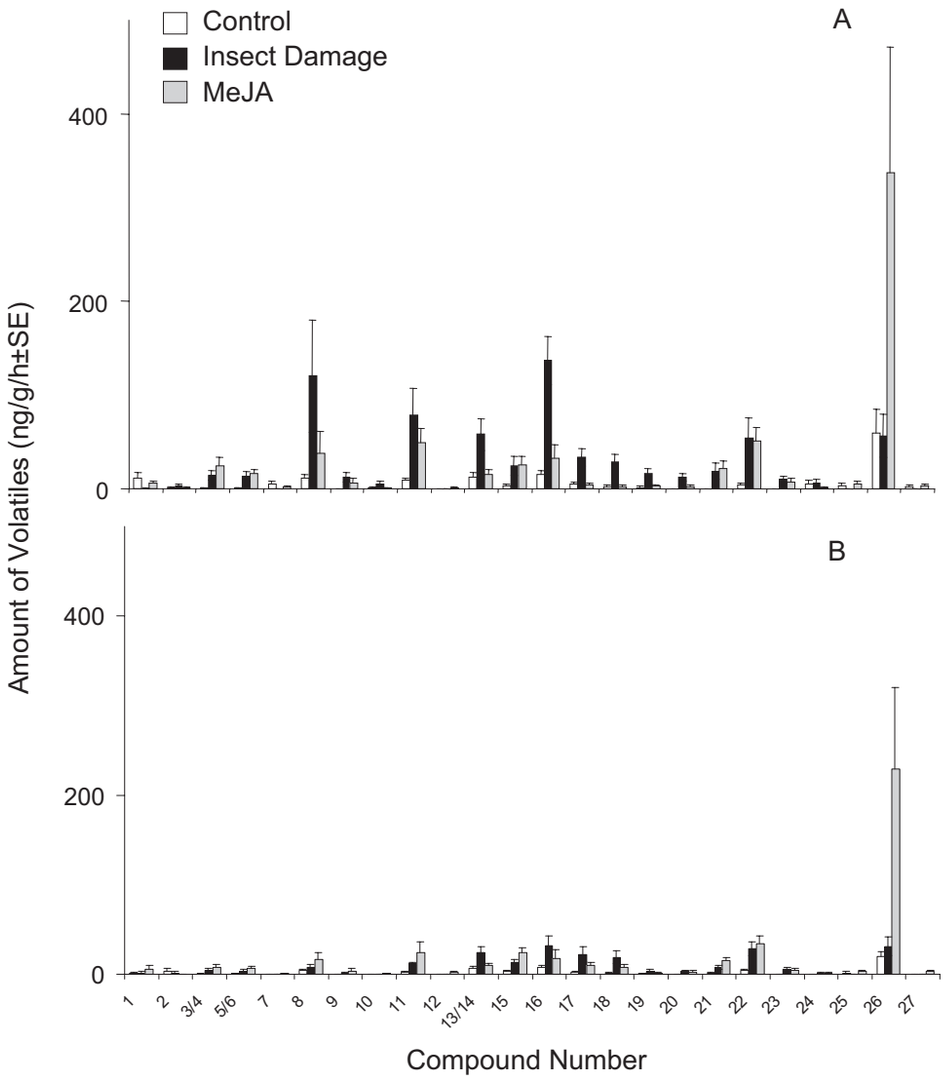

Fig. 1 Amounts of 27 volatile compounds (Table 1) collected from Manchurian ash, *F. mandshurica*. Control plants were either bagged (no insects) or sprayed with Tween-20, insect-damaged plants were fed on by both sexes of adults *A. planipennis*, and MeJA-treated plants were sprayed with a 1.4 mM solution of MeJA. Volatiles were collected during the day (09: 00-18: 00 h) (A) or night (18: 00-09: 00 h) (B)

approximately 5 mm from the antenna. Carbon-filtered and humidified air was delivered at 50 mL/min into one arm of the Y-tube via Tygon tubing. EAGs were measured as the maximum amplitude of depolarization elicited by 1 mL puffs of air delivered through pipette cartridges loaded with various doses of plant volatiles and placed in the second arm of the Y-tube. Cartridges were made by pipetting 0, 0.02, 0.2, 2, and 20 mg of each plant volatile in 20 µL hexane onto 1.4 × 0.5 cm strips of Whatman No. 1 filter paper, allowing 5 min in a fume hood for solvent evaporation, and inserting treated strips into disposable glass Pasteur pipettes. Stimulus puffs were generated in 120 ± 0.02 ms (Stelinski *et al.* 2003) with a clean, hand-held 20 mL glass syringe connected to the pipettes with a 1 cm piece of Tygon tubing. Each plant volatile was delivered in ascending dosage to 10 male and 10 female antennae. Four 1-mL puffs were administered 12 s apart to each antenna at each dosage.

Statistical analysis

A 2-way ANOVA (Systat ver. 10; SPSS Inc. Science, Chicago, IL) tested for differences in volatile emissions where treatment (insect damage and MeJA) and time of emission (day or night), and their interaction, were the independent factors. A significant treatment effect was followed by Tukey multiple comparisons tests. A t-test compared differences in volatile emissions between mechanical damage and controls, as well as the effects of bagging the plants and spraying them with Tween-20 solution. Volatile emissions (ng/g of plant material/h) were transformed by $\ln(\times + 0.05)$ prior to analysis. We summed the number of responding beetles for each treatment and compared the observed distribution of beetles with an expected 50:50 distribution by using G-tests. Data from EAG studies were also subjected to a 2-way ANOVA (SAS System for Windows ver. 8.2; SAS Institute Inc., Cary, NC), with insect sex and volatile dosage as independent variables. Differences among pairs of means between treatments were separated using Tukey tests.

Results

Collection of plant volatiles

We found no differences between volatile emissions from *F. mandshurica* seedlings when bagged or sprayed with Tween-20 solution ($t = 1.28$; df = 3; $P = 0.29$). Thus, data from both control treatments were pooled prior to analysis. These control plants emitted relatively low amounts of volatiles (Figs. 1, 2). The mean amount of daytime volatile emission from control plants was 105.5 ± 19.2 ng/g/h. Emissions from control and mechanically damaged plants (to mimic leaf-area loss by *A. planipennis* herbivory) were

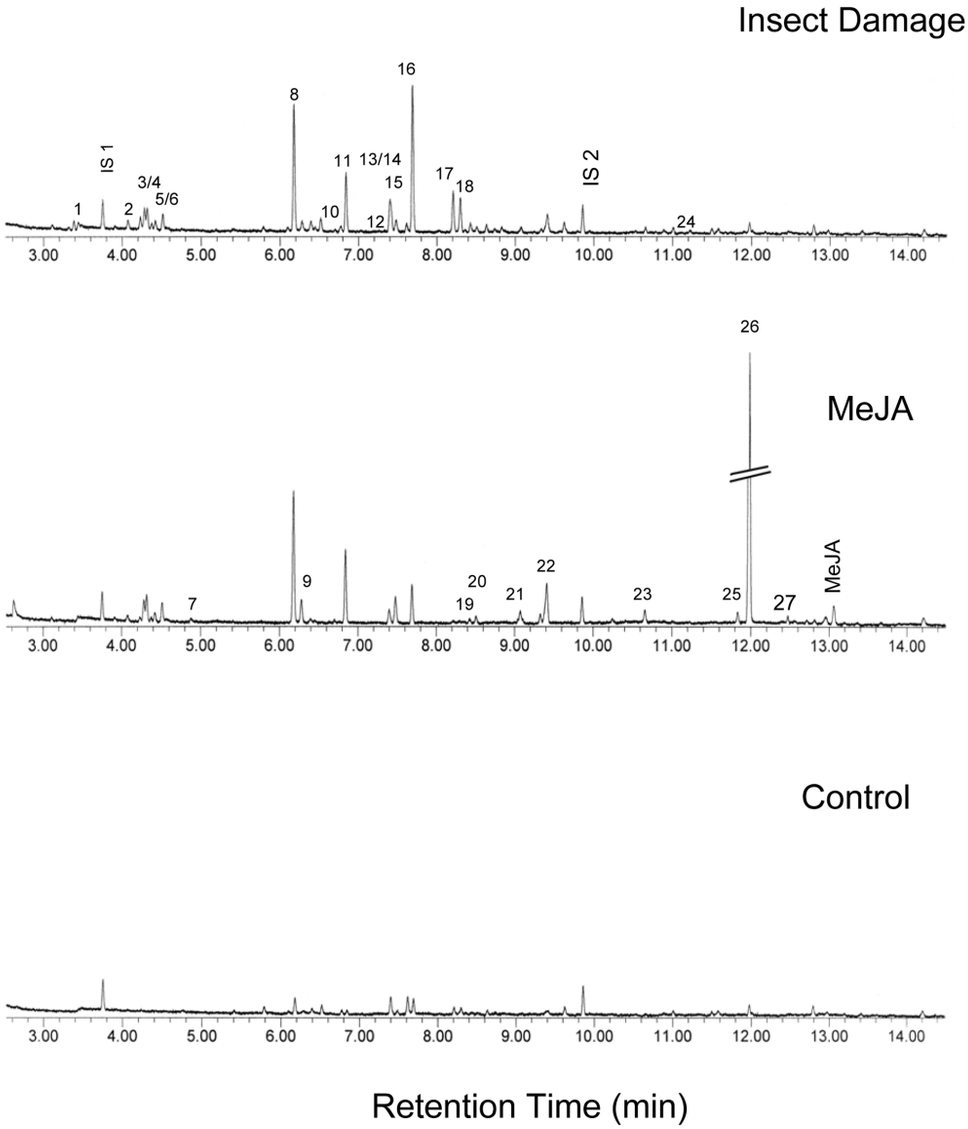

Fig. 2 Chromatograms for daytime (09: 00-18: 00 h) emission of 27 volatile compounds (Table 1) from *F. mandshurica* exposed to damage by adult *A. planipennis*, treatment with a 1.4 mM solution of MeJA, or control. Internal standards: IS 1 = octane, IS 2 = nonyl acetate. GC conditions: 15 m × 0.25 mm i.d., 0.25 µm film DB1 column at 40 °C for 1 min, increasing at 12 °C/min to 180 °C

not significantly different (111.6 ± 10.5 ng/g/h; $t = 1.44$; df = 3; $P = 0.246$).

Feeding by adult *A. planipennis* and exogenous application of MeJA significantly ($F = 13.72$; df = 2,34; $P < 0.001$) and similarly ($t = 0.59$; df = 4; $P = 0.588$) increased total volatile emissions compared to controls (Figs. 1, 2). Compared to controls, feeding by adult *A. planipennis* increased the amount of daytime volatile emissions by 6.8 fold (718.7 ± 186.7 ng/g/h), whereas MeJA treatment increased the amount of emissions 6.3 times (661.5 ± 218.8 ng/g/h). More volatiles were emitted during the day than at night ($F = 4.67$; df = 1,34; $P = 0.013$) (Fig. 1). There was no treatment × time interaction ($F = 0.35$; df = 2,34; $P = 0.601$).

Gas chromatography and mass spectrometry (GC and GC-MS)

We identified 27 compounds emitted from *F. mandshurica* (Fig. 1; Table 1) comprising > 95 % of the total volatile emissions. Compounds emitted in higher amounts from *A. planipennis*-damaged and MeJA-treated plants than from controls included: (*Z*)-3-methyl-butylaldoxime, (*E*)-3-methyl-butylaldoxime, (*Z*)-3-hexen-1-yl acetate, hexyl acetate, (*E*)-β-ocimene, benzene acetonitrile, 3-hexenyl-2-methyl butyrate, indole, and (*Z*)-jasmone ($P \leq 0.05$). Damage by *A. planipennis* increased emissions of linalool, 4,8-dimethyl-1,3,7-nonatriene, indolizine, an unidentified indole-like compound, 3-hexenyl butyrate, and methyl salicylate compared to the MeJA and control treatments ($P \leq 0.05$) (Figs 1, 2; Table 1). The MeJA treatment induced higher emissions of *E*,*E*-α-farnesene than the other two treatments ($P \leq 0.05$). We found no evidence for any insect-derived volatile(s), as the MeJA-treated plants emitted the same compounds as plants damaged by *A. planipennis*.

Behavioural assays

When data for the control, MeJA, and insect damage treatments were pooled, 74 % and 72 % of *A. planipennis* virgin females were attracted towards volatiles emitted

Table 1 Results of ANOVA for effects of treatment (damage by *A. planipennis* and exogenous application of MeJA), time of day (day or night), and their interaction, on volatile emissions of compounds from *F. mandshurica*

		Source of Variance (F values)[2]		
Compound Name and No.	ID[1]	Treatment	Time	Treatment × Time
1. Hexanal	S	$1.26^{n.s.}$	$1.49^{n.s.}$	$0.63^{n.s.}$
2. (*E*)-2-hexenal	S,L	$0.39^{n.s.}$	$0.59^{n.s.}$	$1.03^{n.s.}$
3. (*Z*)-3-hexen-1-ol	S,L			
4. (*Z*)-3-methyl-butylaldoxime[3]	S,L	19.68^{**}	5.23^{*}	$1.52^{n.s.}$
5. (*E*)-2-methyl-butylaldoxime	S,L			
6. (*E*)-3-methyl-butylaldoxime[3]	S,L	18.08^{**}	4.46^{*}	$1.31^{n.s.}$
7. 2-Butoxy ethanol	S,L	$1.45^{n.s.}$	$1.69^{n.s.}$	$1.13^{n.s.}$
8. (*Z*)-3-hexen-1-yl acetate	S,L	3.67^{*}	3.07^{*}	$2.02^{n.s.}$
9. Hexyl acetate	S,L	4.07^{*}	$2.83^{n.s.}$	$1.44^{n.s.}$
10. (*Z*)-ocimene	S,L	$0.48^{n.s.}$	$3.98^{n.s.}$	$1.26^{n.s.}$
11. (*E*)-β-ocimene	S,L	17.18^{**}	12.78^{**}	$0.46^{n.s.}$
12. Linalool oxide	S,L	$2.36^{n.s.}$	$0.01^{n.s.}$	$0.01^{n.s.}$
13. Linalool	S,L			
14. Nonanal[3]	S,L	8.41^{**}	$2.29^{n.s.}$	$0.17^{n.s.}$
15. Benzene acetonitrile	S,L	20.89^{**}	$0.00^{n.s.}$	$0.45^{n.s.}$
16. 4,8-Dimethyl-1,3,7-nonatriene	S,L	6.95^{*}	4.29^{*}	$0.37^{n.s.}$
17. Indolizine[4]	L	11.23^{**}	$0.09^{n.s.}$	$2.11^{n.s.}$
18. Unidentified, Indole-like		18.66^{**}	$1.23^{n.s.}$	$3.14^{n.s.}$
19. (*E*)-3-hexenyl butyrate	S,L	11.20^{**}	5.69^{*}	$1.73^{n.s.}$
20. Methyl salicylate	S,L	13.38^{**}	$2.46^{n.s.}$	$1.26^{n.s.}$
21. (*E*)-3-hexenyl-2-methyl butyrate	S,L	28.26^{**}	$0.02^{n.s.}$	$0.57^{n.s.}$
22. Indole	S,L	24.49^{**}	$0.52^{n.s.}$	$0.14^{n.s.}$
23. (*Z*)-jasmone	S,L	14.89^{**}	$0.42^{n.s.}$	$0.14^{n.s.}$
24. β-caryophyllene	S,L	$0.57^{n.s.}$	$1.63^{n.s.}$	$0.85^{n.s.}$
25. *Z*,*E*-α-farnesene	S,L	4.16^{*}	$0.09^{n.s.}$	$0.03^{n.s.}$
26. *E*,*E*-α-farnesene	S,L	10.55^{**}	$1.84^{n.s.}$	$0.16^{n.s.}$
27. (*E*)-nerolidol	S,L	$2.29^{n.s.}$	$0.26^{n.s.}$	$0.24^{n.s.}$
Totals		14.12^{**}	7.02^{*}	$0.51^{n.s.}$

[1] Identification (ID) was based on comparisons of retention times with authentic standard (S) and spectral data with Wiley275.L and Nist98.L libraries (L).
[2] $^{**} = P \leq 0.01$; $^{*} = 0.01 > P \leq 0.05$; n.s. = not significant.
[3] Compounds (3 and 4), (5 and 6), and (13 and 14) co-eluted.
[4] Tentatively identified, synthetic standard not available.

from insect-damaged and MeJA-treated *F. mandshurica* seedlings, respectively, significantly more than to clean air (G = 10.36; $P = 0.001$) (Fig. 3). Males did not respond significantly to either of the above stimuli (G = 0.43; $P = 0.51$), and neither sex responded significantly to the volatiles from untreated seedlings (Fig. 3).

There was: no bias in the beetles' preference for either arm of the olfactometer (G = 0.51; $P = 0.48$), no response of beetles to MeJA (G = 0.03; $P = 0.85$), and no evidence for chemical attraction of males to virgin females (G = 0.14; $P = 0.71$) or virgin females to males (G = 0.49; $P = 0.48$).

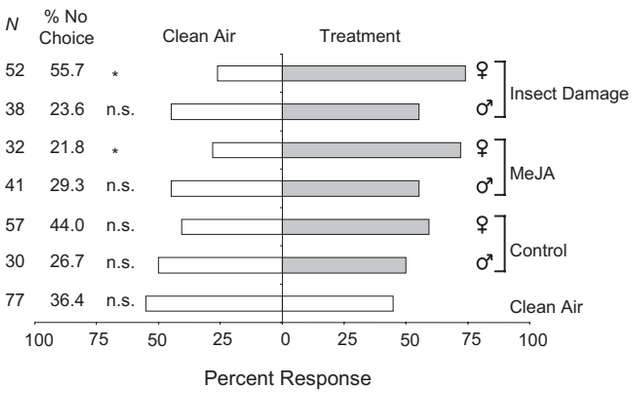

Fig. 3 Responses of walking male and female *A. planipennis* to volatiles from *F. mandshurica* in 2-choice bioassays. Asterisks represent significant differences between choices (G-test, $P \leq 0.05$); n.s. = not significant. % No Choice indicates percent of beetles that remained in central chambers

Gas chromatographic-electroantennogram detection (GC-EAD) analysis

At least 16 compounds consistently elicited an antennal response from male and female beetles (Table 2; Fig. 4). (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-2 and (*Z*)-3-methyl-butylaldoxime, (*E*)-2 and (*E*)-3-methyl-butylaldoxime, and (*Z*)-3-hexen-1-yl acetate elicited the strongest antennal responses at amounts emitted by insect damaged or Me-JA-treated plants. With respect to the aldoximes, GC-EAD responses to injections of 10 ng of synthetic (*Z/E*)-3-methyl-butylaldoxime or (*Z/E*)-2-methyl-butylaldoxime elicited only weak responses to each isomer of these compounds, suggesting that other compounds with similar retention times were present in the extracts. When the slower

Table 2 GC-EAD responses of *Agrilus planipennis* to volatiles from Manchurian ash, *F. mandshurica*

Compound Name and No.	EAD activity[1] Male (n = 6) (5 different extracts)	Female (n = 5) (3 different extracts)
1. Hexanal	+	+
2. (*E*)-2-hexenal	+++	+
3. (*Z*)-3-hexen-1-ol[2]	++++	++
4. (*Z*)-3-methyl-butylaldoxime[3]	++++	++
5. (*E*)-2-methyl-butylaldoxime		
6. (*E*)-3-methyl-butylaldoxime[4]	++	+
7. 2-Butoxy ethanol	+	+
8. (*Z*)-3-hexen-1-yl acetate	+++	++
9. Hexyl acetate	+	+
10. (*Z*)-ocimene	0	0
11. (*E*)-β-ocimene	+	+
12. Linalool oxide	+	+
13. Linalool		
14. Nonanal[4]	+	+
15. Benzene acetonitrile	0	0
16. 4,8-Dimethyl-1,3,7-nonatriene	+	+
17. Indolizine	+	+
18. Unidentified, Indole-like	+	+
19. (*E*)-3-hexenyl butyrate[5]		
20. Methyl salicylate[5]		
21. (*E*)-3-hexenyl-2-methyl butyrate[5]		
22. Indole	0	0
23. (*Z*)-jasmone	0	0
24. β-caryophyllene[5]		
25. *Z*,*E*-α-farnesene	+	+
26. *E*,*E*-α-farnesene	+	+
27. (*E*)-nerolidol[5]		

[1] 0 = no response; + = 0.01-0.03 mV; ++ = 0.04-0.06 mV; +++ = 0.07-0.10 mV; ++++ = > 0.10 mV.
[2] Based on n = 2 male and 1 female; (*Z*)-3-hexen-1-ol separated from the aldoximes when extracts were run at the slower GC program (40 ºC for 1 min, increasing at 8 ºC/min and held for 10 min).
[3] An active peak corresponding to the (*Z*)-2 isomer was also visible in GC-EAD runs.
[4] Compounds (5 and 6) and (13 and 14) co-eluted.
[5] No readings because these compounds could not be identified with certainty in the GC-EAD runs.

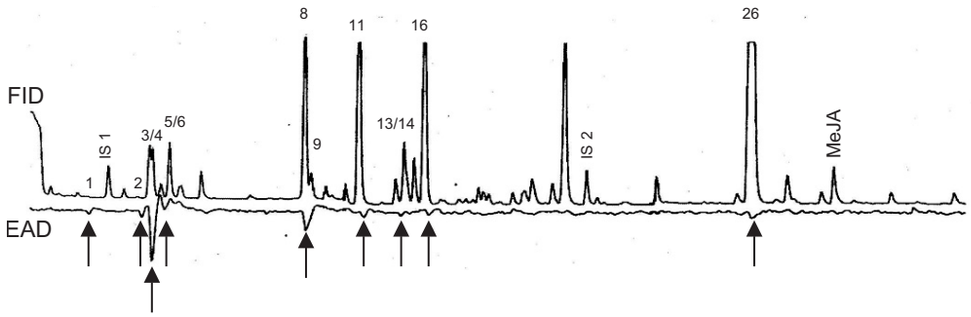

Fig. 4 GC-EAD response from male *A. planipennis* antenna to numbered compounds (Table 1) emitted from MeJA-treated *F. mandshurica*. Arrows indicate antennally-active compounds. Internal standards: IS 1 = octane, IS 2 = nonyl acetate. GC conditions were: 25m × 0.20 mm id, 0.33 µm film HP-1 column, maintained at 60 ºC for 1 min, and then increased at 35 ºC/min to 245 ºC, held for 10 min

GC program was used, (Z)-3-hexen-1-ol was separated from (*Z*)-3- and (*Z*)-2 methyl-butylaldoxime and elicited the largest EAD response of any plant volatile (up to 0.6 mV). Weaker, but repeatable, responses were found to hexanal, hexyl acetate, (*E*)-β-ocimene, linalool (sometimes with nonanal), 4,8-dimethyl-1,3,7-nonatriene, and *E*,*E*-α-farnesene. In agreement with behavioral assays, *A. planipennis* antennae did not respond to MeJA.

EAG dose-response characterizations

EAG responses of female *A. planipennis* to linalool at 2 and 20 mg dosages were greater than male responses (Fs = 91.1 and 59.2, respectively; df = 1, 216; $P < 0.001$). EAG responses by males to hexyl acetate, 2-methyl-butylaldoxime, and 3-methyl-butylaldoxime, at 200 µg, 2 mg, and 20 mg dosages, and to hexenal, *E*-2-hexenal, and *Z*-3-hexen-1-ol at all four dosages were significantly greater than female responses (Fs = 156.3, 91.1, 59.2, and 81.6, 156.3, 91.1, 59.2, respectively; df = 1, 216; $P < 0.001$).

For both sexes, EAG responses to all compounds, except indole, were significantly greater than to solvent controls at 200 µg, 2 mg, and 20 mg dosages (Fs = 18.3, 16.7, 18, 7, respectively; df = 11, 216, $P < 0.01$). Male responses to hexenal, (*E*)-2-hexenal, and (*Z*)-3-hexen-1-ol were greater than to the control at 20 µg dosage (F = 13.5, df = 11, 216,

$P < 0.001$). At 2 and 20 mg dosages, responses of males were highest, but not significantly different ($P > 0.05$), to hexenal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, 2-methyl-butylaldoxime, 3-methyl-butylaldoxime, and hexyl acetate. These responses were significantly greater ($P < 0.01$) than those elicited by the remaining six compounds. Finally, responses of males were significantly greater to (*Z*)-3-hexen-1-ol at 20 and 200 μg dosages than to the 11 other compounds at those dosages ($P < 0.001$) (Fig. 5).

EAG responses of females to linalool and (*Z*)-3-hexen-1-yl acetate, at the 2 and 20 mg dosages, were significantly greater than to the other 10 compounds at those dosages ($Fs = 16.7, 18.7$, respectively; df = 11, 216; $P < 0.01$). EAG responses of females to 2-methyl-butylaldoxime, 3-methyl-butylaldoxime, and hexyl acetate were significantly greater than to hexenal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*E*)-β-ocimene, *E*,*E*-α-farnesene, and (*Z*)-jasmone at the 2 mg dosage ($P < 0.01$). At the highest dosage (20 mg), responses of females to (*Z*)-2-hexenal, 2-methyl-butylaldoxime, and 3-methyl-butylaldoxime were significantly greater than to (*E*)-β-ocimene and (*Z*)-jasmone ($P < 0.01$) (Fig. 5).

Discussion

Volatiles from *F. mandshurica* fed on by adult *A. planipennis* or exposed to MeJA were attractive to virgin female *A. planipennis* in laboratory olfactometers. Several of the released compounds elicited antennal responses in both females and males. The use of induced plant volatiles by foraging *A. planipennis* females in host finding might enable specificity for *Fraxinus* spp. because ash trees often grow surrounded by non-host trees (Chang *et al*. 1996). Field studies by Agius *et al*. (2005) documented that females *A. planipennis* prefer green over white ash (*F. pennsylvanica* and *F. americana* L., respectively) for oviposition, via an unknown short-range mechanism. Attraction of females to induced host-plant volatiles would be of adaptive significance in aiding females on long-range dispersal flights (Taylor *et al*. 2005) to find stressed ash trees for oviposition. Mated females can fly farther than virgin females (Taylor *et al*. 2005), but we tested only the latter. Whether female responses to host volatiles change after mating remains to be determined.

The qualitative similarity in volatile compounds induced by MeJA treatment and *A. planipennis* feeding (Fig. 1) suggests that the octadecanoid-signaling pathway regulates induced volatile production in *F. mandshurica*. However, other signaling pathways may explain the quantitative differences between blends (e.g., Ozawa *et al*. 2000). Because mechanical damage alone did not trigger emission of volatiles in *F. mandshurica*, and feeding by *A. planipennis* induced the highest production, an oral secretion elicitor of plant volatiles may be involved, as is true for the fatty acid-amino acid conjugates in the regurgitant of caterpillars that trigger the octadecanoid pathway in plants (Alborn *et al*. 1997; Halitschke *et al*. 2001). An alternative hypothesis is that mechanical damage of the leaf does not adequately mimic herbivore feeding. For instance, leaves could respond differently depending on the method and duration of mechanical damage (Mithöfer *et al*. 2005). The greater emission of volatiles by *F. mandshurica* during the day than at night period (Fig. 1) is similar to the findings by Loughrin *et al*. (1994), Turlings *et al*. (1998), and Rodriguez-Saona *et al*. (2001) in other insect-host systems. *Agrilus planipennis* is active during daytime (C.R-S. per. obs; Bauer *et al*. 2004), which corresponds to the times of maximum volatile emissions. The nearly identical response of female *A. planipennis* to insect-damaged and MeJA-treated plants (Fig. 3), suggests that females respond broadly to induced-volatile emissions from *F. mandshurica* caused by various triggering factors.

Feeding by *A. planipennis* larvae blocks nutrient transport above the feeding site that can reduce photosynthesis (Urban *et al*. 2004), leading to leaf senescence (Ford and Shibles 1988), and eventually death of the tree (Bauer *et al*. 2004), which may also increase volatile emissions in ash. Because JA promotes leaf senescence (He *et al*. 2002), levels of JA, and thus volatile emissions, should increase in ash trees heavily infested by *A. planipennis* larvae. This hypothesis is supported by field experiments mimicking larval feeding damage by girdling ash trees that resulted in an increase attraction of *A. planipennis* (Poland *et al*. 2005). Attraction of females to previously infested trees would be adaptive because *A. planipennis* larvae develop faster in heavily infested than lightly infested trees (Cappaert *et al*. 2005).

It is unclear why male *A. planipennis* antennae respond to the same *F. mandshurica* volatiles as females, sometimes with a stronger response than females (Fig. 5), yet males did not respond to host-plant volatiles in the olfactometer (Fig. 3). It is possible that the olfactometer did not allow for the full range of host-finding behavior by male *A. planipennis*. Male *A. planipennis* might use plant volatiles as short-distance contact cues or as indicators of the presence of conspecifics. The fact that neither sex was attracted to the other suggests a lack of a sex or aggregation pheromone. The discrepancies between behavioral and electrophysiological responses are not surprising because constituents in the volatile blend from *F. mandshurica* might interact synergistically or antagonistically on female and male *A. planipennis* behavior, respectively, which is not detected when the antennal analysis focuses only on individual compounds. Furthermore, the attraction of arthropods to induced plant volatiles often increases by associative learning (Vet & Groenewold 1990). In our study, larvae and adult *A. planipennis* were reared on a species of ash different from *F. mandshurica*, which could have influenced the beetles' response to host plant volatiles in the choice test.

There were a number of similarities and a few differences obtained by GC-EAD and EAG recording techniques in response to common plant stimuli. Both methods detected sexual dimorphism in antennal responses. Differences in sensitivity between sexes might be indicative of sex-specific differences in the importance of certain volatile cues in host finding (Light *et al*. 1988). For example, females *Lygocoris pabulinus* (L.) (Heteroptera: Miridae) were more sensitive to plant volatiles that guide them to ovipositional sites, while males were more sensitive to insect-derived compounds (Groot *et al*. 1999). Ruther *et al*. (2000) also found that, while antennae of both sexes of the forest cockchafer, *Melolontha hippocastani* F., responded to host plant volatiles, only males were attracted in behavioral assays. Both

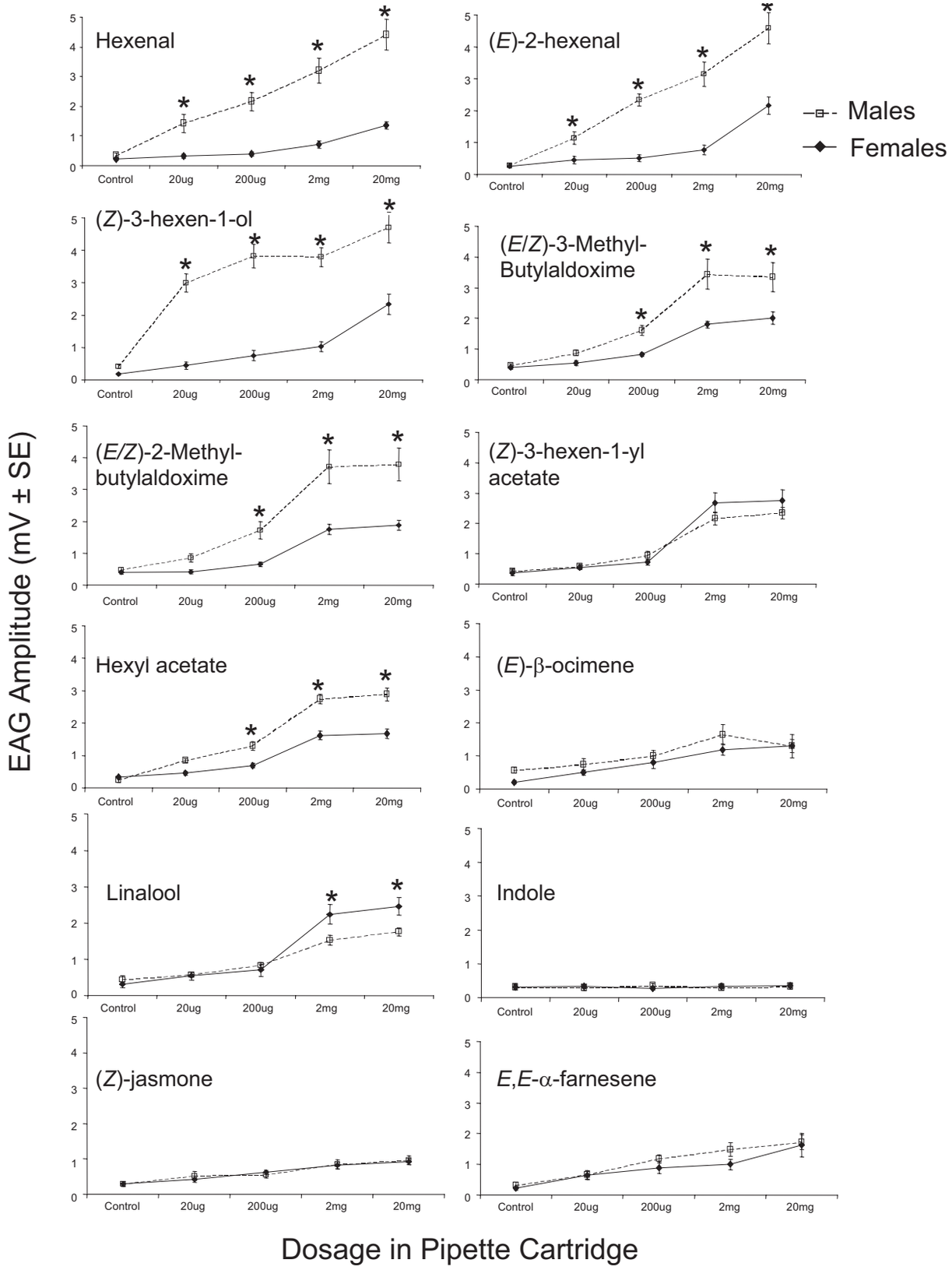

Fig. 5 Unnormalized EAG dose-response profiles for male and female *A. planipennis* to *F. mandshurica* volatiles presented to antennae in ascending order of stimulus dosage. $n = 10$ for each dosage

GC-EAD and EAG techniques recorded weak responses to most terpenoids, including (*E*)-β-ocimene, linalool, and *E*,*E*-α-farnesene, and in both cases there was no response to indole. The major difference between techniques was in response to the synthetic aldoximes: while substantial responses were obtained by EAG, GC-EAD responses were weak.

Both feeding and MeJA induced the production of antennally-active (Figs. 4, 5) green leaf volatiles (GLVs), terpenoids, and nitrogenous compounds, derived from at least three biosynthetic pathways, and often induced by herbivore feeding (Paré & Tumlinson 1999). Even though most GLVs were present in small amounts, all elicited stronger antennal responses than the more abundant terpenoids, suggesting a prominent role in host-plant finding for the former. The two antennally-active nitrogenous compounds, 3-methyl-butylaldoxime and 2-methyl-butylaldoxime, are natural scents of night-active flowers and are attractive to pollinators (Andersson *et al*. 2002). Producing these compounds, as well as indole and indole-like compounds, might be costly to *F. mandshurica* because they are derived from essential amino acids.

The ecological costs of herbivore-induced volatiles may be high, because many herbivore species, especially beetles, are attracted to volatiles induced by conspecifics (e.g. Loughrin *et al*. 1996; Bolter *et al*. 1997; Heil 2004). Because *A. planipennis* is not a pest in Asia (Williams *et al*. 2005; Schaefer 2005), ecological costs of induced volatile emissions in ash might be low in Asia, but high in North America. The emission of inducible volatiles in *Fraxinus* spp. should provide benefits for plant protection that outweigh the metabolic cost of production and the ecological cost of attracting specialist herbivores (Hoballah *et al*. 2004). Because there are no known efficient parasitoids or predators that attack *A. planipennis* either in Asia or North America (Liu *et al*. 2003; Bauer *et al*. 2005), the metabolic and ecological costs would not be offset by attraction of entomophagous insects. The arrival of *A. planipennis* in North America may negate the ecological benefits to green ash in producing linalool, methyl salicylate, and farnesenes that repel larvae of the gypsy moth, *Lymantria dispar* L. (Markovic & Norris 1996).

Since its introduction to North America from Asia, *A. planipennis* has been devastating to ash trees. Development of efficient trapping techniques would greatly improve detection and monitoring programs that currently rely on visual symptoms of trees, which are not evident until at least the second year of attack. We identified several compounds from *F. mandshurica* that, upon further development, might be useful as attractants for *A. planipennis*. Field studies are underway to test this hypothesis.

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