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Sex-Specific Trail Pheromone Mediates Complex Mate Finding Behavior in *Anoplophora glabripennis*

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Abstract *Anoplophora glabripennis* (Motsch.) is a polyphagous member of the Cerambycidae, and is considered, worldwide, to be one of the most serious quarantine pests of deciduous trees. We isolated four chemicals from the trail of *A. glabripennis* virgin and mated females that were not present in trails of mature males. These compounds were identified as 2-methyldocosane and (*Z*)-9-tricosene (major components), as well as (*Z*)-9-pentacosene and (*Z*)-7-pentacosene (minor components); every trail wash sample contained all four chemical components, although the amounts and ratios changed with age of the female. Males responded to the full pheromone blend, regardless of mating status, but virgin females chose the control over the pheromone, suggesting that they may use it as a spacing pheromone to avoid intraspecific competition and maximize resources. Virgin, but not mated, males also chose the major pheromone components in the absence of the minor components, over the control. Taken together, these results indicate that all four chemicals are components of the trail pheromone. The timing of production of the ratios of the pheromone blend components that produced positive responses from males coincided with the timing of sexual maturation of the female.

Keywords Sex trail pheromone · Invasive species · 2-Methyldocosane · (*Z*)-9-Tricosene · (*Z*)-9-Pentacosene · (*Z*)-7-Pentacosene · Mate finding · Coleaptera · Cerambycidae

Introduction

Trail pheromones are best known from eusocial insects, such as ants (Morgan 2009) and termites (Bordereau and Pasteels 2011), and are used primarily to mark the path to a food source. However, trail pheromones also can be gender specific, playing a role in mate finding in a wide range of animals, including insects (Kapranas et al. 2013; Sillam-Dusses et al. 2011; Wang et al. 2002), snails (Ng et al. 2011), snakes (Shine et al. 2005), and even copepods (Yen et al. 2011). Very few sex-specific trail pheromones are known in beetles, with the exception of the cerambycid *Nadezhdiella cantori*, in which females leave trails of the contact sex pheromone on trees when walking, which males use to locate females but this pheromone was not chemically identified (Wang et al. 2002).

Anoplophora glabripennis (Coleoptera: Cerambycidae: Lamiinae) is a long-horned beetle and is ranked among the top quarantine pests in the world (MacLeod et al. 2002). Native to Asia, this beetle is responsible for

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significant losses of high-value shade and timber tree species (Haack et al., 2010). Mate finding in *A. glabripennis* is challenging. Although the adult can fly, it is fairly sedentary, preferring to re-infest its natal host tree until resources are exhausted (Williams et al. 2004). Males are smaller and fly more often (Yasui et al. 2007) and farther than females (Smith et al. 2004; Williams et al. 2004), but are also more likely to remain on a host tree than fly when they are well fed and have just mated (Keena unpublished data). *Anoplophora glabripennis* has a host range of over 43 hardwood tree species in urban and forest habitats in its native and introduced range (Haack et al. 2010; Hu et al. 2009). Thus, the beetle must find a mate among a forest of potential host trees using a weakly attractive long-range sex pheromone (Nehme et al. 2010). This suggests that the use of other chemical cues or signals might be involved in improving the likelihood of finding a mate.

Mate finding and copulation in *A. glabripennis* involves a complex series of behaviors and responses to several chemical cues and signals. A volatile male-produced pheromone, composed of two dialkyl ethers (Zhang et al. 2002), primarily attracts virgin females; attraction is enhanced by plant kairomones, but trap catches in field trials testing addition of these chemicals were low, although the study was performed in an area with a low beetle population (Nehme et al. 2010). After landing on a host tree, both sexes walk along the upper bole and branches of the tree (where they feed), touching the substrate with their mouthparts and antennae (Li et al. 1999). Feeding, for about 10–15 days, is required by females for their ovaries to mature (Li and Liu 1997). Once a male encounters a female, he antennates the female's cuticle and, upon recognizing her as a conspecific through detection of her contact pheromone, quickly mounts and attempts to mate (Zhang et al. 2003). The female-produced contact sex pheromone consists of five monounsaturated cuticular compounds: the alkenes (*Z*)-9-tricosene, (*Z*)-7-pentacosene, (*Z*)-9-pentacosene, (*Z*)-7-heptacosene, and (*Z*)-9-heptacosene. For males, both mate location and mating, in addition to chemoreception, appear to involve vision (He and Huang 1993).

To add to this complexity, laboratory observations of *A. glabripennis* mating behavior suggest that the female deposits a trail pheromone that also is involved in mate location (unpublished data). We frequently have observed that when males are added to a branch after a female has already walked on it, they will walk slowly while palpating the bark as if following a trail directly to the female.

Herein, we report the identification of, and behavioral responses to, a four-component, sex-specific trail pheromone produced by *A. glabripennis* females. In addition, we determined the onset and period of secretion of the pheromone and its relationship to the mating status of females. Finally, we examined how mating status affected behavioral responses of both males and females to the pheromone.

Methods and Materials

Insects Adult *A. glabripennis* used in laboratory studies were from populations maintained on artificial diet at 25°C, 60 % humidity and 16:8 h L:D (Keena 2005). The beetles used in these studies originated from adults that emerged from infested branch sections obtained at quarantine sites in the United States or from larvae extracted from trees in China and shipped on artificial diet; both were transported under permit to the USDA-Forest Service quarantine facility in Ansonia, CT, USA. The infested branch sections were obtained in February 1999 from the Ravenswood, Chicago, Illinois infestation (1,450 adults, 041.58° N and 087.42° W), and in April 1999 from the Bayside, Queens, New York infestation (384 adults, 040.45° N and 073.45° W). Larvae were obtained from Hohhot City, Inner Mongolia, China, in November 2001 (*N*= 286, 040.82° N and 111.60° E). Individuals from all three populations were used to isolate the compounds and determine the timing of pheromone production, but only the Chicago population was used for behavioral bioassays, due to greater availability of adults for our experiments.

Following eclosion, virgin adults were held individually in 950 ml glass jars and kept at 25 °C, 60 % humidity, 16:8 h L:D cycle, with constant ventilation, until used. *Acer saccharum* (sugar maple) or *Acer platanoides* (Norway maple) twigs (3–7 mm diam.), from which leaves had been removed, were added once a week as a food source; twigs were cut fresh weekly and stored in plastic bags at 5 °C. Folded paper towels were placed in the bottom of the jars to collect frass and excess moisture. Two holes (1–2 mm diam.) were drilled in the plastic lids on the jars to allow airflow.

To assure mating status, single pairs of beetles were placed in 3.75 l glass jars for 8 h and observed. Beetles had to mate for at least 3 min (aedeagus extension and insertion) to be considered mated (Keena 2002). After mating, the pair remained together in the jars with Norway maple twigs and a 5×20 cm Norway maple bolt for oviposition. Healthy 11–52 d-old adult beetles were used in the behavioral experiments, while females of different age groups, based on maturation, feeding or mating status, were tested to determine the timing and amount of trail pheromone deposited.

Pheromone Collection To collect chemicals deposited on the substrate by walking beetles, 11–49 d-old virgin males and females, or males and females 48 h after mating, were placed in 100 X 25 mm Petri dishes (NUNC™ Lab-Tek™) lined with filter paper and held at 25 °C, 60 % RH and a 16:8 h L:D cycle for 24 h. After the adults and frass were removed, the Petri dishes with filter paper were sealed with Parafilm and stored at

4 °C until shipped overnight in an ice chest to USDA Agricultural Research Service in Beltsville, MD, USA for processing. To extract chemicals, the filter papers were placed individually in a 20 ml vial and soaked in 5 ml hexane for 24 h. The solutions were stored at −30 °C until analysis. Body-washes were prepared from virgin and mated females from the same colony as used for laboratory bioassays.

Instrumentation All extracts were run as individual samples on an HP 6890 gas chromatograph (GC) equipped with a flame ionization detector (FID) and a 60 m×0.25 mm ID, 0.25 μm film-thickness DB-5 (150 °C and held for 2 min, then heated to 300 °C at 15 °C.min^{-1} and held for 30 min) or a 60 m×0.25 mm ID, 0.25 μm film-thickness DB-Waxetr capillary column (50 °C and held for 2 min, then heated to 250 °C at 15 °C.min^{-1} and held for 15 min, J&W Scientific Inc., Folsom, CA, USA) in the splitless mode with hydrogen (1.4 ml.min^{-1}) as carrier gas. Electronic impact (EI) GC/mass spectrometry (GC/MS) was conducted on an HP 6890 GC coupled to an HP 5973 Mass Selective Detector using a 30 m DB-5 (0.25 mm ID, 0.25 μm film-thickness) capillary column; the temperature program was the same as the GC analyses described above, except helium was the carrier gas. A 70 eV electron beam was employed for sample ionization.

Synthesis of Putative Pheromone Components Long-chain monounsaturated hydrocarbons [(*Z*)- and (*E*)-9-tricosene (*Z*9-C_{23} and *E*9-C_{23})], were synthesized by alkylation of 1-decyne with 1-bromotridecane and subsequent selective reduction by catalytic hydrogenation with Lindlar's catalyst (Oliver et al. 2000) or by sodium in liquid ammonia/THF (Na/NH_3/THF) (Doolittle et al. 1993) to obtain (*Z*)- and (*E*)-olefins (97 % purities), respectively. Other monounsaturated hydrocarbons were synthesized using the corresponding 1-alkynes and bromides in the same manner (Zhang et al. 2003).

2-Methyldocosane (2-Me-C_{22}) was synthesized by alkylation of 4-methylpentyne with 1-bromoheptadecane and subsequent hydrogenation by 5 % palladium on carbon in acetic anhydride using standard procedures. **(1)** Preparation of 1-bromoheptadecane: heptadecanol (2.565 g, 0.0 l mol) was dissolved in dry CH_2Cl_2 (10 ml) and 0.05 ml of pyridine added (20 mol %). The mixture was treated with PBr_3 (0.0 l mol, 2.707 g) at room temperature for 64 h, with thin layer chromatography or GC monitoring. The reaction mixture was cooled in an ice bath, and 10 ml of saturated $NaHCO_3$ were added to quench the reaction. The aqueous layer was extracted with dichloromethane (4×10 ml), and the combined organic layers washed with distilled water until neutrality was reached. The organic layer was dried with Na_2SO_4 and the solvent removed using a rotary evaporator. The crude residue was then flash chromatographed using hexane to produce 1-bromoheptadecane 2.69 g (97 % purity, 85 % yield). EI-MS *m/z* (%): 318 [M^+] (0.25), 239 (1.0), 179 (1.0), 177 (1.0), 165 (2.5), 163 (2.7), 151 (14.5), 149 (15.0), 137 (72.8), 135 (75.3), 113 (7.3), 111 (6.4), 99 (12.6), 97 (20.2), 85 (45.2), 83 (29.1), 71 (67.3), 69 (44.1), 57 (100), 55 (55.6). (**2**) Preparation of 2-methyl-4-docosyne: 4-methyl-1-pentyne (457.5 mg, 1.5 equiv., 5.57 mmol) was dissolved in 10 ml of THF and the mixture cooled to −40 °C under argon or nitrogen. LiBu (2.5 mol/L, 1.5 equiv., 2.23 ml, 5.57 mmol) was added dropwise with a syringe within 0.5 h. The mixture was stirred for 2 h at −20 °C, and then cooled to −40 °C. Two ml of HMPA (11.0 mmol, 3.0 equiv) and 50 mg (0.33 mmol, 3 mol % of 4-methyl-1-pentyne) of NaI were then added. 1-Bromoheptadecane (1.186 g, 3.71 mmol) was added slowly, and then the dry ice bath was removed. The reaction was kept at ambient temperature for 64 h and monitored with GC. The reaction was quenched by adding saturated aqueous NH_4Cl at 0 °C. The water layer was extracted with hexane three times (3×10 ml). The combined extract was successively washed with water and brine, dried with Na_2SO_4, and concentrated under vacuum. The residue was chromatographed on silica gel (50 g) using hexane as a solvent to give 1.23 g of colorless liquid 2-methyl-4-docosyne (98 % purity, 70 % yield). EI-MS *m/z* (%): 320 [$M]^+$ (3.0), 305 (1.0), 277 (1.0), 263 (3.0), 249 (1.0), 235 (1.0), 221 (1.0), 207 (1.0), 193 (1.0), 179 (1.0), 165 (1.0), 151 (3.0), 137 (14.0), 123 (18.0), 109 (31.0), 95 (66.0), 81 (100), 67 (40.0), 55 (27.0). (**3**) Hydrogenation of 2-methyl-4-docosyne: 2-methyl-4-docosyne (610 mg, 1.906 mmol) was dissolved in 25 ml of Ac_2O and 20 mg of Pd-C catalyst were added. The hydrogenation was carried out at room temperature and at 372 kPa. The reaction was stopped by filtering the catalyst. After removing the solvent, 2-methyl-docosane (611 mg) was obtained as a white solid (99 % purity, 98 % yield). EI-MS *m/z* (%): 324 [$M]^+$ (1.0), 309 (4.0), 281 (16.0), 267 (1.0), 253 (1.0), 139 (2.0), 225 (2.0), 211 (3.0), 197 (4.0), 183 (4.0), 169 (5.0), 155 (6.0), 141 (8.0), 127 (11), 113 (16.0), 99 (28.0), 85 (69.0), 71 (88), 57 (100).

11- and 13-Methyl branched hydrocarbons were synthesized by Wittig reaction of the corresponding methyl ketones (prepared by Grignard reactions of corresponding bromides and aldehydes followed by PCC oxidation) with methyltriphenylphosphonium iodide (Maercher 1965) followed by hydrogenation. Saturated hydrocarbons and all other chemicals and solvents were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Dimethyl disulfide (DMDS) derivatives of extracts and synthetic standards were prepared according to standard procedures (Dunkelblum et al. 1985; Zhang et al. 1994).

Pheromone Preparation for Bioassay For behavioral assays, the four pure components were diluted with hexane (96.6 % purity, EMD Chemicals, Gibbstown, NJ, USA) to obtain the ratios and concentrations consistent with the naturally occurring compounds produced by mature females (present in female trails, Fig. 1a, peaks 1, 2, 10, & 11, but not detected

in mature male trails, Fig. 1b). A blend of the two major trail compounds (present in mature female trails, Fig. 1a, peaks 1 & 2, but in negligible amounts in immature virgin female trail, Fig. 1c) were tested in their natural ratio of 1:6 (2-Me-C_{22}:*Z*9-C_{23}), while a blend of the two minor components (present in mature female trail, Fig. 1a, as well as female whole-body wash, Fig. 1d, peaks 10 & 11, but in negligible amounts in immature virgin female trail, Fig. 1c) were tested in their natural ratio of 1:1 (*Z*9-C_{25}:*Z*7-C_{25}). The full four-component pheromone blend was prepared in its natural ratio of 2:12:1:1 (2-Me-C_{22}:*Z*9-C_{23}:*Z*9-C_{25}:*Z*7-C_{25}).

Behavioral Responses to Trail Pheromone Y-shaped Norway maple twigs (~20 cm long main branch, ~10 cm long arms) with the arms at a ~45° angle were used as the choice test arena. Branch bases were no more than 1 cm diam. Branches were 70–100 cm long between the base and fork. The ends of the fork were 14–40 cm apart.

For each twig, ~500 μl of a 5 μg/μl solution [~5 female equivalents (FE)] of the full, four-component trail pheromone were applied evenly to the surface of one of the arms by paint brush (Plaid Enterprises, Inc., Norcross, GA, USA), taking care to cover the entire circumference of the arm, starting at the bottom of the fork of each branch. For the control arm, 500 μl of hexane were applied in the same manner. Separate blends also were made for testing the two major components and the two minor components at the amounts and ratios they occur in the natural full pheromone blend. Branches were dried in a laminar flow hood for 15 min after application. Branches were replaced with a freshly painted and dried branch after being tested with 5 beetles of the same gender and mating status.

For each test, the insect was placed at the bottom of the twig and given 15 min., after beginning its first ascent, to make a choice. *Anoplophora glabripennis* has a tendency to move upwards when placed on a substrate so there was no need to prevent beetles from walking off the twig. A beetle was considered to have made a choice after it had palpated both arms of the twig, and then moved onto one arm so that its entire body (including all six tarsi) was on it. Beetles that just ran quickly up the twig and palpated 0–1 arms were immediately returned to the bottom of the twig for another opportunity to make a choice (the test of each beetle was ended when a choice was made or 15 min. had elapsed since the beginning of the test). Time to choice during each test was calculated as the time from when the beetle was last placed at the bottom of the twig until it made the choice; time spent in previous opportunities during the same test that failed to result in a choice were not included. Each beetle was tested twice with a rest period of ~60 min. between tests; the response was considered valid only if an insect made the same choice in both tests. A "no choice" category was assigned for insects that made two different choices. For the second test, the same branch used in the first test for each set of 5 beetles was re-painted, dried, and the treatment arm flipped 180° so that it was in the opposite position from its orientation in the first test. Each chemical blend was tested to 19 males and 22 females as virgins, and 18 males and 17 females 48–72 h after mating; the same individuals were tested as virgin and mated females.

All insects were tested to the full, major, and minor blends, each separately against the solvent control, to determine if both the major and minor components are necessary components of the pheromone. The same virgin and mated males and females used in the full blend tests were used 24 h later, with at least a 120 min. rest period (the time required to test all individuals twice) allowed between the two blends. Mated males were re-tested again (a third time) after 24 h of isolation for their response to the full trail pheromone blend only; they only were tested once because of their advancing age, to determine if a period of isolation after exposure to the female altered their response.

Behavioral Responses to Major Trail Pheromone Components in the Field in China The two major components of the female trail pheromone, in the same ratio as described above for laboratory bioassays, were also tested in the field in Guang Xia, Ningxia Province, China (38°06′82.40″ N; 105°91′ 60.60″ E) in July of 2007, before we had identified the two minor trail pheromone components. Seven white, rectangular paper stickers (1.5×0.5 cm) were placed ~5 cm apart on the trunk of a willow tree, forming an upward spiral which, from bottom to top, reached approximately 50 % of the circumference of the tree. One hundred μl of a 5 μg/μl solution (~1 FE) of the major pheromone components at the same ratio as described for the laboratory bioassays were dissolved in hexane and applied with a paint brush to coat the surface of the stickers sufficiently without run-off. Six males and six females (of unknown age and mating status) were collected from the field and kept in separate cages until use. After allowing the stickers to dry, beetles were introduced immediately below the first sticker and allowed to walk freely on the trunk. Males were tested first to avoid confounding effects of female trails. No beetles were observed walking on the tree for at least 4 h prior to the start of the experiment. Beetles were assigned scores from 1 to 7, reflecting the number of the 7 stickers walked over by the beetles. We recorded responses, including palpation, arresting, walking, tasting and antennation.

Statistical Analyses Choice data were analyzed by contingency analysis, followed by a probability test with a hypothetical probability set at 50 %, producing *Chi-square* statistics, followed by Fisher's exact test to obtain *P*-values. No-choice results were included in the analyses. Paired *t*-tests were used to compare mean time to choice for each group of beetles, *e.g.*,

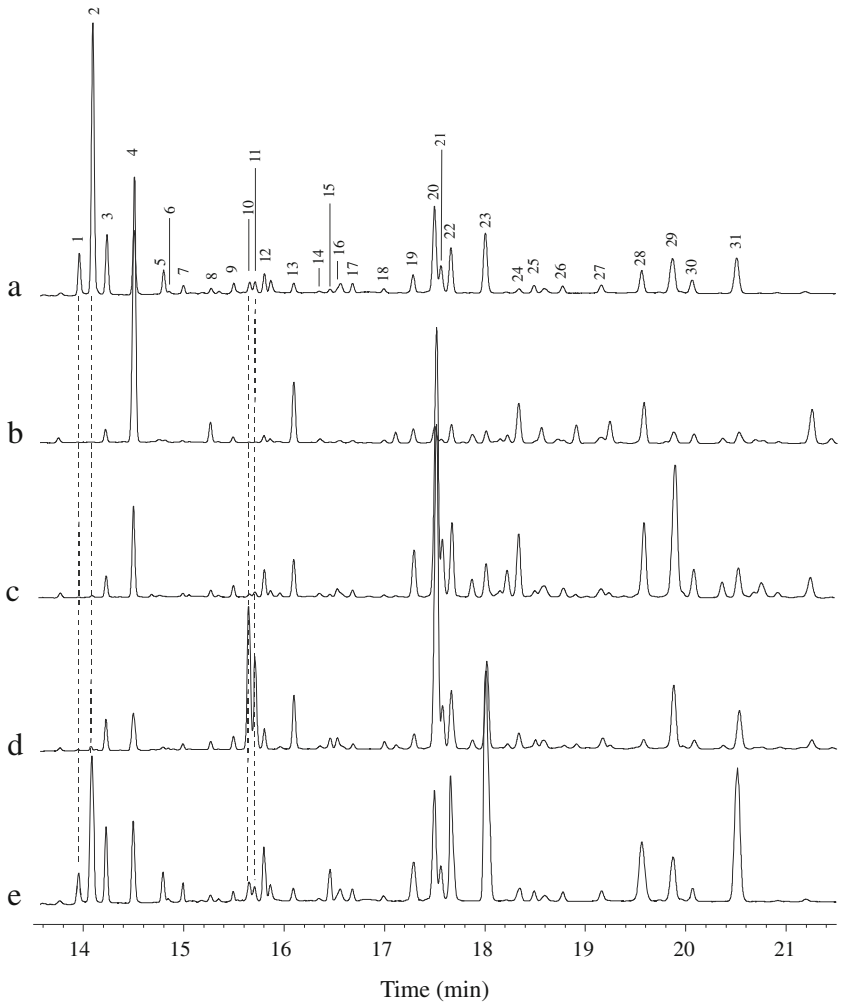

Fig. 1 Gas chromatograms (flame ionization detection) of *Anoplophora glabripennis* hydrocarbons from filter paper trail-washes of mature (20 d-old) virgin females (**a**) *vs.* mature (20 d-old) virgin males (**b**), immature (11 d-old) virgin females (**c**), as well as from whole-body washes of mated (36 d-old) females (**d**) and virgin (49 d-old) females (**e**) on a 60 m DB-5 capillary column. Numbered peaks refer to compounds in Table 1

females before and after mating. The scores (1–7) for the number of stickers walked on by beetles in the field experiment in China were compared between sexes using Wilcoxon's test. Scores for each male and female were also compared to a hypothetical score of 1, because all beetles touched at least the first sticker, using Wilcoxon's two-sample test. All data were analyzed using JMP 10 Pro (SAS Institute Inc.) and R Core Team (2012).

Results

Pheromone Identification We identified four chemicals from the trail wash of *A. glabripennis* mature, virgin females (20 d post-adult eclosion) that were present in only minute amounts in trail wash from immature, virgin females (11 d post-adult eclosion) and completely absent from trails of males (Table 1). These compounds were identified as 2-methyldocosane, (*Z*)-9-tricosene, (*Z*)-9-pentacosene, and (*Z*)-7-pentacosene in an approximate ratio of 2:12:1:1. Thirty one compounds were identified by GC/MS. Analyses of filter paper samples derived from the same-age virgin female and male beetles (20 d-old) showed that they shared a series of compounds (Fig. 1a,b), with the exception of four components (peaks 1, 2, 10, & 11, Fig. 1a) that were associated with female extracts and were undetectable in samples derived from the same age virgin males (Fig. 1b). These four female-specific components accounted for about 17 % of the total deposited on filter papers by virgin females (Table 1); one of the compounds (peak 2) was the most abundant compound in the GC profile (Fig. 1a). Only barely detectable amounts (<0.3 %) of the first two compounds (peaks 1 & 2, Fig. 1a) were detected in samples obtained from younger virgin females (11 d-old; Fig. 1c). They also were detected in adult female cuticular extracts (whole body-washes; Fig. 1d,e, Table 1). These results provide the rationale for referring to compounds comprising peaks 1 and 2 as the major components and those comprising peaks 10 and 11 as the minor components of the trail pheromone blend.

The EI mass spectra of the two major female-specific compounds contained molecular ions at *m/z* 324 (peak 1,

Table 1 Relative abundance of hydrocarbons (mean %±SD) identified from trail and body wash samples of *Anoplophora glabripennis*; trail pheromone components in bold

Peak #[a]	Kovats Index		Compound	Percentage of total hydrocarbon (mean ± SD, *N*=3)					
	DB-5MS	DB-Wax		♀ trail (20 d-old)	♂ trail (20 d-old)	♀ trail (11 d-old)	♀ body-wash (adult virgin)[b]	♀ body-wash (adult mated)[c] *m/z*	Diagnostic MS ions
1	**2261**	**2252**	**2-MeC$_{22}$**[d]	**2.09±1.01**	**nd**	**0.06±0.01**	**1.71±1.09**	**0.15±0.21**	**281 ([M-43]$^+$), 309 ([M-15]$^+$), 324 ([M]$^+$)**
2	**2277**	**2325**	***Z*9C$_{23}$**[d]	**13.24±6.31**	**nd**	**0.20±0.05**	**7.03±0.97**	**3.05±5.09**	**322 ([M]$^+$)**
3	2300	2300	*n*-C$_{23}$[d]	3.13±1.22	1.25±0.19	1.29±0.07	4.27±1.00	3.36±0.94	324 ([M]$^+$)
4	2333	2325	11-MeC$_2$[d]	4.05±0.78	23.29±3.73	3.76±1.61	4.76±1.50	2.36±0.06	168/169, 196/197, 323 ([M-15]$^+$)
5	2371	2368	3-MeC$_{23}$	1.80±0.99	0.41±0.06	0.38±0.15	1.22±0.42	0.53±0.31	309 ([M-29]$^+$), 323 ([M-15]$^+$), 338 ([M]$^+$)
6	2377	2422	*Z*9C$_{24}$[d]	0.29±0.05	0.04±0.02	0.59±0.25	0.44±0.62	0.18±0.06	336 ([M]$^+$)
7	2400	2400	*n*-C$_{24}$[d]	0.79±0.13	0.13±0.01	0.47±0.05	0.92±0.57	1.93±0.52	338 ([M]$^+$)
8	2430	2422	11-MeC$_{24}$	0.67±0.01	1.50±0.36	0.52±0.01	0.49±0.04	0.39±0.06	168/169, 210/211, 337 ([M-15]$^+$)
9	2460	2452	2-MeC$_{24}$	1.28±0.01	0.40±0.11	0.77±0.04	1.00±0.08	0.94±0.71	309 ([M-43]$^+$), 337 ([M-15]$^+$), 352 ([M]$^+$)
10	**2478**	**2524**	***Z*9C$_{25}$**[d]	**1.03±0.37**	**nd**	**0.37±0.14**	**1.12±0.89**	**7.78±4.45**	**350 ([M]$^+$)**
11	**2485**	**2532**	***Z*7C$_{25}$ e**	**1.66±0.86**	**nd**	**0.61±0.29**	**2.05±1.60**	**4.76±1.54**	**350 ([M]$^+$)**
12	2500	2500	*n*-C$_{25}$[d]	1.55±0.10	0.67±0.11	1.59±0.04	2.78±0.60	5.50±0.39	352 ([M]$^+$)
13	2530	2524	11-MeC$_{25}$[d]	1.92±0.65	5.65±0.16	2.07±0.46	1.40±1.01	2.26±0.60	168/169, 224/225, 351 ([M-15]$^+$)
14	2571	2569	3-MeC$_{25}$	0.58±0.03	0.38±0.09	0.56±0.15	0.57±0.25	0.59±0.16	337 ([M-29]$^+$), 351 ([M-15]$^+$)
15	2577	2622	*Z*9C$_{26}$[d]	0.69±0.06	0.12±0.08	0.39±0.17	0.68±0.23	0.61±0.06	364 ([M]$^+$)
16	2584	2631	*Z*7C$_{26}$[d]	1.40±0.18	0.31±0.01	1.03±0.11	1.42±0.99	2.31±0.93	364 ([M]$^+$)
17	2600	2600	*n*-C$_{26}$[d]	1.11±0.02	0.23±0.08	0.61±0.07	2.02±0.55	2.71±0.84	366 ([M]$^+$)
18	2627	2620	12-MeC$_{26}$; 13-MeC$_{26}$	1.00±0.18	0.30±0.04	0.38±0.11	1.05±0.52	1.06±0.32	182,183, 224/225; 196/197, 210/211
19	2660	2654	2-MeC$_{26}$	2.08±0.06	1.10±0.01	2.94±0.64	3.30±0.59	2.93±0.61	337 ([M-43]$^+$), 365 ([M-15]$^+$), 380 ([M]$^+$)
20	2677	2732	*Z*9C$_{27}$[d]	8.80±1.41	1.81±0.18	19.90±0.83	7.20±0.93	21.72±7.88	378 ([M]$^+$)
21	2686	2737	*Z*7C$_{27}$[d]	2.50±0.19	0.48±0.10	3.34±0.01	2.03±0.92	2.87±0.92	378 ([M]$^+$)
22	2700	2700	*n*-C$_{27}$[d]	4.04±0.03	2.36±0.51	4.63±0.76	8.33±1.65	4.78±2.99	380 ([M]$^+$)
23	2729	2728	13-MeC$_{27}$[d]	6.60±1.00	1.43±0.17	3.76±1.59	16.36±6.06	8.20±2.07	196/197, 224/225, 379 ([M-15]$^+$)
24	2722	2773	3-MeC$_{27}$	0.92±0.16	4.96±0.38	4.15±0.69	2.08±0.29	2.03±0.49	365 ([M-29]$^+$), 379 ([M-15]$^+$)
25	2777	2825	*Z*9C$_{28}$[d]	1.07±0.03	2.13±0.10	0.82±0.16	0.59±0.04	0.68±0.17	392 ([M]$^+$)
26	2800	2800	*n*-C$_{28}$[d]	1.07±0.14	0.89±0.73	0.87±0.08	2.03±0.41	1.59±1.66	394 ([M]$^+$)
27	2828	2818	13-MeC$_{28}$; 14-MeC$_{28}$	1.72±0.01	1.85±1.05	1.68±0.95	2.10±0.27	1.09±0.17	196/197, 238/239; 210/211, 224/225
28	2860	2853	2-MeC$_{28}$	2.90±0.33	4.64±0.95	5.45±1.62	4.09±1.65	2.33±0.89	365 ([M-43]$^+$), 393 ([M-15]$^+$)

Table 1 (continued)

Peak #[a]	Kovats Index		Compound	Percentage of total hydrocarbon (mean ± SD, *N*=3)					Diagnostic MS ions
	DB-5MS	DB-Wax		♀ trail (20 d-old)	♂ trail (20 d-old)	♀ trail (11 d-old)	♀ body-wash (adult virgin)[b]	♀ body-wash (adult mated)[c]	*m/z*
29	2877	2927	*Z*9C$_{29}$[d]	4.59±0.13	2.42±0.82	12.89±1.05	3.58±2.09	2.94±0.76	406 ([M]$^+$)
30	2900	2900	*n*-C$_{29}$[d]	1.70±0.16	1.76±0.73	2.31±0.15	1.36±0.32	1.26±1.21	408 ([M]$^+$)
31	2927	2919	13-MeC$_{29}$[d] 15-Me C$_{29}$	4.50±0.55	1.95±0.01	3.23±0.57	8.48±2.32	2.11±0.96	196/197, 252/253; 224/225

Abbreviations: *2-MeC$_{22}$* 2-methyldococane, *nd* not detected

[a] Peak # corresponds to those marked in Fig. 1

[b] Three virgin female beetles (20, 36, and 49 d-old)

[c] Three mated female beetles (28, 36, and 36 d-old)

[d] Compounds were confirmed by comparison with synthetic standards

Fig. 1) and *m/z* 322 (peak 2, Fig. 1), respectively. The spectrum of peak 1 showed a typical fragmentation pattern of a saturated hydrocarbon as well as two significant ions in the high-mass region, *m/z* 309 (M–15) and *m/z* 281 (M–43), which we interpreted as $[M - CH_3]^+$ and $[M - C_3H_7]^+$. It suggested that the 2-methyl branched C_{23} hydrocarbon was the candidate compound. This hypothesis was confirmed by comparison with a synthetic standard. 2-Methyldocosane showed an identical MS spectrum and GC retention times on both polar and non-polar GC columns, compared with the first female-specific component.

The EI-MS of peak 2 (Fig. 1) matched a monounsaturated tricosene spectrum retrieved from the Wiley 275 mass spectral database. The double-bond position of this tentative monounsaturated hydrocarbon was determined by capillary GC/MS analysis of the DMDS derivative of the natural product. One pair of diagnostic methylsulfide fragments at *m/z* 173 and 243, and a molecular ion at *m/z* 416, indicated unsaturation at the 9 position (*i.e.*, Δ9-C_{23} was the likely candidate for the natural product). To confirm the above and to determine geometry, synthetic standards of the (*E*)- and (*Z*)-isomers of Δ9-C_{23} were synthesized and subjected to DMDS analyses. The MS spectra and GC retention times of synthetic *Z*9-C_{23} and its DMDS adduct were indistinguishable from those of the natural product on a DB-5 capillary column. The natural products corresponded to the earlier-eluting DMDS isomers on a DB-5 capillary column, so that the second female-specific compound (peak 2) was established as (*Z*)-9-tricosene.

The EI mass spectra of both minor female-specific compounds (peaks 10 & 11, Fig. 1) contained molecular ions at *m/z* 350, which indicated monounsaturated pentacosenes. Two pairs of diagnostic methylsulfide fragments at *m/z* 173 and 271 (peak 10), and *m/z* 145 and 299 (peak 11), both with a molecular ion at *m/z* 444, indicated that the two compounds had unsaturation at the 9 and 7 positions of the respective molecules. These minor components were identified as *Z*9-C_{25} and *Z*7-C_{25} using the same methods described above for *Z*9-C_{23}. All other compounds derived from the virgin females were identified based on mass spectra, fragmentation patterns, GC retention times, and comparison with synthetic standards, in the same manner as described for the above compounds (Table 1).

The ratio of the major to the minor component was 0.27:1 in 11 d-old females, and 6:1 in 20 d-old virgin and mated females; for virgins, sexually mature (20 d-old) females had 35-fold and 66-fold higher ratios of 2-Me-C_{22} and Z9-C_{23}, respectively, in trail extracts than did younger (11 d-old) beetles. In contrast, the minor components Z9-C_{25} and Z7-C_{25} were in trail extracts of mature virgin females at ratios of only 2.8-fold and 2.7-fold higher, respectively, than in trail extracts of younger virgin females. The ratio of the amount of trail pheromone components (2-Me-C_{22} and Z9-C_{23}) to body wash was much higher for the major than for the minor

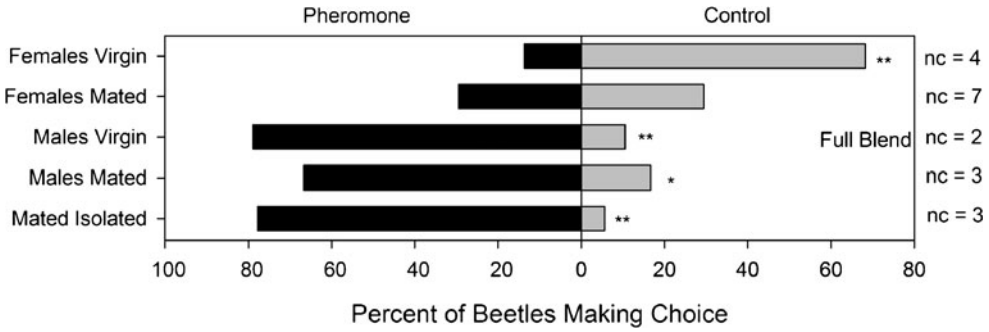

Fig. 2 Percentages of *Anoplophora glabripennis* males and females that chose the trail pheromone-painted branch (*black bars*) over the control branch (gray bars) in a choice experiment using a Y-shaped Norway maple twig. The pheromone was tested in its natural ratio of 2:12:1:1 (2-Me-C_{22}:*Z*9-C_{23}:*Z*9-C_{25}:*Z*7-C25). Nineteen virgin and 18 mated males, 22 virgin and 18 mated females, and isolated mated males were tested separately for each treatment; nc=number of beetles that did not make the same choice twice. Removing no choice from the analysis did not change the significance of results. One and two asterisks indicate $P<0.05$ and $P<0.01$, respectively, for each comparison

components, while the minor components *Z*9-C_{25} and *Z*7-C_{25} were higher in body wash extracts than in the trail deposited on the substrate (Table 1).

Behavioral Responses to the Full Trail Pheromone Blend As beetles climbed up the base of the Y-twigs, they palpated the branch with their mouthparts and antennae, especially when they reached the base of the Y, often trying both arms before choosing; these behaviors often resulted in a consistent choice. Beetles that did not perform this behavior often just ran up the branch and usually made no choice.

There were differences in response to the full pheromone blend as a function of gender and mating status (*Chi-square* and Fisher's exact test: $\chi^2=29.4$, $df=6$, $P<0.001$; Fig. 2). Males, whether virgin (79 %), mated (66.7 %), or mated and isolated from females for 24 h before being retested (78 %), chose the full pheromone blend over the control (Fig. 2, Table 2). In contrast to males, more virgin females chose the control (68.2 %) over the pheromone (13.6 %). Most mated females did not differentiate (29.4 % chose control, 29.4 % chose pheromone, and 41.2 % made no choice).

Table 2 Responses of *Anoplophora glabripennis* adults, by gender and mating status, to the full trail pheromone blend using Y-shaped twigs as a bioassay arena (see Fig. 2)

Bioassay comparison	χ2	*df*	*P*-value (Fisher's exact test)	Response to pheromone[a]
Virgin males *vs.* control	17.8	2	<0.001	+
Mated males *vs.* control	9.0	2	0.011	+
Isolated mated males *vs.* control	16.3	2	<0.001	+
Virgin females *vs.* control	12.1	2	0.002	–
Mated females *vs.* control	0.47	2	0.790	NS

NS no significant choice

[a] (+) = attracted, (−) = repelled

Behavioral Responses to the Major and Minor Pheromone Components Virgin males chose the major trail pheromone component blend over the control 90 % of the time, while mated males did not show a preference ($\chi^2=6.62$, $df=1$, $P=0.005$; Fig. 3, Table 3). Females, regardless of mating status, did not show a preference between the major components and the control.

The minor components produced the opposite behavioral response from that evoked by the major pheromone components. Males did not respond to the minor trail pheromone components regardless of mating status. In contrast, both virgin (73 %) and mated females (72.2 %) chose the minor pheromone components over the control (Fig. 3, Table 3).

Arresting Behavior of Males in Response to the Trail Pheromone Several males arrested (stopped and remained motionless) for up to 10 min after coming into contact with pheromone components. Males then often displayed pheromone-releasing behavior in which they raised their thorax while touching the substrate with the tip of the abdomen while slightly opening their wings (Lacey et al. 2007). We observed this behavior in 26.7 % and 28.5 % of males during testing of the full and major blends of the trail pheromone, respectively, while only two of 19 males arrested when exposed to the minor components. Of the males that chose the full blend *vs.* control or the major components *vs.* control, 87.5 % of them arrested and assumed a pheromone releasing behavioral posture before choosing the trail pheromone (both for full blend and major components: $\chi^2=4.50$, $df=1$, $P=0.034$).

Time To Choice Mating status did not affect male or female time to choice when exposed to the full, major, or minor trail pheromone blends (Table 4). In general, beetles made a choice in 68 sec or less. However, of the males that chose the major trail pheromone components, virgins responded to the pheromone twice as fast as did mated males (19.9±2.65 sec *vs.* 40.8 ±13.2 sec; $\chi^2=5.33$, $df=1$, $P=0.021$).

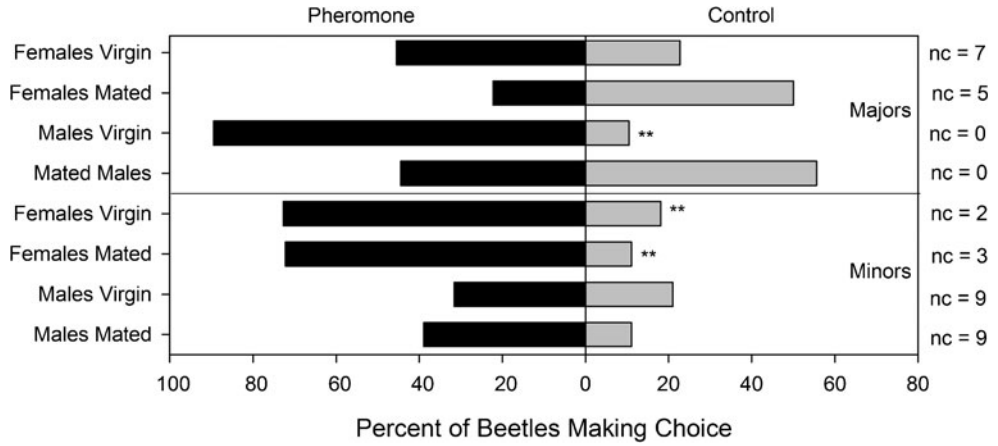

Fig. 3 Percentages of *Anoplophora glabripennis* virgin and mated males, and virgin and mated females that chose the major (*upper panel*) or minor (*lower panel*) trail pheromone components in choice experiments using a Y-shaped Norway maple twig. The major and minor pheromone component blends were tested in their natural ratios. There were differences in behavioral responses among treatments (gender and mating status) for both major and minor pheromone components (majors: χ^2=27.8, *df*=6, *P*<0.001; minors: χ^2=14.6, *df*=6, *P*=0.017). Nineteen virgin and 18 mated males, 22 virgin and 18 mated females, and isolated mated males were tested separately for each treatment (nc=no choice). Two asterisks indicate *P*<0.01 for individual comparisons

Behavioral Responses to Major Trail Pheromone Components in the Field in China Field-collected males (age and mating status unknown) were more likely (Wilcoxon: χ^2=8.67; *df*=1; *P*=0.003) to follow the spiral trail of stickers painted with the major components of the female trail pheromone (4.83±0.60 stickers walked on) in comparison to field-collected females (1.33±0.21 stickers walked on). In addition, male scores were higher than a hypothetical score of 1 (*i.e.*, walked on 1 sticker), which was the baseline met by all beetles regardless of sex (Wilcoxon Signed-rank=10.5; *P*=0.003), while this was not the case for females (*P*=0.174). After touching the first sticker, males exhibited typical searching behaviors, including scanning the bark with their antennae, walking slowly, tasting the stickers with their labial palpi, and grooming. When the tip of an antenna touched a sticker, males deviated their route from walking straight up to moving diagonally toward the next sticker until they explored it with their labial palpi, and then continued to the next sticker. In contrast, females showed no response when they walked on a sticker (Wilcoxon Signed-rank=1.5; *P*=0.5) and instead were more likely to walk straight up the tree.

Table 3 Responses of *Anoplophora glabripennis* adults, by gender and mating status, to the minor and major trail pheromone blends using Y-shaped twigs as a bioassay arena (see Fig. 3)

Bioassay comparison	χ2	*df*	*P*-value (Fisher's exact test)	Response to pheromone[a]
Major components				
Virgin male *vs.* control[†]	11.8	1	<0.001	+
Mated male *vs.* control[†]	0.22	1	0.637	NS
Virgin female *vs.* control	1.73	2	0.422	NS
Mated female *vs.* control	2.33	2	0.311	NS
Minor components				
Virgin male *vs.* control	2.00	2	0.368	NS
Mated male *vs.* control	4.33	2	0.115	NS
Virgin female *vs.* control	15.6	2	<0.001	+
Mated female *vs.* control	12.3	2	0.002	+

NS no significant choice

[a] (+) = attracted

Discussion

The presence of the four hydrocarbons, 2-methyldocosane, (*Z*)-9-tricosene, (*Z*)-9-pentacosene, and (*Z*)-7-pentacosene, in female but not male trail extracts, and the choice of males, but not females, for this blend over the control indicates that these compounds likely constitute a sex-specific trail pheromone, which may function to allow males to follow the trails of females on the substrate they walk on (host trees). The four-component pheromone blend in its naturally occurring ratio was chosen by both virgin and mated males, whereas it appeared to repel females, suggesting that it may function as a spacing (anti-aggregation) pheromone for females. Virgin males also were highly responsive to the two major component blend of pheromone, [2-methyldocosane and (*Z*)-9-tricosene], without the minor components [(*Z*)-9- and (*Z*)-7-pentacosene]. In contrast, mated males did not choose the major components alone. This response may be related to the manner in which the bioassay was done. To obtain mated males for testing, they were held for several days with a female in an enclosed jar. This approach may have resulted in a lack of a response to the major trail pheromone components alone, in contrast to the response to the full pheromone

Table 4 Time to choice (mean±SEM, in sec.) for virgin and mated *Anoplophora glabripennis* adults in response to the female-produced trail pheromone (see Figs. 2 and 3). The full pheromone blend, as well as the major and minor components, were tested separately for each gender and mating status

Group	Virgin	Mated	*t*-test statistics
Full pheromone blend			
Males	50.6±28.5	62.9±30.5	t=0.30, df=28, P=0.770
Females	68.4±19.4	36.4±14.1	t=1.34, df=31, P=0.189
Major pheromone components			
Males	19.9±2.65	40.8±13.2	t=1.17, df=26, P=0.252
Females	24.9±2.40	22.6±3.76	t=0.45, df=26, P=0.252
Minor pheromone components			
Males	14.6±2.22	24.7±5.20	t=1.19, df=23, P=0.247
Females	20.6±2.38	29.2±4.33	t=1.56, df=29, P=0.129

blend, for two, not necessarily mutually exclusive, reasons. First, it is possible that the mated males used were in their refractory period between copulations and were thus likely less responsive to females, *i.e.*, they didn't have sperm ready to transfer because they just mated, and therefore required a stronger signal, in the form of the full pheromone blend, to respond. Secondly, after removing males from the jar, their receptors for the pheromone may have been saturated by trails left by females, reducing their sensitivity, such that the full pheromone blend may be required for the male to track a female after he had recently mated.

The observed behavioral responses to the trail pheromone are consistent with the observed behavior of *A. glabripennis* mate finding and mating behavior in the field, demonstrating the complexity of chemical cues involved in these behaviors. We propose the following scenario of semiochemical mediation of mate-finding behavior. Although it is unclear whether males or females arrive at a host tree first, it is clear that plant volatiles play an important role in host plant location in *A. glabripennis* (Li et al. 2002; Wen et al. 1999), which is consistent with that of most cerambycids (Allison et al. 2004). *Anoplophora glabripennis* males produce a volatile pheromone (Zhang et al. 2002) that attracts primarily virgin females, and which is enhanced in combination with host plant kairomones (Nehme et al. 2010). Once on the same tree, the female-produced trail pheromone, along with visual cues (Li et al. 1999), may be the link that allows a male to find a female on a relatively large substrate. Based on our observations during the bioassays described herein (see Introduction), males appear to detect the female's trail using their mouthparts and antennae. Once a male physically encounters a female, he antennates her cuticle and, upon detection of the contact pheromone, recognizes her as a conspecific female, stimulating copulation (Zhang et al. 2003). After separating from a female, males likely continue to use the trail and contact pheromones to follow females to mate again, as well as mate guard, a common behavior in this species (Morewood et al. 2004).

The ability of males to follow females has several benefits for both sexes. For males, being able to find females easily on a large tree potentially increases his fitness by allowing him to mate with one or more females, as well as guarding the female from further copulations by other males. Once together, pairs go through a series of copulatory bouts, with the male mate guarding in between. Females benefit from deposition of a trail pheromone that allows males to follow and find her; longer time periods spent by the female in copula (which can be achieved if the first male remains with the female long enough and copulates with her multiple times), or by mating with multiple males, increases her fertility, such that a greater proportion of her eggs will hatch (Keena unpublished data).

Virgin females were repelled by the trail pheromone, but mated females were indifferent, suggesting that virgin females may use this pheromone as an anti-aggregation (spacing) pheromone to avoid intraspecific competition for males. It would be advantageous for virgin females to find a male and have a prolonged pair bond without other beetles interfering so that she can receive a full sperm load. Additionally, males that are with a female already will not leave her for another female. Once a female has obtained a sufficient sperm load, there may be an advantage for multiple females to oviposit on the same tree, such as overcoming host defenses by reducing tree fluid pressure through multiple oviposition pits being chewed. Newly eclosed larvae can be drowned by heavy sap flow from host trees (Morewood et al. 2003). Since mated females were indifferent to the full pheromone blend, it is unlikely that the pheromone is used for oviposition marking. Further studies are needed to test these hypotheses. However, responses to partial pheromone components (majors or minors) in bioassays may not be biologically relevant since we always detected all four components in every trail wash sample. However, because ratios of the components change with female age, we cannot rule out that responses to partial blends represent responses to different ratios of the components.

One of the major trail pheromone components, (*Z*)-9-tricosene, and the two minor components, (*Z*)-7-pentacosene, (*Z*)-9-pentacosene, are also constituents of the *A. glabripennis* female contact sex pheromone, which stimulates copulatory behavior in males (Zhang et al. 2003). However, because the major trail pheromone components were consistently far more abundant in female trail wash than in body wash, we hypothesize that they may be produced by a specialized gland(s), although the source of these compounds remains to be investigated.

Monounsaturated and methyl branched hydrocarbons are common cuticular compounds with biological activity in many insects. 2-Methyldocosane is a contact sex pheromone component in the biting midge *Culicoides melleus* (Linley and

Carlson 1978) and the chrysomelid beetle *Chrysochus cobaltinus* (Peterson et al. 2007). It is a cuticular hydrocarbon component in the termites *Neotermes mona* (Haverty et al. 1997) and *Neotermes connexus* (Haverty et al. 2000), and the Hawaiian swordtailed cricket *Laupala pacifica* (Mullen et al. 2008). This compound also is found in Dufour glands of parasitoids of the wheat stem sawfly *Cephus cinctus* and *Bracon cephi* (Gahan) (Bancroft and Smith 2005). However, to our knowledge, 2-methyldocosane has not been reported previously as a semiochemical in the family Cerambycidae.

The ratios of the two major trail pheromone components, 2-Me-C_{22} and *Z*9-C_{23}, deposited by 20 d-old (sexually mature) virgin females compared to 11 d-old virgin females were 35:1 and 66:1, respectively, while the ratios of the two minor components in older to younger virgins were each only about 2.7:1. We suspect that the relatively lower ratio of major: minor components released by younger compared to older virgin females may allow females to avoid encountering males until they are sexually mature. It is also possible that adults likely reach sexual maturity about the same time that they produce the trail pheromone components at the appropriate ratios because adults take 8–14 d following eclosion to sclerotize fully and exit their host tree (Sanchez and Keena 2013), and females then require additional time to mature and feed for their ovaries to develop (Keena 2002; Smith et al. 2002).

Pheromones that elicit both trail following and sex attraction have been reported previously in termites (*e.g.*, Sillam-Dusses et al. 2011). The only previously known example of such a pheromone in the Cerambycidae is that of female *Nadezhdiella cantori,* which leaves trails of her contact sex pheromone on trees when walking, which males use for locating them (Wang et al. 2002).

We have yet to determine how long the trail pheromone produced by *A. glabripennis* females persists on the tree or where it is produced. Fairly rapid disappearance of trail-marking pheromones may benefit a species by avoiding confusion and wasted energy of males following a trail that no longer leads to a mate. Ant foraging trails are known to dissipate quickly and the ability of workers to find the food source depends largely on the reinforcement of the trail by followers (Saran et al. 2007). It is unlikely that the trails produced by female *A. glabripennis,* given the chemical structures involved, would dissipate as quickly as ant trails, but the duration of the biological activity of this pheromone remains to be determined.

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