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SHORT COMMUNICATION

Evidence for the stratification of hydrocarbons in the epicuticular wax layer of female *Megacyllene robiniae* (Coleoptera: Cerambycidae)

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Abstract Contact pheromones mediate mate recognition and play important roles in mating systems of longhorned beetles (Coleoptera: Cerambycidae). One common bioassay of contact chemoreception in cerambycids involves presenting a freeze-killed female to a male in a Petri dish arena. If the male attempts to mate with the female carcass, it confirms that mate recognition signals are present and intact and behavior is not involved. Cuticular hydrocarbons are then stripped from the female with successive solvent washes, rendering her unattractive to males and also resulting in a crude extract containing the cuticular hydrocarbons. To test the bioactivity of the crude extract, the same female is then treated with the extract and presented again to the male. Males of some species, including *Megacyllene robiniae* (Förster), respond less readily to reconstituted females than to those same beetles before they were solvent-extracted. In the present study, we test the hypothesis that the contact pheromone of *M. robiniae*, *Z*9:C_{25}, exists as a layer on the surface of the epicuticle. We used solid phase microextraction (SPME) to sample cuticular hydrocarbons of female beetles after they were freeze-killed, solvent washed, and treated with crude cuticular extracts. We found that extracting cuticular hydrocarbons from females and applying the resulting crude extract back onto the solvent-washed cadaver scrambles the wax layer and decreases the abundance of the contact pheromone presented on the surface of the insect.

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Introduction

The cuticular wax layer of insects is composed of a complex mixture of long-chain fatty acids, methyl esters, aliphatic alcohols, aldehydes, ketones, and hydrocarbons which protect the organism from desiccation. Hydrocarbons often also serve as semiochemicals where a relatively non-volatile signal is required (Howard and Blomquist 2005) and are among the most abundant lipids on the surface of insects. Among longhorned beetles (Coleoptera: Cerambycidae), cuticular hydrocarbons act as female contact pheromones, and mediate mate recognition in species belonging to the subfamilies Prioninae (Barbour et al. 2007; Spikes et al. 2010), Cerambycinae (e.g., Ginzel and Hanks 2003; Ginzel et al. 2003a, b, 2006; Lacey et al. 2008) and Lamiinae (e.g., Wang 1998; Fukaya et al. 2000; Ginzel and Hanks 2003; Yasui et al. 2003; Zhang et al. 2003). The antennae of males contain contact chemoreceptors (Lopes et al. 2005) and a male recognizes a mate only after physically contacting her with his antennae. After this initial antennal contact, males in the subfamily Cerambycinae display a clear progression of behavioral steps that lead to mating: (1) the male orients to (turns toward) the female, (2) stops walking, (3) aligns his body with the female, and (4) mounts her and attempts to couple the genitalia (Ginzel et al. 2003a; Ginzel and Hanks 2005; Lacey et al. 2008).

Our understanding of contact chemoreception in longhorned beetles has increased through the use of bioassays (see Ginzel 2010). In fact, most recent studies have relied on a common bioassay that begins by measuring the mating

response of a male to a freeze-killed female beetle (see Ginzel 2010). If the male attempts to mate with the dead female, it is evidence that mate recognition signals are intact and behavioral cues are not involved in mate recognition. The female is then washed in solvent; a lack of response by the males to the extracted carcass proves that recognition cues were removed. The crude cuticular extract (i.e., solvent wash) is then pipetted onto the carcass, the solvent is allowed to evaporate and the female carcass is presented again to the male. If males display mating behavior toward the solvent-washed female treated with extract, it is evidence that mate recognition is chemically mediated and the cuticular extract contains the contact pheromone.

In some cerambycid species, males display a stronger response toward freeze-killed females than to those same females after they are washed in solvent and treated with crude cuticular extracts (see Ginzel and Hanks 2003; Ginzel et al. 2003a, b, 2006; Lacey et al. 2008). For example, all male *Megacyllene caryae* (Gahan) tested attempted to mate with freeze-killed females, but only 40% of those same males displayed a full mating response, ending with abdominal bending, in response to solvent-washed female carcasses treated with cuticular extract (Ginzel et al. 2006). Moreover, all males of the congener *Megacyllene robiniae* (Förster) responded to freeze-killed females in Petri dish arenas. However, only 66.7% displayed any of the behavioral steps towards those same females after they were solvent-extracted and the crude cuticular extracts had been applied (Ginzel and Hanks 2003), suggesting that the proportion of the contact pheromone to other cuticular compounds on the surface of the wax layer is altered by the extraction and reapplication process. In the current study, we use solid phase microextraction (SPME) to test the hypothesis that the contact pheromone of *M. robiniae, Z*9:C_{25}, is more abundant on the surface of the wax layer of females. We also test the hypothesis that extracting cuticular compounds from freeze-killed female *M. robiniae* and reapplying the crude extracts to solvent-washed cadavers reduces the proportion of the contact pheromone present on the surface of the epicuticle. Unlike other methods (e.g., direct solid sample injection; Bagnères and Morgan 1990; Morgan 1990) for sampling insect hydrocarbons, SPME is a solventless and non-destructive sampling technique that has been used as an alternative method to classical solvent extraction to identify contact pheromones of cerambycids (Ginzel et al. 2003b, 2006; Lacey et al. 2008) and other beetles (see Ming and Lewis 2010). In fact, it appears that wiping a SPME fiber over the cuticle primarily samples the outer surface of the wax layer and yields qualitatively different hydrocarbon profiles than whole-body solvent extraction (Ginzel et al. 2003b) and may more accurately represent those compounds that mediate mate recognition. For example, the contact pheromone of *M. robiniae, Z*9:C_{25}, constitutes ~16% of the total hydrocarbons in hexane extracts of females but 34–36% of the hydrocarbons sampled by SPME, suggesting hydrocarbons that serve as contact pheromones are more abundant on the surface of the wax layer where they are readily accessible to the male antennae.

Materials and methods

Source of beetles

Adult *M. robiniae* were collected into glass vials from goldenrod inflorescences at Tapawingo Park and the Purdue Department of Forestry and Natural Resources Farm, Tippecanoe County, Indiana, between the hours of 1300 and 1600 from mid September through mid October 2007 and 2009. The insects were then brought to the laboratory, housed individually in cylindrical cages of aluminum window screen with 9-cm glass Petri dishes covering top and bottom (300 cm^3), and provided 10% sucrose solution to drink. Beetles were kept on a 16L:8D cycle and only apparently healthy beetles were used in experiments. Male beetles used in bioassays were vigorous and active.

Solid phase microextraction, preparation of whole body extracts, and analysis of cuticular hydrocarbons

We compared the cuticular hydrocarbon profiles of *M. robiniae* females before and after solvent extraction of cuticular compounds, and then after the females were treated with crude cuticular extracts. Specifically, cuticular hydrocarbons were sampled as follows:

1. *Freeze-killed female* A healthy female was freeze-killed (−4°C for 20 min) and then allowed to warm to room temperature (~15 min). The cuticular hydrocarbons of the female were then sampled with SPME and the carcass was presented to a male in a Petri dish arena to test for a mating response as indicated by any of the behavioral steps outlined above.
2. *Solvent-extracted female carcass* If the male attempted to mate with the freeze-killed female, nonpolar compounds were removed from the dead female by immersing her in 1 ml of analytical-grade hexane for 2 min, during which time samples were first vortexed for 20 s. and then sonicated for the remainder of the 2 min wash. This wash step was then repeated with another 1-ml aliquot of hexane and then the solvent washes were combined, and concentrated to 1 ml under nitrogen. The solvent-washed female was then

sampled using SPME to confirm that all cuticular hydrocarbons had been removed, and presented again to the male to test whether he displayed mating behavior. Lack of a response by the male was interpreted as evidence that chemical recognition signals had been removed. Two successive washes in solvent were necessary to completely remove all cuticular hydrocarbons from the carcass and render it unattractive to males.

3. *Solvent-washed female carcass with extract reapplied* (*reconstituted female*) We then gradually pipetted 0.2 female equivalent (FE; which contains ~44 μg of the most abundant compound, nC$_{25}$; see Ginzel et al. 2003a, b) of extract back onto the female carcass and allowed the solvent to evaporate—hereafter referred to as a 'reconstituted' female. The female was then sampled again by SPME (0.2 FE of crude extract was the minimum amount that elicited mating responses from males; Ginzel et al. 2003b). We then presented the female again to the same male and the strength of response of the male to the reconstituted female (step 1–4) was recorded.

The assay was conducted with ten females and only one assay was performed each day in the afternoon between 1300 and 1700 h under ambient laboratory conditions. We sampled the cuticular components using SPME by wiping the length of the fiber (100 μm, polydimethylsiloxane Supelco Inc., Cat. No. 57300-U, Bellefonte, PA, USA) across the elytra ten times, rotating the fiber between wipes. Samples were analyzed by coupled gas chromatography-mass spectrometry (GC–MS) with electron impact ionization (EI, 70 eV) using a Hewlett-Packard (HP) 6890N gas chromatograph (Hewlett-Packard, Sunnyvale, CA, USA) equipped with a DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm film, J&W Scientific, Folsom, CA, USA) in splitless mode, and interfaced to a HP 5975N mass selective detector (MSD), with helium as the carrier gas. The SPME fiber was thermally desorbed in the heated GC injection port (250°C) for 1 min and after a 1 min hold at 50°C the oven temperature was ramped to 240°C at 10°C/min, then from 240 to 300°C at 2°C/min with a hold for 5 min at 300°C. This method was also used to analyze crude extracts prepared as described above, allowing us to compare the hydrocarbon profiles of the crude extract with those of SPME samples of freeze-killed and reconstituted females. Quantitative data presented in Table 1 were produced by these analyses.

Compounds were identified by comparing the retention times and diagnostic ions of compounds with those published in Ginzel et al. (2003b). The abundance of each compound in extracts was calculated as a percentage of the total corrected peak area of all hydrocarbons present in the total ion chromatograms (ChemStation, Version B.03.01; Hewlett-Packard Corp.). The relative proportion of hydrocarbons sampled by SPME from freeze-killed and reconstituted females and those in hexane extracts were compared using multivariate analysis of similarities (ANOSIM; PRIMER software, Clarke and Gorley 2006) with treatment as a factor. ANOSIM tests multivariate data that have an a priori determined factor against a null hypothesis that the factors come from the same population (Clarke and Warwick 2001). The corrected peak area of each compound eluted was a variable in this analysis. We used 9999 bootstrap samples to test the null hypothesis in PRIMER. The influence of treatment on the abundance of the contact pheromone, $Z$9:C$_{25}$, was determined by one-way analysis of variance (ANOVA) followed by a Fisher's least significant difference (LSD) test (StatSoft 2005).

Results and discussion

There were global differences in the hydrocarbon profiles of freeze-killed and reconstituted females and the crude solvent extract (Global $R = 0.22$, $P = 0.001$). In fact, the relative proportions of hydrocarbons in SPME samples of freeze-killed females were significantly different from those in samples of reconstituted females and also those in hexane extracts ($R = 0.351$, $P = 0.001$). There was no quantitative difference in the hydrocarbon profiles from SPME samples of reconstituted females and the extract alone ($R = 0.046$, $P = 0.18$), suggesting that observed differences in the relative abundance of compounds between SPME samples of freeze-killed females and crude extracts is not an artifact of the SPME sampling method. Interestingly, the relative abundance of $Z$9:C$_{25}$, the contact pheromone of *M. robiniae,* was 39% lower in SPME samples of reconstituted females compared to those of freeze-killed females (see Fig. 1, Table 1; means significantly different; ANOVA $F_{2,\ 27} = 11.34$, $P < 0.01$). There was no difference in the relative abundance of $Z$9:C$_{25}$ in crude extract and SPME samples of reconstituted females, however.

In our study, the proportion of alkanes present in the SPME samples of female *M. robiniae* was significantly higher after reconstitution (see Fig. 2; ANOVA $F_{1,\ 18} = 16.52$, $P < 0.001$), while the proportion of alkenes was significantly lower (see Fig. 2; ANOVA $F_{1,\ 18} = 18.41$, $P < 0.001$). The proportion of methyl-branched compounds, however, remained unchanged after extracts were reapplied to solvent-washed females. The cuticular hydrocarbon profiles of female *M. robiniae* are similar across different regions of the body (Ginzel et al. 2003b), suggesting that differences in proportions of hydrocarbons between SPME wipe samples of the elytra and whole-body

Table 1 Cuticular hydrocarbons of freeze-killed and reconstituted female *M. robiniae* sampled by SPME and those in crude solvent extracts alone

Peak Number	Retention Time	Hydrocarbon	% of total hydrocarbons ± 1 SE		
			Freeze-killed	Reconstituted	Solvent extract
1	20.56	nC_{22}	0.28 ± 0.19	0.13 ± 0.04	0.10 ± 0.01
2	21.11	2-meC_{22}	0.44 ± 0.07	0.25 ± 0.04	0.37 ± 0.02
3	21.21	*Z*9:C_{23}	1.80 ± 0.20	0.85 ± 0.19	1.30 ± 0.10
4	21.43	nC_{23}	4.23 ± 0.35	8.00 ± 1.66	5.27 ± 0.38
5	22.05	3-meC_{23}	4.51 ± 0.53	3.64 ± 0.47	4.39 ± 0.46
6	22.25	nC_{24}	1.72 ± 0.19	3.51 ± 0.66	2.77 ± 0.33
7	22.74	4-meC_{24}	0.85 ± 0.18	0.49 ± 0.09	0.69 ± 0.08
8	22.79	2-meC_{24}	1.31 ± 0.19	1.25 ± 0.15	3.17 ± 1.70
9	22.93	*Z*9:C_{25}	33.31 ± 1.87	20.30 ± 2.54	25.46 ± 1.18
10	22.96	*Z*7:C_{25}	5.65 ± 0.41	3.94 ± 0.38	4.63 ± 0.29
11	23.06	nC_{25}	6.63 ± 0.84	15.98 ± 2.07	11.11 ± 1.05
12	23.31	11-meC_{25} 13-meC_{25}	0.37 ± .04	0.28 ± 0.05	0.40 ± 0.04
13	23.62	3-meC_{25}	4.10 ± 0.17	4.25 ± 0.29	4.27 ± 0.20
14	23.80	nC_{26}	0.61 ± 0.09	1.80 ± 0.12	1.31 ± 0.08
15	24.27	2-meC_{26}	2.20 ± 0.38	2.30 ± 0.37	1.93 ± 0.26
16	24.43	*Z*9:C_{27}	8.07 ± 1.23	5.97 ± 0.72	6.42 ± 0.50
17	24.48	Z7:C_{27}	6.80 ± 1.51	5.15 ± 1.24	5.18 ± 1.24
18	24.58	nC_{27}	3.76 ± 0.52	7.01 ± 0.72	4.89 ± 0.30
19	24.85	11-meC_{27} 13-meC_{27}	0.62 ± 0.08	0.78 ± 0.17	0.61 ± 0.12
20	25.21	3-meC_{27}	1.87 ± 0.20	3.33 ± 0.32	2.62 ± 0.31
21	25.43	nC_{28}	0.19 ± 0.03	0.61 ± 0.13	0.54 ± 0.10
22	25.99	C_{29} diene	1.72 ± 0.45	0.70 ± 0.24	1.28 ± 0.31
23	26.06	2-meC_{28}	1.15 ± 0.21	1.40 ± 0.35	1.77 ± 0.39
24	26.25	*Z*9:C_{29}	4.53 ± 0.66	3.85 ± 0.70	3.80 ± 0.68
25	26.45	nC_{29}	0.82 ± 0.13	0.98 ± 0.37	1.88 ± 0.41
26	26.80	11-meC_{29} 13-meC_{29} 15-meC_{29}	1.31 ± 0.15	1.49 ± 0.57	1.33 ± 0.27
27	27.33	3-meC_{29}	0.67 ± 0.17	1.59 ± 0.51	1.66 ± 0.39
28	28.79	nC_{31}	0.32 ± 0.11	0.15 ± 0.06	0.09 ± 0.02
29	29.59	15-meC_{31}	0.19 ± 0.07	0.10 ± 0.03	0.16 ± 0.04
30	30.06	13,17di-meC_{31}	nd	nd	0.57 ± 0.20

Percent of total hydrocarbons represents means ± ten individuals

nd not detected

solvent extracts are not due to hydrocarbon profiles varying across the body of the insect. For example, the relative proportion of the contact pheromone, *Z*9:C_{25}, to other cuticular hydrocarbons is the same on the dorsal and ventral surface of the elytra, thoracic tergites, and abdominal sternites (Ginzel et al. 2003b). Nevertheless, the marked reduction in relative abundance of the contact pheromone in both reconstituted females and hexane extracts suggests that *Z*9:C_{25} is more abundant on the outer surface of the wax layer. Differences in the relative proportion of compounds between SPME samples of freeze-killed females and reconstituted females suggest that solvent extraction and reapplication scrambles their position in the wax layer.

We explored other potential sources for variation in the hydrocarbon profiles of freeze-killed females and reconstituted females. To test whether the extraction process was so vigorous as to contaminate extracts with internal lipids, we first individually extracted three females for 30 s by vortexing them in hexane, and then subjected the same

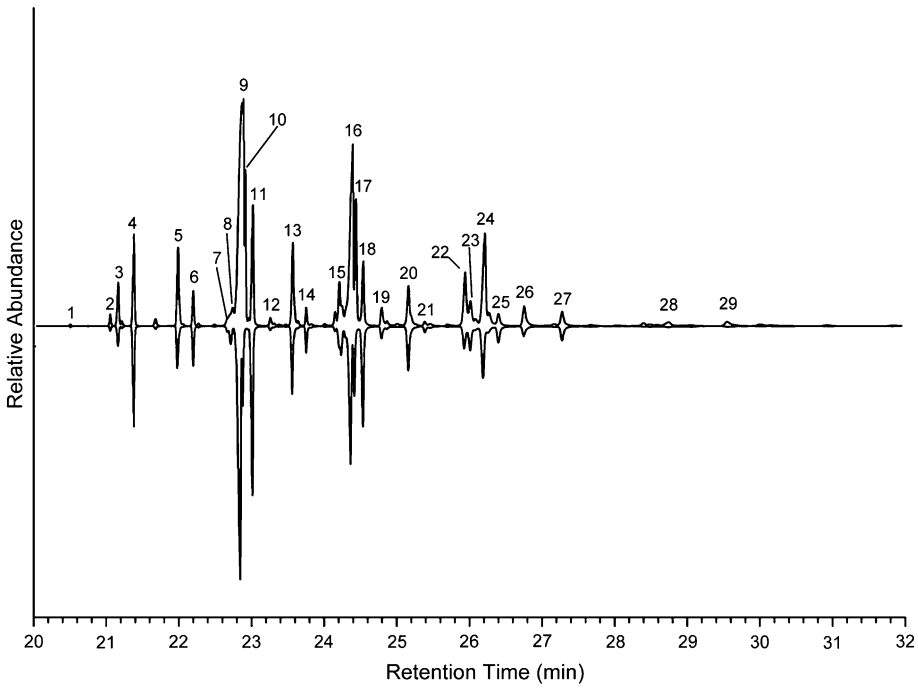

Fig. 1 Representative total ion chromatograms from SPME samples of female *M. robiniae* before solvent extraction (*top*) and after reconstitution (*bottom*) with 0.2 FE of crude extract. Peak numbers correspond to those listed in Table 1

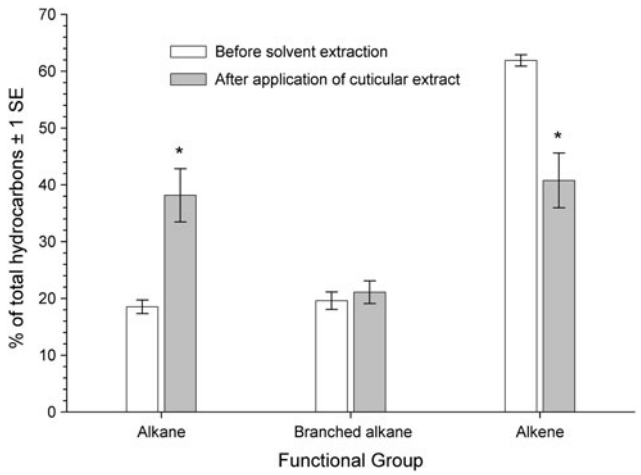

Fig. 2 The contribution of alkanes, methyl -branched alkanes and alkenes to the total hydrocarbons sampled by SPME from the epicuticle of female *M. robiniae* before solvent extraction and after being reconstituted with crude cuticular extracts. An *asterisk* denotes a significant difference (ANOVA, $*P < 0.001$); H_0 = solvent extraction and reapplication of cuticular hydrocarbons does not influence the contribution of each functional group to the total hydrocarbons sampled by SPME

females to a more vigorous 2-min extraction period. We found that the profiles of females washed in solvent for 30 s were not significantly different from those resulting from an additional 2 min wash (data not shown). Differences in hydrocarbon profiles may also be due to the preferential adsorption of some compounds to the glass vial. After extracting hydrocarbons of three females using the methods described above, we poured the extracts from the vials, allowed the remaining solvent to evaporate, and sampled the inside of the vials by SPME. There were no significant differences in the hydrocarbon profiles obtained by wipe sampling the inside of the vial and those of the crude extracts resulting from either the 30 s or 2 min extraction periods (ANOSIM; Global $R = 0.144$, $P = 0.24$; data not shown), demonstrating that differences in hydrocarbon profiles are not due to the vigorous extraction methods or the preferential adsorption of compounds to the glass vial. Moreover, earlier experiments have illustrated that solvent-extracting insect elytra treated with a series of hydrocarbon standards yields both qualitatively and quantitatively similar profiles to those that were applied (Ginzel and Hanks 2002), further suggesting that extraction in a glass vial does not influence hydrocarbon profiles.

Hydrocarbons are thought to be synthesized from specialized cells called oenocytes, and newly formed hydrocarbons are transferred by lipophorin via hemolymph to epidermal cells and ultimately transferred across the cuticle to the surface of the wax layer (Gu et al. 1995; Schal et al. 2001). The mechanism by which this transport and deposition occurs is poorly understood, and it is also unclear how hydrocarbons are arranged on the surface of the insect. Many insects increase or decrease the amount of wax on the cuticle, depending on the environmental and climatic factors, to prevent water loss (Chapman 1998), and compounds may become layered as more or less wax is produced. Small changes in hydrocarbon profiles may also influence mate choice and even mediate assortative mating and lead to reproductive isolation (Peterson et al. 2007).

The physical properties of hydrocarbons may influence their overall abundance and distribution within the wax layer of insects (Gibbs and Crowe 1991; Young et al. 2000) and there has been recent controversy regarding the liquidity of components of the lipid layer at physiological temperatures (Gibbs and Pomonis 1995; Gibbs 2002). It has been suggested that simple alkane–alkene mixtures of pure compounds do not form mixed crystals nor exhibit melting point depression as expected (Gibbs 2002). Rather, individual components retain their melting temperatures (Small 1986) and longer chain saturated hydrocarbons within the mixture melt at higher temperatures than the unsaturated hydrocarbons such as $Z9{:}C_{25}$, the contact pheromone of *M. robiniae* (Gibbs 1995; Ginzel et al. 2003b; Morgan 2004). A double bond introduced into an alkane decreases the melting temperature by ~50°C and a methyl branch (depending on its position) can reduce the melting point of the alkane by as much as 30°C because these branched compounds do not pack as tightly into crystals as the alkanes (Morgan 2004). Accordingly, the insect cuticle may simultaneously contain regions of a permeable alkene-rich melted phase above a less permeable solid phase of crystalline alkanes (Gibbs and Rajpurohit 2010). As temperatures increase, the hydrocarbons in the solid phase dissolve into the melted regions, forming a homogenous liquid layer (Gibbs 2002). However, the proportion of methyl-branched compounds in SPME wipe-samples of reconstituted females in our study did not decrease when compared to freeze-killed beetles, as would be expected if hydrocarbons stratified within the wax layer according to the melting point alone. Nevertheless, the liquidity of surface hydrocarbons at ecologically relevant temperatures could have implications on hydrocarbon-mediated communication in alkene-rich insects. Many contact pheromones of cerambycids are unsaturated compounds (see Ginzel 2010) that tend to have a lower melting temperature and may be in a liquid lipid phase at the surface of the cuticular wax layer where they are more accessible to the male antennae. Clearly, the distribution of contact pheromones and the physical properties of hydrocarbons within the insect epicuticular wax layer warrant further investigation.

Contact pheromones act as primary mate recognition cues for a number of cerambycids (see Ginzel 2010) and visual or tactile cues serve a subordinate role. In the present study, all ten males displayed a step four behavioral response toward reconstituted females, suggesting that tactile cues are either not influenced by solvent extraction and reapplication of cuticular lipids or are not involved in mate recognition. Males of some species even attempt to mate with glass rods or gelatin capsule models to which crude solvent extracts of females have been applied (Kim et al. 1992; Fukaya et al. 1996; Barbour et al. 2007). However, for many cerambycids, there is a critical threshold in contact pheromone concentration below which males lose receptivity (see Ginzel and Hanks 2003; Ginzel et al. 2003a, b, 2006) and males may not respond as readily to reconstituted females in bioassays because the natural ratios of the hydrocarbons have been altered or contact pheromones masked by other compounds in the wax layer. Consequently, it may be necessary to test the bioactivity of crude cuticular extracts at higher concentrations in order to present a biologically relevant concentration of contact pheromone to males in bioassays.

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