7-methylheptacosane is a major component of the contact sex pheromone of the cerambycid beetle *Neoclytus acuminatus acuminatus*

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Abstract. Male *Neoclytus acuminatus acuminatus* (F.) (Coleoptera: Cerambycidae) attempt to mate with females only after touching them with their antennae, suggesting that mate recognition is mediated by contact pheromones in the cuticular wax layer of females. Consistent with that hypothesis, males exhibit similar responses to dead females in laboratory bioassays, but not to solvent-washed dead females with their cuticular hydrocarbons removed. The mating response of males is restored when solvent extracts are reapplied to carcasses of solvent-washed females, indicating that the contact pheromone is present in solvent extracts. Solvent extracts of the female cuticle contain six methylalkanes that are not present in extracts of males, three of which ($7Me\text{-}C_{25}$, $7Me\text{-}C_{27}$ and $9Me\text{-}C_{27}$) constitute almost 40% of the total hydrocarbons. The bioactivity of these three compounds is tested by applying synthetic standards to solvent-washed carcasses of females and presenting them to males. Standards are tested singly, pairwise and as the complete blend; freeze-killed females serve as controls. Males attempt to couple with solvent-washed female carcasses treated with $7Me\text{-}C_{27}$ alone and in combination with $9Me\text{-}C_{27}$ but only the complete blend elicits the same number of mounting and coupling attempts as does the control. These findings suggest that $7Me\text{-}C_{27}$ (7-methylheptacosane) is the major component of the contact sex pheromone of *N. a. acuminatus* and that $7Me\text{-}C_{25}$ and $9Me\text{-}C_{27}$ act as synergists.

Key words. Cuticular hydrocarbon, mate recognition, mating behaviour, methylalkane, solid-phase microextraction.

Introduction

The wax layer on the cuticle of insects provides a waterproof barrier between the insect and its environment, but some of the lipid components may also function as semiochemicals (Howard & Blomquist, 2005). Contact sex pheromones on the cuticle of female longhorned beetles (Cerambycidae) of many species mediate mating responses by conspecific males (Hanks *et al.*, 1996), including species in the primitive subfamily Prioninae (Barbour *et al.*, 2007) and the more advanced subfamilies Cerambycinae (Fukaya *et al.*, 1996; Ginzel *et al.*, 2003a, 2003b, 2005, 2006) and Lamiinae (Wang, 1998; Fukaya *et al.*, 2000; Yasui *et al.*, 2003; Zhang *et al.*, 2003; for phylogenetic relationships, see Farrell & Sequeira, 2004).

Males of the cerambycine species *Neoclytus acuminatus acuminatus* (F.) also appear to recognize females by contact chemoreception, attempting to mate only after contacting females with their antennae (E. S. Lacey, personal observation). This species is endemic to North America (Yanega, 1996), but has been introduced accidentally into Europe and South America (Bense, 1995; Fork, 2004). Adults are diurnal and both sexes aggregate on stressed and dying trees that serve as hosts of the larvae (Linsley, 1964; Waters, 1981;

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Solomon, 1995). Aggregation is mediated by the male-produced pheromone (2*S*,3*S*)-hexanediol (Lacey *et al.*, 2004).

In the present study, the hypothesis is tested that mating responses by male *N. a. acuminatus* are mediated by a contact sex pheromone in the cuticular lipids of females. The identification and synthesis of the pheromone components is reported. Two methods are used to sample the cuticular components, whole-body solvent extraction and wipe-sampling by solid-phase microextraction (SPME; Monetti *et al.*, 1997; Turillazzi *et al.*, 1998; Liebig *et al.*, 2000). Previously, it was found that samples of cuticular lipids taken by wiping SPME fibres across the elytra of females of the cerambycine species *Megacyllene robiniae* (Förster) yield markedly different hydrocarbon profiles than does whole-body solvent extraction (Ginzel *et al.*, 2003a). The contact pheromone of females of that species, (*Z*)-9-pentacosene, is the single dominant peak in SPME samples but is one of three major hydrocarbons in hexane extracts of females. An important component of the contact pheromone of the congener *Megacyllene caryae* (Gahan), (*Z*)-9-nonacosene, is also more abundant in SPME wipe samples of female elytra than in whole-body solvent extracts (Ginzel *et al.*, 2006). These findings suggest that contact sex pheromones are more abundant on the elytra of females where they are readily accessible to the antennae of males, and that wipe sampling by SPME yields a more representative profile of cuticular components that are contacted by males than whole-body solvent extraction (Ginzel *et al.*, 2003a, 2006). This hypothesis is tested again in the present study by sampling cuticular hydrocarbons of female *N. a. acuminatus* by both SPME and solvent extraction, predicting that compounds acting as mate recognition signals are present in higher relative proportions in SPME wipe samples than in whole-body extracts.

Materials and methods

Source of beetles

Adult *N. a. acuminatus* were reared from green ash, *Fraxinus pennsylvanica*, trees that had been colonized naturally by beetles in May 2002. Logs that contained larvae were moved into the laboratory during winter 2002 and stored in a $3 \times 2 \times 1$ m cage of aluminum window screen (ambient conditions: fluorescent lighting, LD 12 : 12 h, 20 °C, 50% RH, until adults emerged after 3–6 weeks). Adults were caged individually in 0.1 m^3 cylinders of aluminum window screen with plastic Petri dishes at top and bottom and provided with water (glass vial with a cotton dental roll, Patterson Dental Supply, South Edina, Minnesota). Beetles used in bioassays appeared to be healthy and active.

Identification of cuticular hydrocarbons

The relative abundances of cuticular hydrocarbons were compared in SPME wipe samples and whole-body solvent extracts by first sampling ten female *N. a. acuminatus* by SPME. Females were held with forceps and the length of a SPME fibre (100 µm, polydimethylsiloxane; Supelco, Cat. No. 57300-U, Bellefonte, Pennsylvania) was wiped across the elytra ten times with enough pressure to maintain contact with the surface of the cuticle without bending the fibre. The fibre was rotated slightly between wipes. The samples were analyzed by coupled gas chromatography-mass spectrometry (GC-MS) at UIUC with electron impact ionization (EI, 70 eV) using a Hewlett-Packard 6890 GC (Hewlett-Packard Corp., Sunnyvale, California) equipped with a DB-5MS capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 µm film; J&W Scientific, Folsom, California) in splitless mode, interfaced to an HP 5973 mass selective detector, with helium carrier gas. The column was programmed from 40°C for 1 min, 10 °C min^{-1} to 300 °C, and then hold for 5 min. Injector temperature was 250 °C and transfer line temperature was 280 ℃. SPME fibres were desorbed in the injection port of the GC at 250 °C for 1 min.

After sampling by SPME, cuticular lipids were extracted from the same ten females. Beetles were freeze-killed at −20 °C for 20 min, then thawed and immersed in 1 mL of hexane (Optima grade, Fisher Scientific, Fair Lawn, New Jersey) for 2 min, with gentle agitation. Extracts were analyzed by GC-MS (same settings as described above). Quantitative data were produced by calculating the corrected areas under peaks of all hydrocarbons that were present consistently in the total ion chromatograms (Chemstation, Version B.02.05; Hewlett-Packard Corp.). Differences between the two sampling methods for female beetles were tested by comparing mean relative abundances of all compounds (corrected area under peak/total area of all peaks) by analysis of variance (PROC GLM; SAS Institute, 2001). The same methods were used to extract cuticular lipids from ten male *N. acuminatus* and to estimate relative abundances of compounds.

Compounds in whole-body extracts of beetles were identified at UC Riverside by GC-MS (same model and column as used at UIUC). The temperature programme was 100 °C for 1 min, 10 °C min^{-1} to 280 °C, and then hold for 20 min. Injector and transfer line temperatures were 280 °C. Hydrocarbons were identified by comparing mass spectra and retention times with those of standards, or from the parent M$^+$ ions and corresponding molecular formulae. Retention times of methylalkanes relative to straight-chain compounds and diagnostic mass spectral fragments demonstrated unequivocally the position of methyl branches (Nelson, 1993; Nelson & Blomquist, 1995). Double bond positions of the alkenes in the extracts of males were determined by treatment of an aliquot with 100 µL of a solution of *m*-chloroperbenzoic acid (2 mg mL^{-1} in methylene chloride) at room temperature for 2 h. The mixture was concentrated just to dryness under a stream of nitrogen, then taken up in hexane and extracted twice with 1 M aqueous NaOH. The resulting hexane solution was dried over anhydrous Na$_2$SO$_4$, concentrated under a stream of nitrogen, and analyzed by GC-MS as described above. Double bond positions in the parent compounds were determined from the diagnostic mass spectral ions from cleavage on either side of

the epoxide function in the derivatives (Hogge *et al.*, 1985).

Synthesis of 7Me-C$_{25}$, 7Me-C$_{27}$ and 9Me-C$_{27}$

Octadecyl bromide (8.33 g, 25 mmol) was purified immediately before use by washing a hexane solution through a 3-cm plug of silica gel with hexane. After evaporation of the solvent, the resulting colourless solid was taken up in 50 mL of dry terahydrofuran. Ten millilitres of the solution was added to Mg turnings (1.22 g, 50 mmol, ground with a mortar and pestle immediately before use) in a dry three-necked flask under Ar, and a few crystals of iodine and 100 μL of dibromoethane were added. After the Grignard reaction started, as demonstrated by the warming of the mixture and the disappearance of the brown colour of the iodine, the mixture was stirred at 35–45 °C and the remaining solution of the bromide was added dropwise over 1.5 h. When the addition was complete, the mixture was stirred at 45 °C until the bromide had been consumed (45 min). The resulting warm solution was cannulated into a clean dry flask under Ar, and 2-octanone (2.82 g, 22 mmol) was added dropwise over 30 min. The resulting mixture was stirred at ambient temperature overnight. The mixture was then poured into 1 M KH$_2$PO$_4$ buffer and extracted with hexane. The hexane extract was washed with water and brine, dried over anhydrous Na$_2$SO$_4$, concentrated and the residue subjected to Kugelrohr distillation (oven-temperature to 140 °C, 0.05 mm Hg) to remove most of the octadecane byproduct. The resulting semi-solid mixture of 7-methylpentacosan-7-ol (95% pure by GC) was used without further purification. MS (EI,70 eV; *m/z*, relative abundance): 367 (7, M$^+$-15), 364 (9, M$^+$-18), 298 (15), 297 (75), 130 (9), 129 (100), 127 (11), 126 (16), 125 (10), 111 (54), 97 (37), 83 (46), 71 (43), 69 (78), 57 (84), 56 (58), 55 (76), 43 (93), 42 (12), 41 (56).

The crude 7-methylpentacosan-7-ol was taken up in 50 mL of benzene, 100 mg of *p*-toluenesulphonic acid was added, and the mixture was refluxed with a Dean-Stark trap for 3 h. After cooling, hexane was added and the mixture extracted with dilute aqueous NaHCO$_3$ and brine. The organic layer was then dried over Na$_2$SO$_4$ and concentrated. The residue was taken up in hexane and passed through a 2 cm plug of silica gel, eluting with hexane, yielding approximately 6 g of a mixture of C$_{26}$ alkenes. After concentration to approximately 50 mL, 0.5 g of 5% Pd on C was added and the mixture stirred for 2 h under a slight positive pressure of H$_2$. The mixture was filtered through Celite® (Fisher Scientific, Fairlawn, New Jersey) and concentrated, and the residue dissolved in 100 mL of hot acetone, then cooled in a refrigerator at 4 °C. Filtration and air drying yielded 5.54 g of 7Me-C$_{25}$ as a low melting (melting point 25 °C) white solid, > 99.5% pure by GC. MS: 351 (2, M$^+$-15), 281 (12), 280 (10), 252 (4), 113 (15), 112 (33), 99 (13), 97 (10), 85 (30), 83 (14), 71 (80), 70 (13), 69 (14), 57 (100), 56 (16), 55 (26), 43 (72), 41 (32).

9Me-C$_{27}$ was made in analogous fashion by substitution of 2-decanone for 2-octanone in the first reaction (purity > 98%). MS: 379 (2, M$^+$-15), 281 (2), 280 (7), 252 (2), 141

(9), 140 (20), 127 (5), 113 (7), 112 (4), 111 (7), 99 (15), 97 (11), 85 (48), 83 (11), 71 (60), 70 (11), 69 (16), 57 (100), 56 (17), 55 (30), 43 (91), 42 (10), 41 (38).

7Me-C$_{27}$ was prepared similarly by reaction of the Grignard reagent prepared from 1-bromoeicosane and 2-octanone, followed by dehydration and catalytic hydrogenation as described above (purity > 99%). MS: 394 (M$^+$, trace), 309 (9), 308 (7), 280 (2), 113 (12), 112 (26), 99 (9), 97 (8), 85 (22), 83 (12), 71 (68), 70 (11), 69 (15), 57 (100), 56 (15), 55 (26), 43 (81), 42, (10), 41 (34).

Bioassays of hexane extracts of females

Dose-response tests were conducted to confirm that male *N. a. acuminatus* rely on a contact sex pheromone to recognize females and to determine quantities of pheromones necessary to elicit responses from males. Preliminary observations revealed that males display a clear progression of behavioural steps after contacting females, analogous to the series of behaviours reported for related species (Ginzel *et al.*, 2003a, b, 2005, 2006): the male stopped walking (= arrestment); aligned his body with the female; mounted her; and attempted to couple the genitalia. The assessment of behavioural responses to females was cumulative: males needed to perform all four steps to mate with a female (Ginzel *et al.*, 2003a, 2006).

The responses of individual males to females that had been subjected to the sequential treatments described below were tested.

1) *Freeze-killed (−20 °C for 20 min) female*. The carcass was thawed to room temperature and presented to an individual male. An attempt to mate was evidence that recognition cues were intact and that a behavioural response by the female was not necessary for mate recognition.
2) *Extracted female carcass*. Cuticular components were removed by immersing the same female carcass in 1 mL hexane for 2 min, with gentle agitation. The extracted female was air-dried for 10 min to allow hexane to evaporate and then presented again to the same male. Lack of a response by the male was evidence that chemical recognition cues had been removed.
3) *Reconstituted female*. Hexane extract was gradually pipetted onto the extracted female carcass. Hexane was allowed to evaporate and the treated female carcass then was presented to the same male. A response (at least arrestment) was taken as evidence that the recognition signal was present in the cuticular extract. To determine the threshold of male sensitivity to recognition signals present on the female, extracted females were treated initially with 0.1 female equivalent (10% of the crude extract) and then presented to males. Extract was added to the female in 0.1 female equivalent increments until males responded or the entire extract had been applied.

A trial was scored as a 'response' if males displayed at least arrestment immediately after first contacting a female with his antennae. A trial was scored as 'no response' if the male
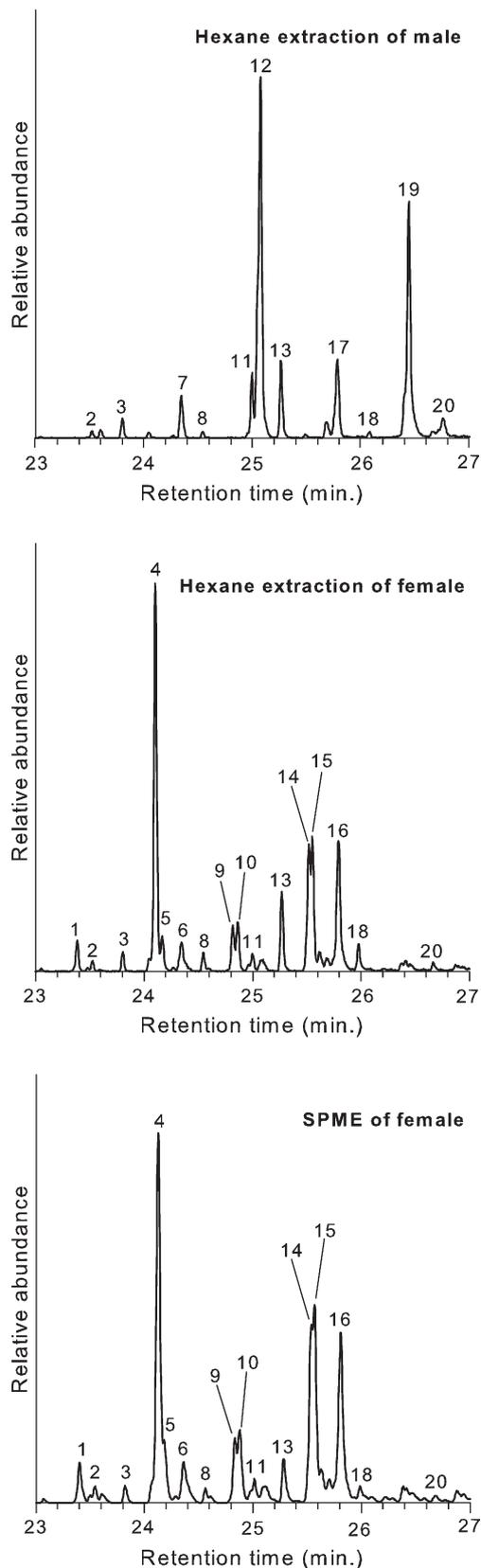
showed none of the expected behaviours after initial antennal contact, but rather continued to walk after contacting the female with his antennae. Bioassays were conducted in clean glass Petri dish arenas lined with filter paper, under ambient laboratory conditions (see above), between 11.00 and 17.00 h on 3 days in December 2002. The responses of 27 males were tested, videotaping bioassays for subsequent analysis. The numbers of males responding to reconstituted females were compared with the numbers responding to freeze-killed females with Fisher's exact test (Sokal & Rohlf, 1995).

Bioassays of hydrocarbon standards

A second bioassay tested the activity of female-specific compounds. Solutions of synthetic 7Me-C_{25}, 7Me-C_{27} and 9Me-C_{27} were prepared in hexane at concentrations approximating those in the hexane extracts of female *N. a. acuminatus* (12, 3 and 6 μg, respectively, per one female equivalent; quantified by comparing peak areas in integrated total ion chromatograms with that of an internal standard, nC_{20}). Each of 20 males was first presented a freeze-killed female, then cuticular hydrocarbons were removed from the same female and males were presented with the extracted female. Next, each male was presented, in random order, with extracted females that had been treated with all possible combinations of hydrocarbon standards: 7Me-C_{25}, 7Me-C_{27}, or 9Me-C_{27} separately, in solutions containing pairwise combinations, and with all three compounds combined. Bioassays were conducted between 11.00 and 17.00 h on 4 days in March and April of 2003 (five males per day; fresh control and treatments on each day) under ambient laboratory conditions. Responses of males to treatments were analyzed with Fisher's exact test (Sokal & Rohlf, 1995) by comparing numbers of males showing the complete mating response to treatments with those to freeze-killed females.

The initial strategy for identifying the contact pheromone was to synthesize and test all female-specific branched-chain alkanes, beginning with the most abundant. This general strategy proved effective in identifying the contact pheromone of the cerambycine *Xylotrechus colonus* (F.) (Ginzel *et al.*, 2003b, 2005). The first three compounds of female *N. a. acuminatus* that were synthesized and tested, 7Me-C_{25}, 9Me-C_{27} and 7Me-C_{27}, together comprised over 40% of the total hydrocarbons in the cuticular profile (Fig. 1; Table 1). Because the activity of these three synthetic compounds, when applied to extracted female carcasses, equalled that of freeze-killed females (see Results), the remaining minor components were not synthesized and tested.

Fig. 1. Representative total ion chromatograms of whole-body hexane extracts of an adult male (top) and female (middle) *Neoclytus acuminatus acuminatus*, and solid-phase microextraction (SPME) wipe sample of female cuticle (bottom). DB-5 capillary column, 40 °C for 1 min, 10 °C min^{-1} to 300 °C, and hold for 5 min. Numbers above peaks refer to compounds in Table 1.

Table 1. Cuticular hydrocarbons of male and female *Neoclytus acuminatus acuminatus*.

| | | % Total hydrocarbons $\pm$ SE[a] | | | |
| | | Hexane extracts | | SPME | |
Peak number[b]	Hydrocarbon	Male	Female	Female[c]	Diagnostic ions
1	6Me-C_{24}	ND	3.62 ± 0.35	4.67 ± 0.40	337 (M^+-15), 98/280
2	2Me-C_{24}	0.06 ± 0.06	1.17 ± 0.16	0.90 ± 0.21	337 (M^+-15), 309
3	nC_{25}	2.59 ± 0.25	2.62 ± 0.15	1.65 ± 0.12*	352 (M^+)
4	7Me-C_{25}	ND	24.5 ± 0.89	25.5 ± 0.69	351 (M^+-15), 112/280
5	5Me-C_{25}	ND	3.59 ± 0.36	4.71 ± 0.41	351 (M^+-15), 85/308
6	3Me-C_{25}		3.20 ± 0.17	3.83 ± 0.22*	366 (M^+), 337
7	3Me-C_{25}	3.15 ± 0.21			366 (M^+), 337
	Δ10-C_{26}[d]				364 (M^+), 83, 97, 111
8	nC_{26}	1.24 ± 0.21	2.96 ± 0.26	1.36 ± 0.11*	366 (M^+)
9	8Me-C_{26}	ND	3.46 ± 0.10	4.83 ± 0.13*	365 (M^+-15), 126/280
10	6Me-C_{26}	ND	3.75 ± 0.34	5.06 ± 0.41*	365 (M^+-15), 98/308
11	2Me-C_{26}	4.56 ± 0.57	4.41 ± 0.52	4.16 ± 0.41	365 (M^+-15), 337
12	Δ10- and Δ12-C_{27}	33.9 ± 1.48	ND	ND	378 (M^+), 83, 97, 111
13	nC_{27}	14.4 ± 1.02	12.0 ± 1.04	4.78 ± 0.41**	380 (M^+)
14	9Me-C_{27}	ND	9.84 ± 0.61	13.8 ± 1.01*	379 (M^+-15), 140/280
15	7Me-C_{27}	ND	4.44 ± 0.87	5.82 ± 0.59	379 (M^+-15), 112/308
16	3Me-C_{27}		13.1 ± 0.61	14.5 ± 0.53	394 (M^+), 379, 365
17	3Me-C_{27}[d]	9.34 ± 0.43			394 (M^+), 379, 365
	Δ10-, Δ12- and Δ14-C_{28}				392 (M^+), 83, 97, 111
18	nC_{28}	3.10 ± 0.28	3.85 ± 0.19	1.75 ± 0.12*	394 (M^+)
19	(Z)-14-C_{29}[e]	25.2 ± 1.38	ND	ND	406 (M^+), 83, 97, 111
20	nC_{29}	0.83 ± 0.12	2.79 ± 0.32	1.89 ± 0.29*	408 (M^+)

[a]Percent of total hydrocarbons represents means for ten individuals. ND, not detected; SPME, solid-phase microextraction.
[b]Peak numbers are in order of elution and correspond to those in Figure 1. DB-5 MS capillary column, 40 °C for 1 min, 10 °C min^{-1} to 300 °C, hold 5 min.
[c]Asterisk indicates statistically significant difference between means for hexane extracts and solid-phase microextraction samples of females (analysis of variance: *$P < 0.05$, **$P < 0.001$).
[d]Male-specific alkenes coeluted with methylalkane, summed abundances are for both compounds. Double bond positions were determined from diagnostic mass spectral ions after epoxidation of the alkenes.
[e]Double bond position and geometry confirmed with a standard of epoxide of (Z)-14-29:H.

Results

Identification of cuticular hydrocarbons

Hexane extracts of females contained consistently 16 hydrocarbon components (Fig. 1; Table 1), including the complete series of straight-chain alkanes from C_{25} to C_{29} and 11 branched alkanes with single methyl groups. Seven of the methylalkanes were present in detectable amounts only in extracts of females. These included 6Me-C_{24}, 7Me-C_{25}, 5Me-C_{25}, 8Me-C_{26}, 6Me-C_{26}, 9Me-C_{27} and 7Me-C_{27}. No alkenes were detected in extracts of females. Hexane extracts of male *N. a. acuminatus* contained nine saturated hydrocarbons, including the same straight-chain alkanes that were present in extracts of females, four methylalkanes, and several C_{26}-C_{29} alkenes with co-eluting positional isomers (Table 1). The C_{27} and C_{29} alkenes comprised nearly 60% of total hydrocarbons in males (Fig. 1; Table 1).

Chromatographic profiles of hydrocarbons in SPME wipe samples of female *N. a. acuminatus* were qualitatively similar to those of whole-body hexane extracts (Fig. 1; Table 1).

All components present in SPME samples also were present in hexane extracts. The most conspicuous differences between sampling methods were the significantly greater proportions of four of the seven female-specific methylalkanes (3Me-C_{25}, 8Me-C_{26}, 6Me-C_{26} and 9Me-C_{27}) in SPME samples, and greater proportions of all *n*-alkanes in hexane extracts (Table 1).

Synthesis of 7Me-C_{25}, 7Me-C_{27} and 9Me-C_{27}

Authentic standards of 7Me-C_{25}, 7Me-C_{27} and 9Me-C_{27} were synthesized in straightforward fashion by reaction of 2-alkanones with a Grignard reagent to give methyl-branched tertiary alcohols. Acid-catalyzed dehydration to a mixture of alkenes, followed by catalytic hydrogenation gave the required hydrocarbons in multigram quantities. These low-melting point solids could be readily purified by recrystallization from acetone at 4 °C. Furthermore, this strategy had the advantage that, if necessary, the tertiary alcohol intermediates could be readily separated from any

nonpolar impurities (e.g. dimers from the Grignard reagent) by liquid chromatography, simplifying the eventual purification of the final products.

Bioassays of hexane extracts of females

In the initial bioassay, all males ($n = 27$) responded (behavioural step 1, at least) to freeze-killed females, confirming that mate recognition signals were intact. None of the males responded to hexane-extracted females, demonstrating that the recognition signal had been removed. All males again responded to female carcasses to which the hexane extracts had been reapplied (arrestment, at least; response not different from response to freeze-killed females; Fisher's exact test, $P > 0.05$). These results supported the hypothesis that females have a contact sex pheromone that can be removed by solvent extraction. Males responded to treated female carcasses after application of an average ($\pm$ 1 SE) of 0.73 ± 0.18 female equivalents of crude extract.

Bioassays of hydrocarbon standards

In bioassays with synthetic standards, only the three-component blend of $7Me\text{-}C_{25}$, $7Me\text{-}C_{27}$ and $9Me\text{-}C_{27}$ elicited arrestment in males at a level equal to their response to freeze-killed females (Table 2). Similar percentages of males progressed through the whole behavioural sequence to attempt connecting the genitalia when presented with freeze-killed females or to female carcasses that had been treated with the three-component blend. Females treated with $7Me\text{-}C_{27}$ alone or in combination with $9Me\text{-}C_{27}$ also elicited attempts from males to copulate.

Discussion

The present results support the hypothesis that mating responses by male *N. a. acuminatus* are mediated by a contact sex pheromone in the cuticular lipids of females. The activity of $7Me\text{-}C_{27}$ as a single component suggests that it is a major component of the contact sex pheromone of females. Combination of this compound with $9Me\text{-}C_{27}$ and $7Me\text{-}C_{25}$ results in a greater arrestment response, suggesting that these other compounds also contribute to the overall activity. Both $7Me\text{-}C_{25}$ and $7Me\text{-}C_{27}$ are cuticular hydrocarbons of other cerambycid species (Tanigaki *et al.*, 2007) and $9Me\text{-}C_{27}$ is apparently a component of the contact sex pheromone of yet another cerambycid species (Fukaya *et al.*, 2000). All three of these compounds are present in the wax layer of some beetle and ant species (Brophy *et al.*, 1983; Akino, 2002), suggesting that they may be common cuticular hydrocarbons of insects.

The role of the four minor methylalkanes that are specific to female *N. a. acuminatus* remains to be investigated. Nevertheless, the fact that males respond as strongly to the

Table 2. Responses of male *Neoclytus acuminatus acuminatus* ($n = 20$) to freeze-killed females and to hexane-washed females that had been treated with one female equivalent of 7Me-C25, 7Me-C27, or 9Me-C27 separately, in pairwise combinations, and with all three compounds combined.

Compound(s)	Percent of males responding per step in behavioural sequence[a]			
	Step 1[b]	Step 2	Step 3	Step 4[c]
Freeze-killed female (control)	100	100	75	35
7Me-C$_{25}$	20**	10	10	0**
7Me-C$_{27}$	40**	40	15	10
9Me-C$_{27}$	15**	10	0	0**
7Me-C$_{25}$+7Me-C$_{27}$	40**	40	15	0**
7Me-C$_{25}$+9Me-C$_{27}$	20**	15	5	0**
7Me-C$_{27}$+9Me-C$_{27}$	70*	70	35	20
7Me-C$_{25}$+7Me-C$_{27}$ + 9Me-C$_{27}$	85	85	55	40

[a]Responses of males to treatments versus control were tested with Fisher's exact test (*$P < 0.05$, **$P < 0.001$).
[b]Numbers of males responding to treatments by reaching behavioural step 1 were compared with responses to controls (H_0 = treatment and freeze-killed female do not differ in activity).
[c]Numbers of males responding to treatments by reaching behavioural step 4 were compared with responses to controls (H_0 = treatment and freeze-killed female do not differ in activity).

blend of $7Me\text{-}C_{25}$, $7Me\text{-}C_{27}$ and $9Me\text{-}C_{27}$ as they do to freeze-killed females suggests that the remaining compounds might play only a minor role, if any, in mate recognition. The incomplete response of some males to freeze-killed females (less than the four-step response) suggests that mating is either inhibited because chemical cues are altered in the freezing process or because males require some behavioural or physiological response from females for mating to occur at natural rates.

It should be emphasized that the three active compounds are chiral. It would be very difficult to determine the enantiomers that the insects are producing because of current limitations in analytical methods and the small amounts of material available from the insects. To our knowledge, there are no chiral stationary phase GC or LC columns that are capable of resolving enantiomers of long-chain methyl-branched hydrocarbons. Nevertheless, the insects respond to racemic standards, suggesting that activity is not strongly hindered by the possible presence of unnatural enantiomers, assuming that the insects produce only one of the two possible enantiomers of each compound.

Contrary to our expectation, the dominant contact sex pheromone component of *N. a. acuminatus*, $7Me\text{-}C_{27}$, is not significantly more abundant in SPME samples than in hexane extracts. However, biologically active $9Me\text{-}C_{27}$ is present at higher abundance in SPME wipe samples than in hexane extracts, as are two other methylalkanes whose roles in mate recognition have not been tested. These findings support the notion that SPME yields a more representative profile of components actually encountered by the antennae of males,

and so may provide a clearer indication of potential semio-chemicals present in the wax layer than does solvent extraction. SPME also has the advantage of providing hydrocarbon profiles of living insects, so that individuals can be sampled repeatedly.

Mate and host location by *N. a. acuminatus* is similar apparently to that of many species in the subfamily Cerambycinae, with both sexes being attracted to host volatiles and male-produced sex pheromones, and males recognizing females by contact chemoreception (Lacey *et al.*, 2004, 2007, 2008; Ginzel & Hanks, 2005). The limited data that are available suggest that the chemical structures of volatile sex pheromones in the Cerambycinae are highly conserved, with the result that some species that share common hosts may be attracted to each others' pheromones (Hanks *et al.*, 2007). By contrast, the contact pheromones of cerambycids appear to be much more species-specific (Ginzel *et al.*, 2003a, 2003b, 2005, 2006), and thus may play a critical role in reproductive isolation of closely related, sympatric species.

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