Identification of an Important Component of the Contact Sex Pheromone of *Callidiellum rufipenne* (Coleoptera: Cerambycidae)

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ABSTRACT Adult male and female *Callidiellum rufipenne* (Motschulsky) (Coleoptera: Cerambycidae) aggregate on severely stressed or fallen trees and cut logs of cypress species (Cupressaceae) that are the hosts of their larvae. Our studies showed that male *C. rufipenne* actively search for females on these trees and only respond to females after contacting them with their antennae. Removing cuticular hydrocarbons from fresh carcasses of females with solvent rendered the carcasses unattractive to males, but activity was restored when the solvent extract was reapplied. These findings suggest that contact pheromones mediate mate recognition in this species. Bioassays of fractions of the extracts determined that the branched chain hydrocarbon fraction was primarily responsible for activity. 5,17-Dimethylnonacosane was a key sex-specific component in extracts of females, and synthetic 5,17-dimethylnonacosane elicited a strong mating response from males. We conclude that this compound is an important component of the contact sex pheromone of *C. rufipenne* if not the sole component. Solvent extracts of both sexes also contained 9-pentacosyne and 9-heptacosyne. To our knowledge, this is only the second report of alkynes in the cuticular lipids of insects.

KEY WORDS 5,17-dimethylnonacosane, 9-pentacosyne, 9-heptacosyne, cuticular hydrocarbons, mating behavior

The epicuticular wax layer of insects is a complex mixture of long-chain hydrocarbons, fatty acids, alcohols, esters, aldehydes, and ketones that protects insects from desiccation (Gibbs 1998). It is also increasingly clear that some of these chemicals have additional roles as contact sex pheromones (Howard and Blomquist 2005). The compounds that act as sex pheromones may be present only in the wax layer of females (Carlson et al. 1998, Ginzel et al. 2003, Lacey et al. 2008), or alternatively, differences between the sexes in the relative abundance of compounds may constitute the sex-specific signal (Bartelt et al. 1986, Howard and Blomquist 2005).

Within the large beetle family Cerambycidae, female-produced contact sex pheromones have been identified for three species in the tribe Monochamini of the subfamily Lamiinae (Fukaya et al. 1996, 1997, 2000; Zhang et al. 2003) and three species in the tribe Clytini of the Cerambycinae (Ginzel et al. 2003, 2006; Lacey et al. 2008). *Callidiellum rufipenne* (Motschulsky) (tribe Callidiini, Cerambycinae) is native to Japan, but it has been introduced into several other areas of the world, including northeastern North America (Maier 2007). It was discovered in Connecticut in 1998 (Maier and Lemmon 2000) and currently ranges from Massachusetts to North Carolina in the United States, primarily in coastal areas (Maier 2007). In the United States, it has been reared from eight species in four genera of the Cupressaceae (Maier 2007). The adults are diurnal, ≈2 cm long, and are active for ~1 mo in the spring (for biology, see Shibata 1994, Maier 2008). Adults apparently do not feed and live ≈18 d in the wild. The species is protandrous, and the primary sex ratio is ≈1:1. Both males and females mate repeatedly. Adult females oviposit on recently felled or severely stressed trees.

We report here the results from field and laboratory studies of the mating behavior of *C. rufipenne.* In particular, compounds in cuticular extracts of adult male and female *C. rufipenne* were analyzed and fractionated, and the roles of various cuticular hydrocarbons as contact sex pheromones was investigated.

Materials and Methods

Field Site and Source of Specimens. Freshly cut logs of arborvitae (*Thuja occidentalis* L.) were piled in a yard in an urban neighborhood of New Haven, CT, on 15 March 2007. Adult *C. rufipenne* flew to these logs, where they mated and oviposited, from 22 April to 9 May 2007 (C.E.R., personal observation). The logs were left in situ over the winter. Beginning 30 January 2008, they were brought into the laboratory in batches

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of three to four to provide a steady supply of adult beetles throughout the spring. Adult *C. rufipennis* began emerging from the logs on average 1 wk after they were brought indoors. Voucher specimens were placed in the collection of The Connecticut Agricultural Experiment Station.

Beetles that were to be used for analysis of cuticular hydrocarbons were collected directly into glass vials to avoid contamination and frozen at −20°C. Beetles used in mating trials and bioassays were housed individually in 9-cm plastic petri dishes and provided water (8-ml vial with a cotton roll). Petri dishes with beetles were kept in a dark incubator at 15°C. Beetles used in mating trials and bioassays were isolated in dishes for at least 24 h before being tested and were used in bioassays only once per day. Age of adult males was not controlled, rather, to ensure that males were vigorous, each was presented with a freshly thawed, freeze-killed female carcass immediately before each trial. Males that failed to respond to the female were not used in experiments.

Mating Behavior and the Role of Contact Chemoreception. Observations of mating behavior in the field were supplemented by videotapes of three pairs of beetles mating on arborvitae logs (22 April 2007). These observations strongly suggested that males identify females by contact chemoreception with their antennae. We confirmed the role of contact chemoreception with the following bioassay ($N = 20$ different pairs of beetles), videotaping all trials (Handycam, HDR-SR1; SONY, San Diego, CA).

1. We characterized the sequence of behaviors that were involved in mating by placing one beetle of each sex in an arena (12-cm-diameter clear glass bowl inverted on filter paper). The bowl was coated with liquid Teflon (Anti-Ant; Farnam Pet Products, Phoenix, AZ) to limit movement of beetles, except for a 3-cm-wide band for observation and videotaping. The arena was under natural light from a window supplemented with light from a 60-W incandescent bulb and under ambient laboratory temperatures (≈20°C). The study was conducted with 20 pairs of beetles between 0900 and 1500 hours. Recordings of the interactions between males and females, leading up to and culminating in copulation, were used to confirm that antennal contact was necessary for mating. Seven of the 20 pairs assayed were allowed to complete mating, and these tapes were further analyzed to characterize the typical sequence of mating behavior.
2. After a male successfully coupled with the female, she was removed and killed by freezing at −20°C for at least 20 min. The female carcass was warmed to room temperature and presented to the same male. An attempt to mate was taken as evidence that recognition cues were intact and that a behavioral response by the female was not necessary for mate recognition. Once a male contacted a living female with his antennae, a clear progression of behaviors led to copulation: (1) the male oriented to (turned toward) the female and followed her if she was walking; (2) he grasped the female with his front legs and aligned his body with hers; and (3) he mounted her and attempted to couple the genitalia. This assessment of behavioral responses was cumulative: males must perform steps 1–3 in sequence.
3. A trial was scored as a "response" if the male displayed at least the second step (aligning his body with the female) on contacting the female carcass with his antennae. Males rarely reached step 2 without attempting to mate (step 3). A trial was scored as "no response" if the male did not reach the second step of the behavioral progression after contacting the female with his antennae or tarsi three times.
4. Dead females that elicited a response from males were immersed for 2 min in two successive 1-ml aliquots of hexane (Optima Hexanes; Fisher, Fair Lawn, NJ). The combined extracts were concentrated under a stream of purified nitrogen to the 1 ml in a calibrated vial. The extracted female carcass was presented again to the same male. If the male responded by attempting to mate, the female carcass was extracted again by sonication in hexane for 3 min and again presented to the male (Sonicator model FS30; Fisher). The process was repeated until the male no longer attempted to mate with the female after touching her with his antennae at least three times, confirming that the contact pheromone had been removed and that other cues were not sufficient to elicit a response from the male.
5. We pipetted 0.1 female equivalent (FE) of crude extract (0.1 ml) evenly over the surface of the extracted female and allowed the solvent to evaporate. The treated female carcass was presented again to the same male to confirm that the extract had restored activity. If the male did not respond to the treated female, we added 0.1 FE of extract incrementally until the male did respond or a maximum of 1 FE was reached.

Fractionation of Cuticular Extracts. We fractionated the cuticular hydrocarbons of *C. rufipenne* as a first step in isolating the active components. We collected crude extract for fractionation and bioassays by extracting 50 females together in two 5-ml aliquots of hexane. A portion of the extract (20%) was set aside to be used as a positive control in bioassays, and the remainder was concentrated to ≈0.5 ml and fractionated as follows. The tapered end of a disposable Pasteur pipette was fitted with a small plug of glass wool, the pipette was loaded with 400 mg of silica gel impregnated with 10% by weight $AgNO_3$ (+ 230 mesh; Sigma-Aldrich, Milwaukee, WI), and the pipette was placed in a drying oven at 125°C for 4 h. The pipette was cooled in a closed glass test tube and rinsed with 2 by 1 ml hexane to pack the silica gel bed, discarding the eluate. The concentrated crude extract was loaded onto this column, which was eluted sequentially with 3 ml hexane, 3 ml 20% cyclohexene in hexane, and 3

ml ether, yielding fractions containing saturated hydrocarbons, unsaturated hydrocarbons, and more polar compounds, respectively. One half of the saturated hydrocarbons fraction (20 FE) was concentrated to dryness under a stream of nitrogen and taken up in 3 ml isooctane. Powdered 5-Å molecular sieve (300 mg, previously dried by heating to 140°C under vacuum) was added, and the resulting slurry was stirred for 48 h at room temperature. The mixture was filtered through a plug of Celite (Fisher), rinsing with isooctane, yielding a fraction containing only saturated branched chain hydrocarbons.

Bioassay of Fractions of the Crude Cuticular Extract, and a Synthetic Cuticular Component. We used the following bioassay to test the activity of the four fractions containing saturated hydrocarbons (branched and unbranched), unsaturated hydrocarbons, polar components, branched hydrocarbons, and synthetic 5,17-dimethylnonacosane (see below). A freeze-killed female was extracted as described above and tested with three different males to ensure that the contact pheromone had been removed. If any of the three males responded to the female, she was subjected to further extractions, testing her against three different males after each extraction, until none of males responded to her.

Fractions of the cuticular extracts of females, and synthetic 5,17-dimethylnonacosane, were bioassayed by pipetting hexane solutions of fractions onto a solvent-washed female carcass. We used 0.2 FE female equivalents of each fraction because males responded well to this dosage of crude extract (see Results). To test the activity of synthesized 5,17-dimethylnonacosane, 100 ng was applied in 10 μl hexane, which approximated the amount in 0.25 FE of *C. rufipenne* extract. For all bioassays, controls consisted of a second solvent-extracted female carcass to which an equivalent amount of hexane had been applied. We presented the control and chemically treated carcasses to individual males simultaneously, on opposite sides of the glass bowl arena, with their positions randomized to control for location effects. Before each assay, males were presented with two positive controls: a thawed, freeze-killed female and a solvent-extracted female treated with 0.2 FE of crude extract. Males that did not respond to both controls were discarded.

For all bioassays, we presented each of at least three treated female carcasses to 5–10 different males (N = 20–28 males) and videotaped the males to facilitate interpretation of their responses. We scored responses on a scale of 0–3, as described above. For each bioassay, we tested differences between treatments in the numbers of males responding with Fisher exact tests (Sokal and Rohlf 1995).

Identification of Cuticular Hydrocarbons. We collected extracts of cuticular hydrocarbons by separately rinsing five male and five female beetles in two 1-ml aliquots of hexane. The resulting extracts were analyzed by coupled gas chromatography (GC)–mass spectrometry (MS) using a Hewlett-Packard (HP) 6890 GC interfaced to an HP 5973 mass selective detector (Avondale, PA). The GC was fitted with a DB5-MS column (30 m by 0.25 mm ID, 0.25-μm film thickness; J&W Scientific, Folsom, CA), programmed at 100°C/1 min, 10°C/min to 280°C, and hold for 20 min. Injections were in splitless mode, with injector and transfer line temperatures of 280°C. Helium carrier gas was used, in constant pressure mode. Aliquots of the composite extracts from 50 beetles were analyzed using the same conditions.

Most cuticular hydrocarbons were identified by a combination of retention time comparisons versus straight-chain alkane standards and interpretation of their mass spectra. Specifically, the molecular ion indicated the total number of carbons in the molecule, methyl-branched hydrocarbons gave enhanced, diagnostic ions at branch points that allowed the positions of the methyl branches to be determined, and the presence of methyl branches resulted in diagnostic shifts in retention times versus straight-chain standards (Nelson 1993, Nelson and Blomquist 1995).

To identify the two alkyne compounds in the unsaturated hydrocarbons fraction (see Results), an aliquot of the fraction from females was concentrated to dryness under a stream of nitrogen and analyzed by ^{1}H NMR as a $CDCl_3$ solution on a Varian INOVA-400 spectrometer (400 MHz). The recovered sample was concentrated to dryness and taken up in 1 ml hexane, and the solution was transferred to a septum-capped 1.5-ml vial. To determine the position of the alkyne, the triple bond was stereoselectively reduced to the corresponding alkene, and the alkene was epoxidized. The resulting epoxide gave diagnostic mass spectral fragments from cleavage on either side of the epoxide, allowing the position of the alkyne in the parent compound to be determined (Hogge and Millar 1987). Thus, ≈1 mg of Lindlar catalyst was added, and the vial was flushed with H_2 and sealed, with a balloon filled with H_2 attached. The mixture was stirred vigorously for 5 min and filtered through a pad of Celite to remove the catalyst. After concentration, the crude product, containing alkenes and ≈20% alkanes from overreduction, was treated at room temperature for 2 h with *m*-chloroperbenzoic acid in CH_2Cl_2 (100 μl of a 10-mg/ml solution). The reaction mixture was concentrated under a stream of nitrogen and partitioned between 1 ml pentane and 200 μl of 0.25 M aqueous NaOH. The pentane layer was dried over anhydrous Na_2SO_4 and analyzed by GC-MS.

Synthesis of Compounds

Synthesis of 5,17-Dimethylnonacosane. Tetrahydrofuran (THF) was distilled from sodium/benzophenone ketyl under argon. ^{1}H- and ^{13}C-NMR spectra were recorded with a Varian INOVA-400 spectrometer (400 and 100.5 MHz, respectively), in $CDCl_3$ solution. Mass spectra of synthetic intermediates were obtained with a HP 5890 GC interfaced to an HP 5970 mass selective detector. The GC was fitted with a DB5-MS column (30 m by 0.25 mm ID) programmed from 50 to 280°C at 10°C/min. Solutions of crude products were dried over anhydrous Na_2SO_4 and con-

centrated under partial vacuum by rotary evaporation, unless otherwise stated. Crude products were purified by vacuum flash chromatography on silica gel (230–400 mesh; EM Science, Gibbstown, NJ). Reactions with air- or water-sensitive reagents were carried out in oven-dried glassware under argon. Yields have not been optimized.

1,9-Diiodononane. A solution of 1,9-nonanediol (3.2 g, 20 mmol) and Et_3N (7 ml, 50 mmol) in 100 ml of CH_2Cl_2 was cooled to −10°C in an ice-salt bath, and methanesulphonyl chloride (3.5 ml, 45 mmol) was added dropwise over 10 min. The resulting slurry was stirred 1.5 h in the ice bath and poured into 150 ml cold water. After separation of the layers, the aqueous layer was extracted again with CH_2Cl_2, and the combined extracts were washed with saturated $NaHCO_3$, water, and brine, dried, and concentrated. Toluene (10 ml) was added, and the resulting oil was pumped under vacuum to azeotrope off the remaining Et_3N. The crude product (7.12 g), which crystallized as the solvent was removed, was taken up in 100 ml acetone, and the solution was chilled in an ice bath. Powdered NaI (15 g, 100 mmol) was added in three portions over 15 min, and the resulting yellow slurry was stirred at room temperature overnight and warmed to 60°C for 3 h. The mixture was cooled, and most of the acetone was removed by rotary evaporation. The residue was taken up in 100 ml hexane, washed with saturated $NaHCO_3$, water, and brine with a small amount of $Na_2S_2O_3$ added (to reduce traces of iodine to iodide), dried, and concentrated. The resulting pale yellow oil (7.81 g, 95%) gave one spot on silica gel TLC (hexane) and was used directly. ^{1}H NMR: δ 1.27–1.31 (m, 6H, 3 × CH_2), 1.38–1.43 (m, 4H, 2 × $CH_2CH_2CH_2$ I), 1.83 (overlapped tt, 4H, 2 × CH_2CH_2I, J = 7.2 Hz), 3.20 (t, 4H, 2 × CH_2I, J = 6.8 Hz). ^{13}C NMR: δ 7.46, 28.62, 29.38, 30.64, 33.71. MS: *m/z* 253 (5, M^+-I), 211 (6), 197 (11), 183 (11), 169 (12), 155 (22), 141 (2), 125 (6), 97 (2), 83 (23), 69 (49), 55 (83), 41 (100).

12-Iodododecan-2-one. A solution of acetone dimethylhydrazone (1.1 g, 11 mmol; TCI America, Portland, OR) in THF (80 ml) was cooled to −78°C under argon, and butyllithium (4.6 ml, 2.6 M in THF, 12.0 mmol) was added dropwise. After stirring for 2.5 h at this temperature, 1,9-diiodononane (3.8 g, 10 mmol) was added dropwise, and the mixture was warmed to room temperature and stirred overnight. The mixture was cooled in an ice bath, quenched with 2 M HCl (30 ml), and again stirred overnight at room temperature to hydrolyze the hydrazone. The mixture was diluted with water (40 ml) and extracted with ethyl acetate (2 by 50 ml). The combined organic phases were washed with aqueous $NaHCO_3$ (30 ml) and brine (30 ml), treated with anhydrous Na_2SO_4 and decolorizing charcoal, filtered through a plug of Celite, and concentrated. The residue was purified by vacuum flash chromatography (5% ethyl acetate in hexane), yielding 1.05 g of 12-iodo-dodecan-2-one (45% yield, based on 25% recovered starting material). ^{1}H NMR: δ 1.28–1.33 (m, 10H, 5 × CH_2), 1.36–1.41 (m, 2H, $CH_2CH_2CH_2$I), 1.55–1.61 (m, 2H, $CH_2CH_2COCH_3$), 1.82 (overlapped tt, 2H, CH_2CH_2I, J = 6.8 Hz), 2.14 (s, ^{3}H, CH_3CO), 2.42 (t, 2H, CH_2CO, J = 7.6 Hz), 3.19 (t, 2H, CH_2I, J = 7.2 Hz). ^{13}C NMR: δ 7.52, 24.05, 28.70, 29.35, 29.52, 30.08, 30.67, 33.74, 44.00, 209.5. MS: *m/z* 310 (M^+, trace), 183 (5), 155 (2), 141 (1), 127 (1), 109 (5), 95 (4), 83 (11), 71 (14), 69 (15), 58 (36), 55 (24), 43 (100), 41 (29). The spectral data matched that previously reported (Bérubé et al. 2006).

15-Iodo-5-methylpentadec-4-ene. Butyltriphenylphosphonium bromide (2.0 g, 5.08 mmol) was dissolved in THF (20 ml) under argon. The solution was cooled in an ice bath, butyllithium (1.86 ml, 2.9 M in THF, 5.4 mmol) was added dropwise, and the solution was stirred for 30 min. 12-Iodododecan-2-one (1.05 g, 3.39 mmol) was added dropwise, the ice bath was removed, and the mixture was stirred overnight. The reaction was quenched with saturated aqueous NH_4Cl (25 ml), extracted with hexane (3 by 25 ml), backwashed with brine, dried, and concentrated. The residue was purified by vacuum flash chromatography (hexane), yielding 0.51 g of a mixture of stereoisomers of 15-iodo-5-methylpentadec-4-ene mixed with the bromide analogs from SN2 displacement of the iodide with the bromide from the Wittig salt (≈43% yield for the combined iodides and bromides). The mixture was used immediately in the next step. MS: *m/z* (iodide), 308 (M^+-42, 3), 293 (3), 252 (5), 210 (1), 155 (3), 111 (4), 97 (11), 83 (37), 69 (47), 55 (100), 41 (58). (bromide analog), 302 (M^+, 3), 304 (M^+ + 2, 3), 123 (1), 111 (3), 109 (3), 98 (10), 83 (30), 70 (47), 55 (100), 41 (55). The mass spectra for the bromide and iodide isomer pairs were very similar.

14-Methyloctadec-14-en-2-one. Butyllithium (0.76 ml, 2.9 M in THF, 2.2 mmol) was added dropwise to a solution of acetone dimethylhydrazone (0.2 g, 2.0 mmol) and THF (11 ml) cooled to −78°C, under argon. After stirring for 2.5 h at −78°C, the mixture of isomers of 15-iodo-5-methyl-pentadec-4-ene and 15-bromo-5-methylpentadec-4-ene (0.51 g, 1.46 mmol) was added, and the reaction was warmed to room temperature and stirred overnight. The mixture was cooled in an ice bath, quenched with 2 M HCl (9 ml), and stirred overnight to hydrolyze the hydrazone. The mixture was diluted with water (10 ml) and extracted with ether (3 by 20 ml). The combined ether phases were washed with brine, dried, concentrated, and vacuum flash chromatographed (1%, and then 5% ethyl acetate in hexane), yielding 0.26 g (64%) of a 52:48 mixture of the stereoisomers of 14-methyloctadec-14-en-2-one. ^{1}H NMR: δ 0.90 (t, 3H, $CH_3CH_2CH_2CH$, J = 7.6 Hz; overlapping triplets from the *Z*- and *E*-isomers), 1.27–1.38 (m, 20H, 10 × CH_2), 1.58, 1.68 (d and br s, 3H, allylic CH_3, J = 1.2 Hz, from the *Z*- and *E*-isomers), 1.93–2.02 (m, 4H, 2 × allylic CH_2), 2.14 (s, 3H, CH_3CO), 2.42 (t, 2H, CH_2COCH_3, J = 7.2 Hz), 5.12 (br t, 1H, alkene CH, J = 6.8 Hz). MS: *m/z* 280 (M^+, 3), 264 (1), 183 (3), 138 (4), 125 (6), 109 (15), 98 (29), 83 (29), 69 (42), 56 (45), 55 (84), 43 (100), 41 (48). The mass spectrum for the other isomer was virtually identical.

5,17-Dimethylnonacosa-4,17-diene. A solution of n-dodecyltriphenylphosphonium bromide (0.099 g, 0.193 mmol) and THF (3 ml) was cooled in an ice bath

under argon, and butyllithium (0.067 ml, 2.9 M in THF, 0.193 mmol) was slowly added. After stirring for 30 min, 14-methyl-octadec-14-en-2-one (0.036 g, 0.13 mmol) was added, and the mixture was warmed to room temperature and stirred overnight. The reaction was quenched with saturated aqueous NH_4Cl (5 ml) and extracted with hexane (3 by 7 ml), and the combined hexane layers were washed with brine, dried, and concentrated. The product was purified by vacuum flash chromatography (hexane), yielding 0.019 g (34%) as an approximately equal mixture of the four stereoisomers of 5,17-dimethylnonacosa-4,17-diene. ^{1}H NMR: δ 0.87–0.92 [m, 6H, $CH_3CH_2CH_2CH$, $CH_3(CH_2)_{10}CH$], 1.28–1.39 (m, 38H, 19 × CH_2), 1.59 (s, ^{3}H, allylic CH_3), 1.68 (s, ^{3}H, allylic CH_3), 1.95–2.03 (m, 8H, 4 × allylic CH_2), 5.12 (br t, 1H, alkene CH J = 7.2 Hz), 5.36 (t, 1H, alkene CH, J = 4.8 Hz). MS: *m/z* 432 (M^+, 11), 334 (2), 290 (2), 247 (2), 207 (3), 152 (4), 137 (4), 125 (3), 111 (9), 97 (21), 96 (26), 83 (33), 81 (37), 69 (46), 55 (100), 43 (66). The mass spectra for the other three isomers were very similar.

5,17-Dimethylnonacosane. Five percent palladium on carbon (≈0.005 g) was added to a solution of the mixed isomers of 5,17-dimethylnonacosa-4,17-diene (0.044 g, 0.102) in hexane (2 ml), and the mixture was stirred for 3 h under hydrogen atmosphere. The mixture was filtered through a plug of celite, giving a quantitative yield of 5,17-dimethylnonacosane. ^{1}H NMR: δ 0.84 (d, ^{3}H, CH_3CHCH_2, J = 6.4 Hz), 0.85 (d, ^{3}H, CH_3CHCH_2, J = 6.8 Hz), 0.87–0.91 (m, 6H, 2 × CH_3), 1.08–1.27 (m, 52H, 2 × CH, 25 × CH_2). MS: *m/z* 436 (M^+, trace), 421 (1), 379 (2), 267 (2), 196 (6), 169 (2), 155 (2), 141 (3), 127 (6), 113 (8), 99 (12), 85 (43), 71 (52), 57 (94), 43 (100), 41 (31).

Results

Mating Behavior and the Role of Contact Chemoreception. In the field, adult male and female *C. rufipenne* were most active on host material during the middle of the day (≈1100–1500 hours), particularly on warm, sunny days. Males actively patrolled the surface of the host material, exploring it with their outspread antennae. Females moved about more slowly, probing for potential oviposition sites under bark crevices with their ovipositors.

In laboratory and videotaped observations in the field, males responded to females only after contacting them with their antennae. Males did not approach females in a manner that would suggest that they were attracted from a distance by either visual or olfactory cues. After contacting a female, the male turned toward her, and attempted to grasp her with his first two pairs of legs while he aligned his body with hers. Females often ran from males, with the male still clinging to the tergum of the female with his forelegs, sometimes palpating her tergum. When the female stopped walking, the male lifted his abdomen by extending his hind legs, curled it under his body, and attempted to couple the genitalia, while continuing to palpate the tergum of the female. Once the genitalia were connected, the male pulled backward sharply with his abdomen, causing the ovipositor to extend to its full length. Females usually remained sedentary during copulation, with the male palpating the tergum while stroking it with the forelegs and pumping his abdomen.

Average copulation time was 249 ± 24 s in the laboratory (N = 7). After withdrawing his aedeagus, the male remained astride the female, grasping her tergum with his forelegs. He remained in this half-mount position as the female walked, suggesting mate guarding (Thornhill and Alcock 1983). On larval hosts, females probed the bark with their ovipositors as they walked. Males remained with one female for as long as 20 min in the field, fighting off other males that approached. If a male was separated from a female, he would attempt to resume the mate-guarding position. On two occasions, we observed that a mate-guarding male switched to a different female after contacting her with his antennae and attempted to copulate with the new female.

All 20 males attempted to mate with freshly killed females after contacting them with their antennae in laboratory arenas. Several sequential extractions with solvent usually were required for females to be rendered unattractive to males (range, 4–8; mean = 5.7 ± 1.330 SD), suggesting that males were quite sensitive to the contact pheromone of females. However, female carcasses that were subjected to too many washes with solvent became brittle, and males would no longer respond to them, even after application of 1.0 FE of crude extract. This finding suggests that qualities other than contact pheromones are required to elicit responses from males.

In 19 of 20 cases, application of crude extract to solvent-extracted female carcasses restored their attractiveness to males, and each male showed the full progression of mating behaviors. Eleven males responded to females that were treated with 0.1 FE of the extract, five males required 0.2 FE of extract to respond, and three required 0.3 FE or more before responding (mean = 0.17 ± 0.12 SD). These results confirmed that male *C. rufipennis* recognize females using a contact sex pheromone that was extractable with hexane.

Bioassay of Fractions of the Crude Cuticular Extract, and a Synthetic Cuticular Component. Males responded with the full range of mating behaviors to the saturated hydrocarbons fraction and the branched chain hydrocarbons fraction but not to the unsaturated hydrocarbon and polar compound fractions (Table 1). These results suggested that the critical component(s) of the contact pheromone consisted of saturated, branched chain hydrocarbons. Because 5,17-dimethylnonacosane was the only relatively large and sex-specific component of the branched hydrocarbon fraction of female extracts (see below), it was a logical candidate to synthesize and test as a contact sex pheromone. Males responded to female carcasses treated with a solution of synthetic 5,17-dimethylnonacosane (mixture of all four possible stereoisomers; Table 1), confirming that this compound was sufficient to elicit the mating response.

Table 1. Number of male *C. rufipenne* responding to hexane-extracted dead females (controls) and to hexane-extracted dead females treated with fractions of crude extract and synthetic 5,17-dimethylnonacosane

Fraction/compound	*N*	Number of males responding		χ^2 statistic (*P*)
		Control female	Treated female	
Saturated hydrocarbons[a]	20	2	20	24.9 (<0.001)
Unsaturated hydrocarbons[a]	20	2	5	1.56 (0.21)
Polar compounds[a]	20	0	1	1.03 (0.31)
Branched saturated hydrocarbons[a]	24	1	20	32.4 (<0.001)
5,17-Dimethylnonacosane[b]	28	6	18	10.5 (0.001)

[a] Tested at doses of 0.2 FE.
[b] Tested at a dose of 0.25 FE.

Identification of Cuticular Hydrocarbons. Hexane extracts of adult male and female *C. rufipenne* were dominated by branched-chain alkanes of 24–31 carbons, with lesser amounts of straight-chain alkanes (Fig. 1; Table 2). The relative amounts of the various compounds seemed to be more variable in females than in males, and extracts from both sexes appeared qualitatively similar, with the exception that extracts from females contained one compound, 5,17-dimethylnonacosane, that was sex specific. Overall, no compounds more polar than hydrocarbons were detected in the extracts under the GC-MS conditions used for the analyses.

The unsaturated hydrocarbons fraction of extracts from both males and females also contained two homologous compounds with mass spectra suggesting that they were C_{25} and C_{27} compounds with two sites of unsaturation. These compounds were the only compounds in this fraction, and they were more abundant in extracts from females than males. Each eluted slightly after the corresponding saturated alkane on the DB-5 GC column, suggesting that they might be conjugated dienes, because nonconjugated dienes elute before the saturated analogs on this column (J.G.M., personal observation). However, they did not react with the powerful dienophile 4-methyl-1,2,4-triazoline-3,5-dione, indicating that a conjugated diene was probably not present. Thus, these compounds had to be either allenes or alkynes. They were conclusively identified by data from several methods. First, ^{1}H NMR analysis of the unsaturated hydrocarbons fraction showed no alkene or allene protons, but there was a distorted triplet at 2.11 ppm, as would be expected for CH_2 groups on either side of an internal alkyne. The recovered fraction was partially reduced with Lindlar catalyst and H_2, followed by epoxidation with *m*-chloroperbenzoic acid. The mass spectrum of the resulting more abundant C_{25} epoxide exhibited a weak molecular ion at m/z 366 (2% of base peak at m/z 69), and strong diagnostic fragments at m/z 155 (40%) and 253 (37%), unequivocally placing the triple bond in the parent alkyne in the nine position, and identifying it as 9-pentacosyne. The mass spectrum of the epoxide derived from the less abundant C_{27} alkyne showed analogous diagnostic ions at m/z 155 and 281, indicating that the parent alkyne was 9-heptacosyne.

Discussion

The mate searching behavior of male *C. rufipenne* and the role of contact pheromones are consistent with many other species of the Cerambycidae (Akustu and Kuboki 1983, Hanks et al. 1996, Ginzel and Hanks 2003, Zhang et al. 2003, Crook et al. 2004, Lopes et al. 2005, Wang and Chen 2005, Wang and Davis 2005, Ginzel et al. 2006, Ibeas et al. 2008). Antennal contact with females treated with 5,17-dimethylnonacosane usually was sufficient to elicit the full repertoire of mating behavior from males. However, other branched chain compounds in the unsaturated hydrocarbons fraction may also contribute to the overall activity. It seems less likely that components from one or more of the other fractions of the crude cuticular extracts may be part of the contact pheromone, because the branched chain fraction seemed to be similar in activity to both the crude extract and the saturated alkanes fraction (i.e., both branched and unbranched alkanes). Most of the contact pheromones of cerambycids that have been identified to date are composed of a blend of long-chain hydro-

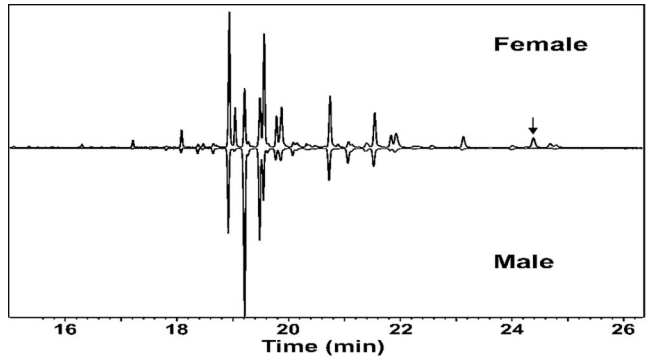

Fig. 1. Representative gas chromatograms of hexane extracts of cuticular lipids of female (top trace) and male (bottom, inverted trace) *C. rufipenne*. The arrow indicates the peak from 5,17-dimethylnonacosane.

Table 2. Relative proportions of compounds in hexane extracts of freeze-killed male and female *C. rufipenne*

Peak no.	Ret. time	Hydrocarbon	Percent relative to 11Me- + 13Me-C25 ± SD[a]		Diagnostic ions[b]
			Female	Male	
1	17.21	C23	4.2 ± 4.1	0.7 ± 0.6	324
2	17.54	9Me-, 11Me-C23	0.2 ± 0.2	0.4 ± 0.4	140/224, 168/196, 323 (338)
3	17.82	9,13-DiMe-C23	0.2 ± 0.2	1.1 ± 0.7	140/239, 168/211, 337 (352)
4	18.09	C24	14.2 ± 9.9	3.1 ± 1.2	338
5	18.39	10Me-, 11Me-, 12Me-C24	4.2 ± 0.8	3.2 ± 0.6	154/224, 168/210, 182/196, 337 (352)
6	18.48	6Me-C24	3.0 ± 2.0	1.2 ± 0.5	98/281, 337 (352)
7	18.66	9,13-DiMe-C24	4.5 ± 1.6	4.6 ± 1.4	140/253, 182/211, 351 (366)
8	18.95	C25	146 ± 109	52.9 ± 14.1	352
9	19.05	9-pentacosyne	46.2 ± 14.5	2.6 ± 1.7	81, 95, 109, 123, 137, 348
10	19.24	11Me-, 13Me-C25	100	100	168/224, 196, 366
11	19.30	11,13-DiMe-C25	7.6 ± 2.7	6.0 ± 1.7	168/239, 196/211, 365 (380)
12	19.51	9,13-DiMe-C25	75.4 ± 24.2	75.0 ± 18.7	140/267, 196/211, 380
13	19.57	3-Me-C25	150 ± 115	45.7 ± 12.5	337, 366
14	19.79	C26	33.2 ± 22.8	9.9 ± 3.5	366
15	19.88	3,7-DiMe-C25	60.8 ± 38.3	20.9 ± 8.4	127/281, 351, 365 (380)
16	20.09	11Me-, 12Me-, 13Me-C26	9.5 ± 2.3	5.4 ± 0.6	168/238, 182/224, 196/210 (380)
17	20.32	4Me-C26	5.9 ± 6.8	0.8 ± 0.6	Enlarged 71, 337 (380)
18	20.74	C27	58.9 ± 50.1	19.8 ± 8.2	380
19	20.87	9-heptacosyne	5.6 ± 3.4	0.2 ± 0.4	81, 95, 109, 123, 137 (376)
20	21.08	11Me-, 13Me-C27	12.7 ± 2.7	9.9 ± 2.3	168/252, 196/228 (394)
21	21.40	11,15-DiMeC27	9.5 ± 5.3	6.5 ± 2.3	168/267, 196/239 (408)
22	21.55	3Me-C27	62.3 ± 57.0	19.8 ± 9.3	365, 379 (394)
23	21.84	C28	12.2 ± 11.6	2.1 ± 2.5	394
24	21.92	3,9-, 3,11-, 3,13-, 3,15-DiMe-C27	28.5 ± 18.1	7.4 ± 4.9	155/280, 183/252, 209/224, 239/196, enlarged 379, 393 (408)
25	24.02	11,15-DiMe-C29	3.7 ± 3.6	3.0 ± 1.1	168/295, 224/239, 421 (436)
26	24.37	5,17-DiMe-C29	33.4 ± 4.8	0	85/379, 196/267, 436
27	24.80	3,11-DiMe-C29	4.5 ± 4.7	1.0 ± 1.1	183/280, 407 (436)

[a] Means for five individuals. Amounts relative to the combined peak from 11Me- and 13Me-C25, the largest peak in extracts from 8 of the 10 individuals analyzed.

[b] Molecular ions shown in parentheses were not seen but were inferred from the mass spectral fragment ions and the retention times relative to straight-chain alkanes.

carbons (Ginzel et al. 2003, Zhang et al. 2003, Lacey et al. 2008), but some also contain lactones and ketones (Yasui et al. 2007).

It also must be emphasized that the synthetic compound was a mixture of four stereoisomers, whereas the insects probably produce only a single stereoisomer. The biological activities of "unnatural" stereoisomers of volatile sex attractant pheromones have been widely explored, and in many cases, it has been shown that "unnatural" stereoisomers can strongly antagonize the response to attractant pheromones (reviewed by Mori 2007). However, the possible effects of contact pheromone stereoisomers has received minimal attention (but see Shibata et al. 2002, Carlson et al. 2005). In one of the few studies that specifically addressed the effects of contact pheromone stereoisomers, Fukaya et al. (1997) found that the cerambycid beetle *Psacothea hilaris* (Pascoe) was relatively insensitive to the enantiomeric composition of methyl-branched hydrocarbons, whereas the stereochemistry of alkene bonds was critically important, as might be expected given the substantial differences in the shapes of isomers containing either *Z* or *E* double bonds.

5,17-Dimethylalkanes seem to be relatively common in insect cuticular hydrocarbons, having been found in several insect taxa (Juarez et al. 2001, Sullivan 2002, Howard and Baker 2003, Steiner et al. 2006). Furthermore, 5,17-dimethylalkanes have been implicated as possible contact sex pheromone components in two parasitoid wasp species (Sullivan 2002, Steiner et al. 2006). In contrast, to our knowledge, the identification of 9-pentacosyne and 9-heptacosyne represents only the second report of alkynes in the cuticular lipids of insects. The former compound, 9-pentacosyne had been found previously in cuticular extracts of the ant *Platythyrea punctata* (Hartmann et al. 2005). For our study species, although these alkynes were present in much greater amounts in extracts of females than those of males, the lack of behavioral responses to the unsaturated hydrocarbons fraction, which consisted solely of these two compounds, suggests that they do not play a role in mate recognition in this species.

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