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ROLE OF CONTACT PHEROMONES IN MATE RECOGNITION IN *Xylotrechus colonus*

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Abstract—Adult male and female rustic borers, *Xylotrechus colonus* F. (Coleoptera: Cerambycidae), aggregate on cut logs and fallen trees that are the hosts of their larvae. Our studies show that male *X. colonus* actively search for females, and only respond to them after contacting them with their antennae. Stripping cuticular hydrocarbons from females with solvent rendered them unattractive to males, suggesting that males did not recognize females by mechanoreception alone. Reapplying solvent extract to washed females restored their attractiveness to males, confirming the role of cuticular hydrocarbons in mate recognition. Female cuticular hydrocarbon extracts contain *n*-pentacosane, 9-methylpentacosane, and 3-methylpentacosane, components that were either absent or present in very small amounts on males. We demonstrate that the contact pheromone is a blend of these three cuticular hydrocarbons.

Key Words—Cerambycidae, longhorned beetle, mating behavior, cuticular hydrocarbon, *n*-pentacosane, 9-methylpentacosane, 3-methylpentacosane, methyl-branched alkane.

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INTRODUCTION

Among the Coleoptera, sex pheromones have been identified for few species other than bark beetles and weevils (Mayer and McLaughlin, 1991). It has long been thought that longhorned beetles, Cerambycidae, rely on pheromones for mate location (Linsley, 1964). However, there is convincing evidence of long-range pheromones for only a few species in this family (Hanks, 1999). Hanks (1999) suggested that reproductive strategies and behavior of longhorned beetles appear to be associated with the condition of the larval host plant. Sex pheromones that operate over short or long distances are produced by either males or females of some species whose larvae feed in healthy, marginally weakened, or dead host plants (Hanks, 1999). For species that attack severely stressed or moribund trees, however, the sexes apparently are brought together by their mutual attraction to the larval host. Once on the host, males of these "stressed host species" actively search for mates, using their antennae to recognize females by contact chemoreception (Hanks, 1999).

Cerambycid species that use sex pheromones that operate over a distance include three species of the genus *Xylotrechus* in which the males produce the pheromone: Asian species *X. pyrrhoderus* (Bates) and *X. chinensis* Chevrolat, and the African *X. quadripes* Chevrolat (Iwabuchi, 1982, 1988; Sakai et al., 1984; Iwabuchi et al., 1987; Kuwahara et al., 1987; Hall et al., 1998). The North American species *Xylotrechus colonus* F., however, shows behaviors consistent with the stressed host strategy of Hanks (1999): both sexes are attracted by volatile compounds of larval hosts, and there is no evidence of long-range pheromones (Ginzel, 1999). Adult *X. colonus* are crepuscular and the larvae develop in recently killed and stressed deciduous trees of many species, primarily hickory (Yanega, 1996). The objectives of our study were to: (1) characterize the mating behavior of *X. colonus*, (2) identify the types of cues involved in mate recognition, and (3) identify and bioassay specific cuticular hydrocarbons that serve as contact pheromones.

METHODS AND MATERIALS

Field Site and Source of Specimens. Field studies were conducted at Allerton Park, a University of Illinois Natural Area in Piatt County, Illinois, USA. We studied mating behavior of *X. colonus* on two hickory trees, *Carya glabra* (Mill) Sweet, that had been felled on May 1 and July 9, 1998. The same trees, and others felled later, were the source of beetles for all of our laboratory studies. Beetles were collected directly into glass vials without handling to avoid contaminating their cuticular hydrocarbons. In the laboratory, we individually housed beetles in cylindrical cages (9 cm diam. $\times$ 12 cm tall) of aluminum window screen with 9-cm glass Petri dishes covering top and bottom. Caged beetles were provided

10% sucrose solution in an 8-ml feeder vial into which was inserted a 4-cm long cotton dental wick (Patterson Dental, South Edina, Minnesota, USA). Feeders were replaced at least every three days. Beetles used in mating trials and bioassays had been isolated in these cages for at least 24 hr and were only used in bioassays as long as they were active and vigorous.

Mating Behavior. To characterize the mating behavior of *X. colonus* and identify cues used in mate location, we observed beetles in the field as well as in laboratory arenas. In the field, we observed mating behavior of beetles on the felled hickory trees on warm evenings during May through August 1998. We observed $\sim$100 interactions among beetles of which 20 were videotaped (Panasonic AG-465 Proline S-VHS video camera). To study mating behavior in the laboratory during the same time period, we paired females and males in glass Petri dishes (9 cm diam. $\times$ 2 cm tall) lined with filter paper (No. 1, Whatman, Maidstone, England). Dishes were cleaned with acetone between trials and allowed to air dry. Twenty pairs of beetles in these arenas were videotaped in late afternoons under ambient light from exterior windows. The beetles became active when overhead lights were turned off.

We studied videotapes of beetles from field and laboratory studies to determine whether one sex showed directed movements towards the other sex from a distance, evidence that mate location might be mediated by either volatile pheromones or vision. We predicted (based on Hanks, 1999) that males would respond to females only after contacting them with their antennae, suggesting the use of contact pheromones. We also studied videotapes to characterize the copulatory behavior of *X. colonus*.

Role of Contact Chemoreception. We confirmed that male *X. colonus* used contact pheromones to recognize females via bioassay. Individual females were freeze killed (-4°C for 20 min), allowed to warm to room temperature, and presented to individual males in Petri dish arenas to confirm that males would recognize dead females and attempt to mate, demonstrating that recognition cues were intact. Cuticular hydrocarbons of females were removed by immersing them in two 1-ml aliquots of hexane for 2 min each; aliquots were combined and concentrated to 1 ml under nitrogen. Dead-washed females were then presented individually to the same males to confirm that males no longer responded (did not stop to inspect females with their antennae or otherwise orient to them), demonstrating that chemical cues had been removed and mating was not elicited by mechanoreception alone. To prove that the extract contained the pheromone, we pipetted 0.1 female equivalent (FE) of extract (0.1 ml) on each original dead-washed female, entirely coating the body with the solution, allowed it to evaporate, and presented the female again to males to confirm that the recognition cue was restored. If males did not respond, we incrementally added 0.1 FE (0.1 ml of extract) to each dead-washed female, to a maximum of 1 FE, and recorded the FE level at which males responded.

In these bioassays, conducted in late afternoons between July 10 and 22, 1998, under laboratory conditions, each of 10 dead females was presented to three different males ($N = 30$ males). We observed and videotaped the behavior of individual males toward females (dead, dead-washed, or dead-washed extract-treated) in a Petri dish arena. After a male contacts a living female with his antennae, a clear progression of behaviors leads to copulation: (1) the male stops walking, (2) aligns his body with the female, (3) mounts her, and (4) bends abdomen to connect claspers. We recorded behaviors of each male for 5 min after initial antennal contact with the female, and a trial was scored as a "response" if the male displayed at least the first behavior (stopping), and we also recorded subsequent behaviors. A trial was recorded as "no response" if the male showed none of these behaviors, but rather continued to walk after first contacting the dead female. Responses of males to dead-washed extract-treated females were compared to their response to dead-washed females using a χ^2 goodness of fit test (Sokal and Rohlf, 1995).

Identification of Female Contact Pheromone. To identify the compounds that served as contact pheromones (see Results), we extracted 10 females and males by individually immersing them in hexane as described above. Extracts were initially analyzed by electron ionization-mass spectrometry with a Hewlett-Packard 5973 mass spectrometer interfaced to a HP 6890 gas chromatograph, using a HP-5MS (cross-linked 5% phenylmethyl siloxane) capillary column (30 m $\times$ 0.25 mm, 0.25 μm film thickness) in splitless mode with helium as the carrier gas (6.1 psi head pressure, linear velocity $\sim$36 cm/sec). Oven temperature was ramped from 200 to 240°C at 5°C/min, then from 240 to 300°C at 2°C/min. Injector temperature was 250°C, and the transfer line temperature was 280°C.

We examined chromatograms for consistent female-specific peaks that might serve as contact pheromones. These compounds were tentatively identified by interpretation of their mass spectra by the methods of Blomquist et al. (1987) and Nelson and Blomquist (1995) using a Varian 3400 capillary gas chromatograph interfaced with a Finnigan 4023 mass spectrometer and INCOS data system, using a 30 m $\times$ 0.32 mm DB-5 column with helium as the carrier gas (8 psi head pressure, linear velocity $\sim$30 cm/sec). Column temperature was ramped from 200 to 280°C at a rate of 3°C/min.

The identities of three female-specific compounds (see Results) were confirmed by comparing retention times and mass spectra of insect-produced compounds with those of standards. An $\sim$100% pure standard of *n*-pentacosane (*n*-C_{25}) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). 9-Methylpentacosane (9-MeC_{25}) was synthesized by G. Pomonis (approximate purity 85%) using the methods of Pomonis et al. (1989). 3-Methylpentacosane (3-MeC_{25}) was obtained from the cuticle of American cockroaches, *Periplaneta americana* (L.), by the methods of Dwyer et al. (1981). Approximately 100 late-instar and adult cockroaches were extracted with hexane, the solvent was removed

under nitrogen, and the hydrocarbons were isolated by elution of the concentrated extract through a column of Florosil with hexane. The saturated hydrocarbons were separated from alkenes by elution with hexane through a BioSil A column impregnated with 10% (w/w) silver nitrate. Straight-chain hydrocarbons were removed from the resulting eluate by inclusion in 5Å molecular sieves as described in O'Conner et al. (1962) leaving 3-MeC_{25} (approximate purity: 97%).

Testing Activity of Isolated Compounds. We prepared hexane solutions of the hydrocarbon standards in concentrations that approximated the original extracts of female *X. colonus* ($\sim 2.5 \times 10^{-3}$ mg/μl, see Results). We tested the activity of standards with the following bioassay, testing the three female-specific compounds (*n*-C_{25}, 9-MeC_{25}, and 3-MeC_{25}) separately, in solutions containing pairwise combinations, and with all three compounds combined into one solution (combinations were 1 FE of each standard in 1 ml of hexane, approximating ratios of compounds in the original extract; see Results). Freeze-killed females were individually presented to single males in a Petri dish arena to confirm that males would attempt to mate. For each test, we presented each of at least five dead females to two or three different males ($N = 16$ to 24 males). Females were then washed in hexane to remove cuticular compounds (as described above). Dead-washed females were again presented to males; a lack of response confirmed that contact pheromones had been eliminated. Finally, we pipetted 1 ml of synthetic standards, containing 1 FE of individual standard or 1 FE of each component in a blend, onto dead females, allowed solvent to evaporate, and they were again offered individually to the same males. We rated the response of males as already described and videotaped each step of the bioassays to confirm responses. The proportion of males responding to dead-washed, hydrocarbon-treated females was compared to their proportional response to dead-washed females using the χ^2 test (Sokal and Rohlf, 1995). Due to delays in obtaining standards, these bioassays were conducted from 1999 to 2001.

RESULTS

Mating Behavior. In the field, adult male and female *X. colonus* appeared on hickory logs just before sunset. They became active as light levels decreased, with males running up and down the log surface with their antennae spread, apparently searching for females. Females remained stationary, or walked slowly, probing the bark surface with their ovipositors.

In both field and laboratory studies, every male responded to a female only after contacting her with his antennae ($N = 20$ for both experiments); no male directly approached a female in a manner that would suggest they were cued by vision or volatile attractants that operated over a distance. Once a male touched a female with his antennae, he immediately mounted her, grasping her

pronotum or elytra with his forelegs, and apparently biting or palpating her tergum. All of the females were receptive to mating. The male then bent his abdomen to connect with the female's genitalia, and extracted the ovipositor by extending his hind legs, raising the abdomen, and pulling back. During copulation, females generally were immobile, or walked slowly for short distances. Average copulation time in the laboratory was 21.1 ± 13 sec ($N = 10$). After withdrawing his aedeagus, the male remained astride the female, grasping her dorsum with his forelegs. He remained in this position as the female walked, a behavior consistent with mate guarding (Thornhill and Alcock, 1983). On larval hosts, females probed the bark with their ovipositors. Pairs mated repeatedly.

Role of Contact Chemoreception. All 30 *X. colonus* males attempted to mate with freshly killed females ($N = 10$) in a Petri dish arena but did not respond to the same females after they had been washed in hexane, suggesting that a contact pheromone had been removed by the solvent and that recognition was not cued solely by mechanoreception. However, 23 of 30 males attempted to mate with dead-washed extract-treated females ($\chi^2 = 25.8$, $P < 0.001$), and each showed the full progression of mating behaviors: stopped walking, aligned his body with the female, mounted, and bent abdomen to connect genitalia. Mating behavior was elicited by 0.1 FE of extract in 57% of these beetles, and 0.2 FE in the remaining 43%. After attempting to mate for a few seconds, males abandoned the dead-washed extract-treated females. These findings are further evidence that chemical cues play an essential role in mate recognition.

Identification of Female Contact Pheromone. Gas chromatograms of cuticular extracts of female and male *X. colonus* showed consistent differences (Figure 1, Table 1). Peak number 2 (Figure 1, Table 1), which was absent in males, was identified as 9-methylpentacosane based on its retention time of about 27.3 equivalent chain lengths (ECL) and diagnostic ions at m/z 140 and m/z 252/253, which arise from cleavage on either side of the methyl branching group (Figure 2, top). A small amount of 11-methylpentacosane was also present in this peak as indicated by ions at m/z 168/169 and m/z 224/225. Peak number 4 (Figure 1, Table 1), also absent in males, was identified as 3-methylpentacosane based on its ECL of about 27.7 and diagnostic ions of M-29 at m/z 337 and M-57 at m/z 309 (Figure 2, bottom). Spectra for other hydrocarbon components were interpreted in a similar manner (Table 1). Males shared many hydrocarbons with females, but differed primarily in having greater amounts of the 11-, 13-, 15-MeC_{25} (peak 16, Figure 1, Table 1). We focused on the three early-eluting peaks that were present in females and in small amounts or absent in males, *n*-C_{25}, 9-MeC_{25}, and 3-MeC_{25}, assuming males used female-specific compounds to distinguish between the sexes. We omitted 11-MeC_{25} because it was present only in trace amounts in female extracts. Comparison of peak areas with an internal standard showed that the concentration of each of the three compounds was $\sim 2.5 \times 10^{-3}$ mg/μl. Identification of these

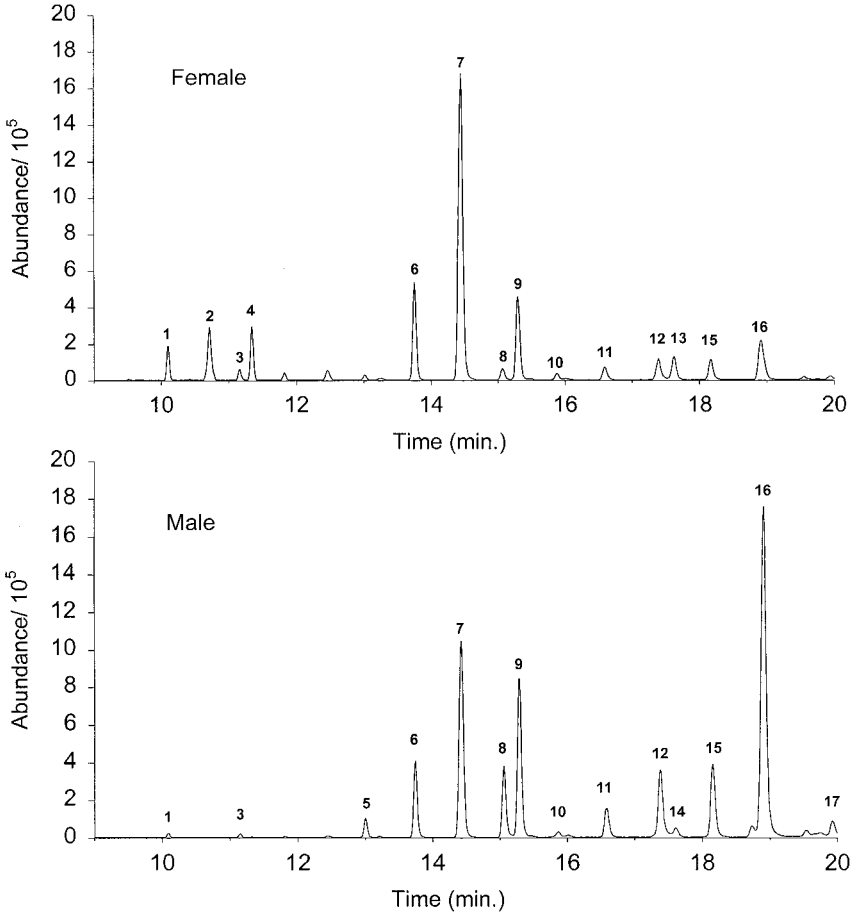

FIG. 1. Representative gas chromatograms of hexane extracts of a *Xylotrechus colonus* female (top) and male (bottom).

compounds was confirmed by comparing retention times and mass spectra with standards.

Testing Activity of Isolated Compounds. In bioassays, males showed a weak response only to *n*-C_{25} when pure compounds were added individually to dead-washed hydrocarbon-treated females (Table 2). Pairwise combinations of 1 FE of each compound did not elicit a response by males (Table 2). However, males responded to dead-washed hydrocarbon-treated females that received the blend containing 1 FE of all three components in 1 ml of hexane, showing the full sequence of mating behaviors (Table 2).

TABLE 1. CUTICULAR HYDROCARBONS OF FEMALE AND MALE *Xylotrechus colonus*[a]

Peak	Hydrocarbon	Female	Male	Diagnostic ions
1	*n*-C_{25}	+	+	352 (M^+)
2	9-MeC_{25}	+	−	140, 252/253, 366 (M^+)
2	11-MeC_{25} (trace)	+	−	168/169, 224/225
3	2-MeC_{25}	+	+	323, 351, 366 (M^+)
4	3-MeC_{25}	+	−	309, 337, 366 (M^+)
5	3-MeC_{26}	−	+	337, 365, 380 (M^+)
6	*n*-C_{27}	+	+	380 (M^+)
7	11,13-MeC_{27}	+	+	168/169, 196/197, 224/225, 252/253 (M^+)
8	2-MeC_{27}	+	+	351, 279, 394 (M^+)
9	3-MeC_{27}	+	+	337, 365, 394 (M^+)
10	*n*-C_{28}	+	+	394 (M^+)
11	13-MeC_{28}	+	+	196/197, 238/239
11	12, 11-MeC_{28} (trace)	+	+	168/169, 182/183, 252/253, 266/267
12	C_{29}:1	+	+	406 (M^+)
13	C_{29}:1	+	−	406 (M^+)
14	3-MeC_{28}	−	+	351, 379, 408 (M^+)
15	*n*-C_{29}	+	+	408 (M^+)
16	11, 13, 15-MeC_{29}	+	+	168/169, 196/197, 224/225, 252/253
17	C_{31}:1	−	+	434 (M^+)

[a] Peak numbers correspond with those in Figure 1; "+" indicates compound is present and "−" indicates it is absent. 11-MeC_{25} and 12, 11-MeC_{28} coeluted in trace amounts with other compounds. Peaks 12 and 13 represent isomers of the same alkene.

DISCUSSION

Adult *X. colonus* of both sexes are attracted by volatiles emanating from cut hickory logs in olfactometer bioassays (Ginzel, 1999), suggesting that mate location is mediated by plant volatiles, rather than long-range sex pheromones. This reliance on plant volatiles for mate location has also been observed in other cerambycid species whose larvae require stressed or dying hosts, including *Hoplocerambyx spinicornis* (New.) (Beeson and Bhatia, 1939), *Monochamus alternatus* Hope (Ikeda et al., 1980; Okamoto, 1984), and *Phoracantha semipunctata* F. (Hanks et al., 1996, 1998). Males of these species use their antennae to locate females on the larval host, and the elongate and filamentous structure of their antennae appear well suited to this purpose (Hanks et al., 1996; Hanks, 1999).

Male *X. colonus* searched for females by running on the bark surface with their antennae outstretched and only responded to a female after contacting her with their antennae. Our bioassays demonstrated that the contact pheromone is comprised of three compounds: *n*-pentacosane, 9-methylpentacosane, and 3-methylpentacosane. Males only responded to the complete blend of these compounds, and the blend elicited the same mating behaviors that males exhibited in response to crude extract, confirming that no other compounds were necessary for

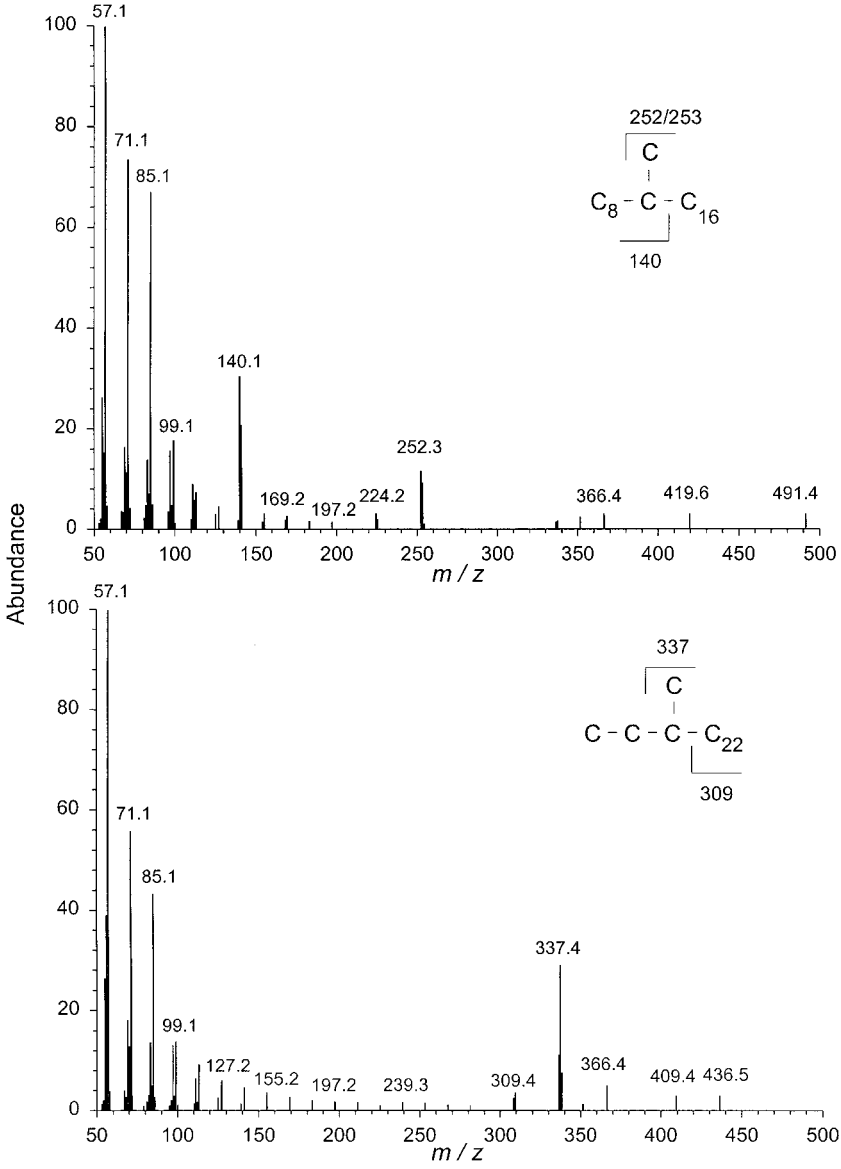

FIG. 2. EI mass spectra of 9-methylpentacosane (top) and 3-methylpentacosane (bottom), showing origins of major fragments.

TABLE 2. RESPONSE OF MALE *Xylotrechus colonus* TO SOLVENT-WASHED DEAD FEMALES (CONTROL) AND THE SAME FEMALES TREATED WITH STANDARDS OF SUSPECTED CONTACT PHEROMONES

Compound[a]	Males (N)	Response to washed females[b] (%)	Response to compound(s) (%)	No response to compound(s) (%)	(χ^2 statistic (P)[c]	Behavior of responding males
nC$_{25}$	24	0	21	79	5.6 (<0.05)	stopped walking
9-MeC$_{25}$	24	0	8.3	91.7	2.1 (>0.1)	stopped walking
3-MeC$_{25}$	21	0	4.8	95.2	1.0 (>0.1)	stopped walking
nC$_{25}$ + 9-MeC$_{25}$	19	0	11	89	2.1 (>0.1)	stopped walking
nC$_{25}$ + 3-MeC$_{25}$	18	0	11	89	2.1 (>0.1)	stopped walking
9-MeC$_{25}$ + 3-MeC$_{25}$	16	0	0	100	0 (1)	none
nC$_{25}$ + 9-MeC$_{25}$ + 3-MeC$_{25}$	24	0	71	29	26.3 ($P < 0.001$)	stopped walking, aligned body with female, mounted, bent abdomen

[a] Single compounds were tested at a dose of 1 FE/ 1 ml hexane and mixtures were tested at a concentration of 1 FE of each component in 1 ml hexane.
[b] See Methods and Materials for definition of "response" and "no response."
[c] Percentages of males responding to compounds were compared to their response to solvent-washed females using the χ^2 test.

mate recognition. The lack of response of males to dead-washed females rules out the exclusive use of mechanoreception in mate recognition, but it may work in concert with chemoreception.

Such hydrocarbon components of the lipid layer on the cuticle are involved in mate recognition in many insect species (Howard and Blomquist, 1982; Nelson, 1993), and may be saturated or unsaturated, terminally branched monomethyl alkanes, or internally branched monomethyl-, dimethyl-, and trimethylalkanes (Lockey, 1980), and tetramethylalkanes (Nelson et al. 1988). Straight chain alkanes, particularly those of odd carbon number, are ubiquitous among insects (Lockey, 1980), including *n*-pentacosane, *n*-heptacosane, and *n*-nonacosane, which were present on the cuticle of male and female *X. colonus*. The structural variability of these hydrocarbons allows for the diversity of pheromones necessary for species specificity, and their chemical stability and low volatility make them suitable as contact pheromones (Dani et al., 2001).

Although the insect cuticle often contains a complex mixture of hydrocarbons, only a few may comprise the contact pheromone of a species (Howard, 1993). Active compounds are sometimes present in both sexes, but may be more abundant on the cuticle of one sex. For example, (*Z*)-14-nonacosene, (*Z*)-13-nonacosene, and (*Z*)-13-heptacosene are present on both sexes of the fly *Musca autumnalis* De Geer, but more abundant on females for which they serve as the sex pheromone (Uebel et al., 1975). Similarly, both sexes of *X. colonus* have *n*-pentacosane, but it was far more abundant on females than on males. In general, hydrocarbon chain length was associated with beetle sex to some degree: female *X. colonus* had greater amounts of the C_{25} to C_{27} methylalkanes, while males had greater amounts of the C_{27} to C_{29} methylalkanes (Figure 1, Table 1). In fact, males had longer chain analogs of two of the female-specific compounds that comprised the contact pheromone: *n*-C_{25} and 3-MeC_{25} (Table 1). Sex-based differences in alkyl chain length also occur in the tsetse fly, but females have greater amounts of longer chain methylalkanes than do males (Nelson and Carlson, 1986).

The role of contact pheromones in cerambycids apparently was first suggested by Heintz (1925), who reported that males of some flower-visiting species responded to females only after contacting them with their antennae and that antennectomized males could not find mates. Males of other cerambycid species show behaviors that suggest they use contact pheromones. For example, male *M. alternatus* attempt to copulate with glass rods treated with a solvent extract of females (Kim et al., 1992). Male *Anoplophora malasiaca* (Thomson) show a similar response to female extract on glass rods, and cuticular hydrocarbons apparently are constituents of the contact pheromones (Fukaya et al, 2000). Male *Psacothea hilaris* (Pasc.) respond to gelatin capsules treated with extract of the female cuticle that contains the primary component (*Z*)-21-methyl-8-pentatriacontene (Fukaya et al., 1996, 1997). The structure of that pheromone is similar to the three compounds that male *X. colonus* responded to in our study.

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