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EVIDENCE FOR A SEX PHEROMONE IN BARK BEETLE PARASITOID *Roptrocerus xylophagorum*

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Abstract-Male *Roptrocerus xylophagorum* (Ratzeburg) (Hymenoptera: Pteromalidae) exhibited courtship and mating behaviors including wing fanning, antennation, mounting, and copulation attempts when exposed to glass bulb decoys coated with a whole-body extract of females in hexane, acetone, or methanol. Activity of extract-treated decoys declined gradually over one week. Males responded much less strongly to freeze-killed female cadavers extracted with solvents than to unextracted cadavers; treatment of extracted cadavers with female extract restored male responses. The pheromone was found to be equally present over the surface of both the abdomen and head/thorax of females, and the origin of the pheromone could not be conclusively localized to any single body region. The activity of pheromone on females increased between day 1 and days 3–5 following eclosion; otherwise, pheromone activity was not significantly affected by either female age or mating. Males were arrested within the zone of a glass surface on which females had walked, suggesting that the pheromone might be substrate-borne. Recent exposure to females reduced male responsiveness, but responsiveness was fully restored after a few hours of male isolation from females. When hexane extracts of whole females were fractionated on silica gel, the pheromone's activity was largely recovered with the first, most nonpolar fraction. Female extracts and fractions were analyzed by coupled gas chromatography-mass spectrometry. Cuticular hydrocarbon alkanes were identified as the extract components whose concentrations correlated best with male responses. Evidence of the pheromone's long persistence, low volatility, low polarity, and presence over the insect's entire body surface further supported the hypothesis that the pheromone was composed of one or more cuticular hydrocarbons.

Key Words-parasitoid, sex pheromone, ***Roptrocerus xylophagorum,*** Pteromalidae, bark beetle, Scolytidae, cuticular hydrocarbons, semiochemicals.

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INTRODUCTION

Sex pheromones are apparently widespread in the parasitic Hymenoptera and have been shown to occur in at least 16 families (Eller et al., 1984; Swedenborg and Jones, 1992a; Cormier et al., 1998). These pheromones mediate mate-finding and mating behaviors, including long-range orientation of males to females (Swedenborg and Jones, 1992a; Fauvergue et al., 1999; Jewett and Carpenter, 1999), concentration of male searching activity on substrates contacted by females (Kamano et al., 1989; Kainoh et al., 1991; Pompanon et al., 1997; Fauvergue et al., 199X), and short-range mate recognition and stimulation of male courtship behaviors (Vinson, 1978; Yoshida, 1978; Simser and Coppel, 1980; Takahashi and Sugai, 1982). Interest in parasitoid sex pheromones has been fueled in part by the practical needs of integrated pest management and the benefits offered by pheromone-baited traps as an efficient means for sampling and monitoring parasitoid populations in the field (Lewis et al., 1971; Powell, 1986; Reed et al., 1994; Jewett and Carpenter, 2001).

Roptrocerus xylophagorum (Ratzeburg) is an external parasitoid of the larvae and pupae of at least 12 economically important species of bark beetles (Coleoptera: Scolytidae) in North America, Australia, and Europe (Bushing, 1965; Mills and Schlup, 1989; Sullivan et al., 2000). My observations of male sexual behavior suggested the presence of a sex pheromone in this species; however, males did not demonstrate anemotaxis or other long-range orientation to live females in a Y-tube olfactometer. The following studies were devised to test the hypothesis that *R. xylophagorum* uses a close-range sex pheromone and to identify some of this pheromone's functional and chemical properties.

METHODS AND MATERIALS

Insects. Parasitoids were obtained from a stock culture initiated with insects from Alabama, Georgia, and Louisiana. Voucher specimens from this culture have been deposited at the University of Georgia Museum of Natural History. Parasitoids were collected daily from the window-illuminated side of a screen cage as they emerged from bolts of loblolly pine, *Pinus taedu* L., infested with the bark beetle, *Ips grandicollis* (Eichhoff) (Sullivan et al., 1999). Following collection, males and females were housed together in 250-ml Erlenmeyer flasks containing two to three light-duty paper wipers and stored in an incubator at 8°C, 40–90% relative humidity, and a 14L:10D photoperiod. Flasks were provisioned with honey and distilled water and removed from the incubator for approximately 1 hr daily to allow parasitoids to feed, take water, and mate. Male courtship behavior toward live females was observed inside the flasks or small glass enclosures and recorded.

Unless otherwise noted, parasitoid males and females utilized in bioassays had been collected from emergence cages three to five days previously. Ninety

percent of these females were found to have live spermatazoa in their spermathecae (N = 30). One day prior to use in bioassays, males were isolated from females into separate Erlenmeyer flasks held as above but at 25 ± 2°C.

Bioassays. Male responses to surrogates of live females were observed in an olfactometer consisting of a glass tube (15 mm x 5 mm ID) sealed on one end with a disk of Teflon screening (O.l-mm mesh). Each female surrogate was mounted singly on an insect pin inserted lengthwise through a cork such that the surrogate was 6–9 mm from the cork's tapered end. Surrogates were either glass female "decoys" or the cadavers of freeze-killed female parasitoids. Glass decoys consisted of borosilicate glass bulbs (3-4 mm long x 1.5 mm diam.) constructed by melting the ends of Drummond 5-μl micropipets in a Bunsen burner (Figure 1). For cadaver surrogates, females were killed in a deep freezer and mounted lengthwise on insect pins by gluing their legs to the pin's shaft with a small amount of Elmer's Glue-All. After the glue had dried, extracts were applied to surrogates as a 10-μl aliquot delivered from either a micropipet or a Hamilton syringe. The extract was delivered as evenly as possible across the surface of each female surrogate. Normally, glass decoys were assayed two days following application of test extracts; cadavers were tested one day following mounting and application of test extracts to permit extraction solvents to evaporate completely. Unless noted otherwise, individual surrogate preparations were tested in four separate trials (each with a different male) and then replaced. No significant reduction in male responses was observed between the first and fourth trials with the same surrogate ($P = 0.136$, signed-rank test, $N = 201$).

At the beginning of each trial, a male parasitoid was gently aspirated into the glass tube using a length of flexible plastic tube (-30 cm long x 6 mm ID) sealed to the screened end of the glass tube. A mounted female surrogate was then presented to the male by inserting it into the open end of the glass tube. The surrogate's mounting cork was pushed firmly into the tube's mouth, thus stabilizing the surrogate within the glass tube and preventing male escape. The behavior of the enclosed male was then observed with a dissecting microscope under fluorescent laboratory lighting for 2.0 min.

For each trial, a response score was recorded as the sum of single points awarded for each of three observed behaviors: (1) climbing onto the surrogate while antennating its surface ("mounting"), (2) rapid vibration of the wings either while adjacent to or mounted on top of the surrogate ("fanning"), and (3) repeated thrusting of the exserted genitalia against the surrogate ("attempted copulation"). Males were tested once and then discarded. Individual experiments consisted of sequential assays of a related set of treatments grouped to allow statistical comparisons. Within each experiment, treatments were assayed in random order and with equal replicates of each treatment executed at any single testing session. Experiments were conducted between hours 4 and 11 of photophase at ambient laboratory conditions: 2.5 ± 2°C and 30–60% relative humidity. Separate olfactometer tubes

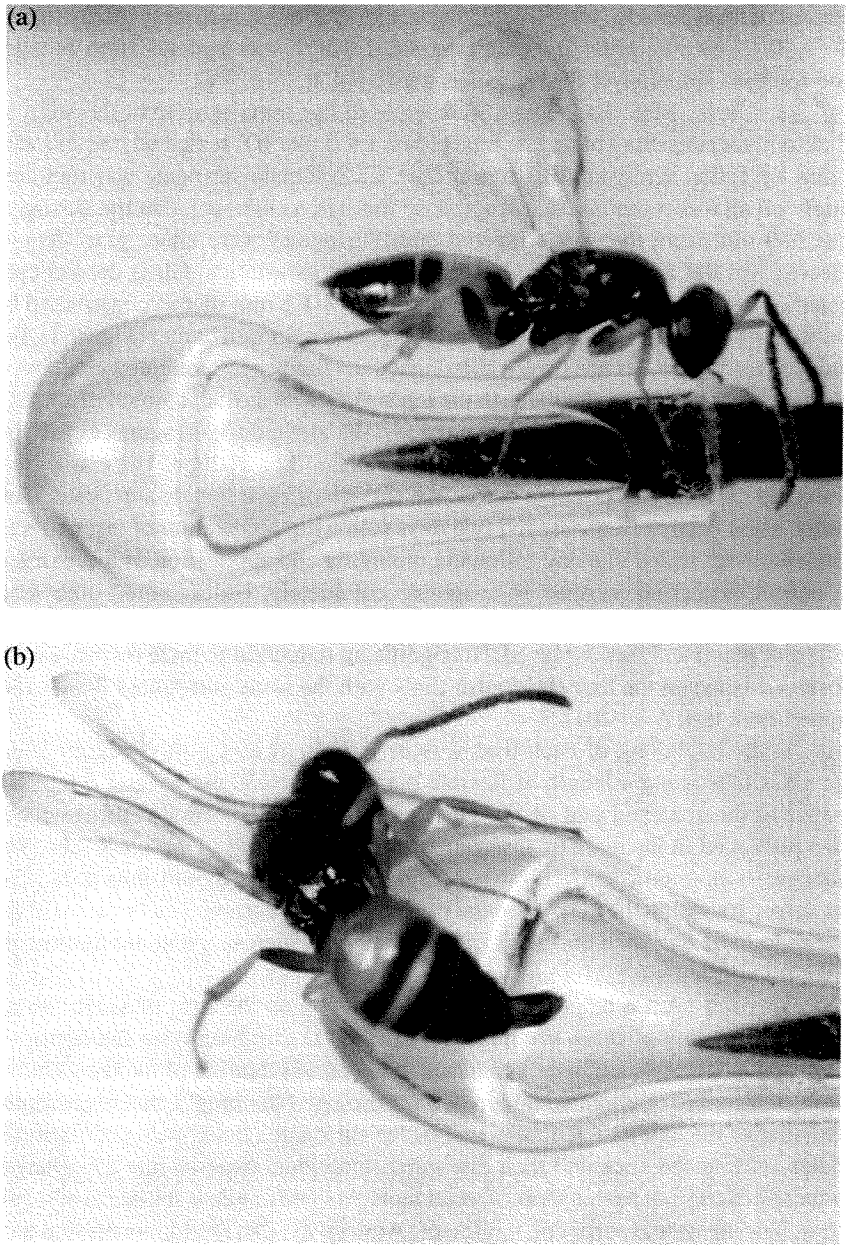

FIG. 1. Male *R. xylophagorum* exhibiting sexual behaviors in response to a glass bulb treated with a whole-body acetone extract of *R. xylophagorum* females: wing fanning (a) and copulation attempt (b).

were assigned to the different surrogate treatments, and olfactometer tubes were sonicated in a 1: 1 acetone-ethanol mixture and oven dried for >2 hr at 120°C between test sessions. Experiments and treatments are described below.

Pheromone Stimulation of Male Sexual Behaviors. In the olfactometer, males (N = 20) were presented with female cadavers that were either (1) not manipulated, (2) extracted to remove all traces of pheromone, or (3) extracted and then treated with one female equivalent (1 FE) of female extract. Female extract was obtained by steeping 10 freezer-killed females in 50 μl acetone for 15 min, then rinsing the insects with another 50 μl acetone and combining the rinse with the original extract (1 FE = 10 μl extract). Cadavers used in treatments 2 and 3 above were further extracted: twice in 10 ml hexane, acetone, and 95% ethanol for > 1 hr each with gentle agitation on a shaker table.

Pheromone Characteristics. In separate experiments, males were presented with: (1) glass decoys treated with either a hexane, acetone, or methanol extract (1 FE) of whole parasitoids, (2) decoys treated with sequential dilutions of a 1 FE acetone extract of whole females (1 .0, 0.1 , 0.01, 0.001, or 0.0001 FE), or (3) decoys that had been treated with a 0.01 FE acetone extract and then stored in a dark cabinet in the laboratory for 2-6 hr, or one, two, four, or seven days (N = 20, 20, and 32, respectively).

Source of Pheromone. Isolated body regions were extracted sequentially to distinguish between pheromone that may have spread across the entire body surface from its site of origin and that present in exocrine organs within or beneath the cuticle. The abdomens of 10 freezer-killed females were separated from heads/thoraces with a scalpel, and the two body regions were placed into separate vials and extracted three times in acetone: (1) 5 min with 100 μl, then (2) 15 min with 100 μl, and finally (3) thoroughly macerated with a glass rod and extracted with 1 .O ml for 15 min. Prior to application to decoys, extracts 1 and 2 were diluted 1/10 in acetone (0.1 FE = 10 μl). Extract 3 was spun at 2000 rpm *in* a clinical centrifuge to remove particulates and then used undiluted. In the olfactometer, males (N = 20-28) were presented with glass decoys treated with one of the three extracts of either body region. Each extraction was tested as a separate two-treatment experiment.

Effect of Female Mating and Age on Pheromone Production. Parasitoid pupae were dissected from I. grandicollis-infested pine bolts from the stock culture, placed into gelatin capsules, incubated in the dark at 29°C and 70–80% relative humidity, and inspected daily. On the day of eclosion, females were either extracted immediately or transferred in groups of up to eight individuals into housing flasks as described previously. Half of these flasks contained 3-5-day-old males (N = 12–15) collected from the emergence cage; the remaining flasks did not contain males. Flasks were maintained at 25 ± 2°C, 40–80% relative humidity, and a 14L:10D photoperiod for three to five days, and then the females were removed and extracted. To estimate the proportion of mated females in the mixed-sex flasks at the time of extraction, 18 females were dissected live and the spermathecae examined.

Individual parasitoids were extracted whole in acetone (2 x 100 μl) for 15 min (0.05 FE = 10 μl). Males (N = 32) were presented with glass decoys treated with the extract of: (1) a 1-day-old virgin male, (2) a l-day-old virgin female, (3) a 3- to 5-day-old virgin female, (4) a 3- to 5-day-old female from a mixed-sex flask, or (5) a female that had naturally emerged three to five days earlier from a pine bolt in the stock culture. Eight decoys each bearing the extract of an individual insect were assayed per treatment (four males tested per decoy).

In a separate experiment, males (N = 32) were presented with cadavers of females that had emerged from culture bolts either 3-5 or 14-16 days earlier. Females in the 14 to 16 day category were removed from the 8°C incubator at five days and then maintained in culture flasks at 25 ± 2°C, 40–80% relative humidity, and a 14L: 10D photoperiod for an additional 9-11 days. Females in the three to five-day category were kept consistently at 8°C prior to freezing and mounting.

Effect of Recent Female Contact on Male Responsiveness to Pheromone. Males were isolated from females for varying durations prior to being assayed for their response to decoys treated with 0.1 FE acetone extract of whole females. Male test subjects were held in housing flasks with ~50 females before being isolated into clean flasks. Males (N = 30) experiencing four different separation intervals were assayed: 0 hr (removed from mixed-sex flask immediately before each trial), l-2 hr, 5-9 hr, and 18-36 hr. The same decoy was used in up to eight trials.

Activity of Fractions of Female Extract. A hexane extract of whole females was fractionated using low-pressure liquid chromatography, and the resulting fractions were bioassayed. A Pasteur pipet was plugged at its tip with silanized glass wool and then wet-packed with silica gel (1.0 g, 70-230 mesh, Merck grade 7754, conditioned > 1 hr at 130°C) in 40% ethyl ether in hexane. The packed column was rinsed with 10 ml hexane prior to loading with sample (50 μl of a 100-μl hexane extract of 10 females, i.e., 5 FE). Fractions were eluted sequentially with 5 ml of hexane; 5% ether in hexane; 30% ether in hexane; ether; acetone; and methanol. Prior to application to decoys, each fraction was evaporated under nitrogen to a volume of 0.5 ml, and a portion of the original extract was diluted 1/10 in acetone (so that 10 μl = 0.1 FE for all extracts and fractions). Males (N = 16) were presented with glass decoys treated with each of the six fractions singly, the six fractions in combination (10 μl of each), or the original, unaltered extract.

Female Deposition of Pheromone on Surfaces. Ten females were confined for 0.5 hr inside a 4-cm-diam. plastic Petri dish bottom covered with a 15-cm-diam. glass plate. During this time, females walked across and/or stood motionless on the glass surface. Control glass plates were prepared similarly but with no females present. Two concentric circles were drawn on the reverse side of each plate: the inner circle marked the position of the dish rim and the outer was 7 cm diam. The Petri dish and females were removed, and the glass plate was suspended vertically against a white surface (dish side out). Within 1 hr of female removal, individual

male parasitoids were released onto the plate surface (inside the bottom third of the inner circle) and observed for 5 min. The total time each male spent in motion within the outer circle was measured with a stopwatch. Four female-exposed and blank plates were assayed with 6–10 males each.

Chemical Analyses. Portions (100 μl each) of the extracts and fractions utilized as decoy baits were concentrated under nitrogen to approximately 2-5 μl, and 1 μl was then injected splitless into a Hewlett-Packard 6890 gas chromatograph coupled to a model 5973 mass-selective detector (GC-MS). The column was a Hewlett-Packard HP-1 (polydimethylsiloxane; 50 m x 0.2 mm ID, 0.1 1-μm film thickness); the temperature program was 60°C for 1 min, then 10°C/min to 200°C, then 6°C/min to 310°C, then isothermal for 20 min. Each sample was spiked with two internal standards (11 ng 1,3,5-triphenyl benzene and 9 ng a-humulene) prior to concentration. Components of the extracts-fractions were identified by their mass spectra and retention time matches to known standards when available. Each component was quantified by dividing the integration area for one of its diagnostic ions by the integration area of a diagnostic ion of one of the internal standards. The following decoy baits were analyzed: acetone extract of 1-day-old virgin males, acetone extracts of 1-day- and 3- to 5-day-old virgin females, hexane extract of naturally emerged, presumably mated females, and all six silica gel fractions of the hexane extract.

Statistical Analyses. Response scores for the two to four males tested with each surrogate were averaged, and these means were utilized as individual data points in statistical analyses. Significant differences among treatment means were identified with the nonparametric Kruskal-Wallis ANOVA and Mann-Whitney rank sum tests. The Student-Newman-Keuls test was employed for all-pairwise comparisons among treatments ($\alpha = 0.05$; SigmaStat, 1997). Associations between the concentrations of bait constituents and male responses to those baits (i.e., the mean response score for that bait) were assessed with Spearman's rank order correlation (SigmaStat, 1997).

RESULTS

Male Sexual Behavior. Courtship behavior by males toward live females consisted of a characteristic succession of steps. A male would initiate courtship either after chance antenna1 contact with a female (during what appeared to be nondirected, random movements) or after coming within -2 cm of a female. The male would approach with accelerated, sinuous movements while fanning his wings almost continuously. He would then mount the female, typically from the side, while making constant antenna1 contact. Once on top, the male aligned his body above the female's with his head directly above hers. Sometimes the male would pivot his body several times before settling in the typical alignment. The

aligned male then initiated a predictable sequence of behaviors: (1) a brief burst of wing fanning, (2) a nodding motion of the head in which the mouthparts were extruded and stroked forward and upward above the female's antennae, and (3) a motionless pause approximately equal in duration to behaviors 1 and 2. Step 2 was often accompanied by sweeping movements of the male's antennae slightly towards one another and upward; occasionally the male's antennae would contact and noticeably move the female's antennae. This complete sequence was typically repeated two to five times and immediately followed either by male attempts at copulation or abandonment of the female. If copulation attempts failed, often the male would reposition himself above the female and recommence the sequence.

Bioassays. In the glass-tube olfactometer, males responded to female cadavers with wing fanning, mounting, and copulation attempts. Cadavers extracted with a series of organic solvents elicited relatively few male responses, but their activity was largely restored after treatment with 1 FE acetone extract (Figure 2). Males responded to extracted cadavers with mounting and antennation only; only the nonextracted and extract-treated cadavers elicited wing fanning and copulation attempts.

Glass bulb decoys treated with 1 FE extract likewise stimulated wing fanning, mounting, and copulation attempts (Figure 1). However, methanol extracts of females elicited significantly lower levels of response than either hexane or acetone extracts (Figure 3). Acetone extracts of females induced male responses in a dose-dependent manner within the concentration range of 0.0001 FE to 1 FE (Figure 4).

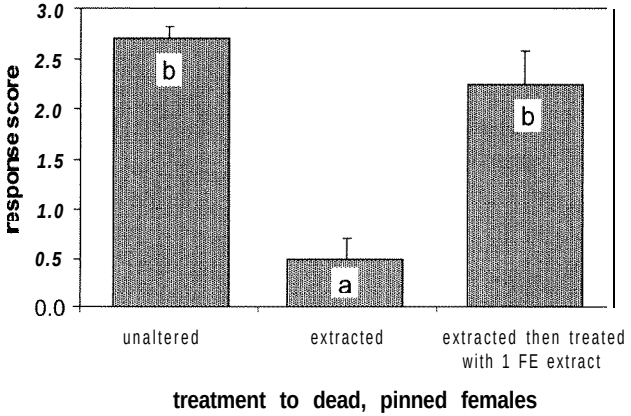

FIG. 2. Mean response scores (+SE) of individual male *R. xylophagorum* (N = 20) reacting to pin-mounted female cadavers that were either not manipulated. extracted repeatedly in organic solvents, or extracted and then treated with whole-body extract (1 FE) of a female. Means with the same letter are not significantly different (Student-Newman-Keuls test; $\alpha = 0.05$).

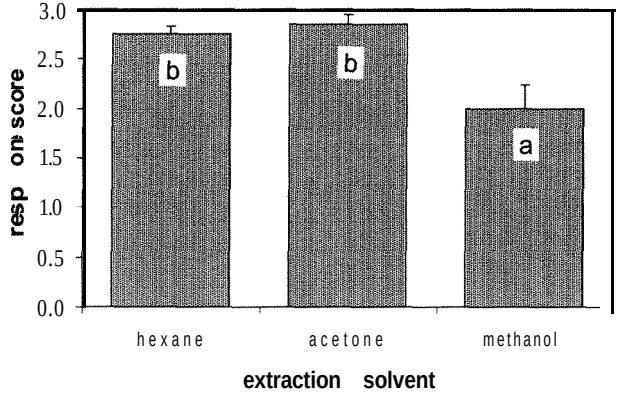

FIG. 3. Mean response scores (+SE) of individual male *R. xylophagorum* (N = 20) reacting to glass bulb decoys treated with either a hexane, acetone, or methanol extract (1 FE) of a female. Means with the same letter are not significantly different (Student-Newman-Keuls test; $\alpha = 0.05$).

Glass decoys treated with 0.01 FE acetone extract gradually lost activity over a seven-day period (Figure 5).

Male responses to decoys treated with the initial extracts of either abdomens or heads/thoraces of females were essentially identical, while decoys treated with the second consecutive extract of heads/thoraces had a marginally higher level of activity than the second consecutive extract of abdomens ($P = 0.053$; Figure 6).

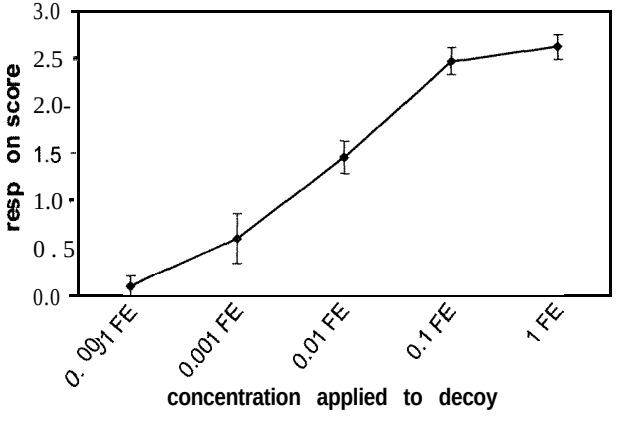

FIG. 4. Mean response scores (±SE) of individual male *R. xylophagorum* (*N* = 20) reacting to glass bulb decoys treated with the serial dilutions of an acetone extract of females (0.0001–1.0 FE).

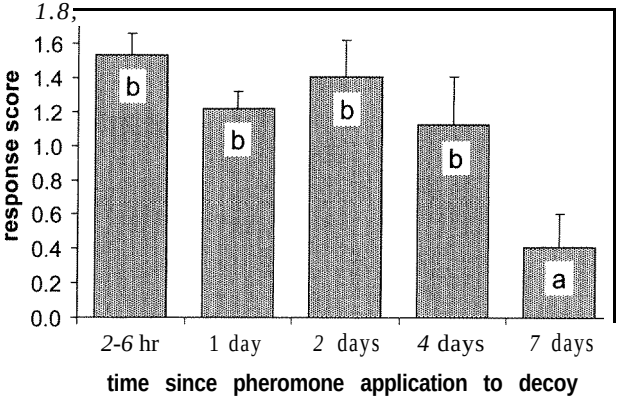

FIG. 5. Mean response scores (+SE) of individual male *R. xylophagorum* ($N = 32$) reacting to glass bulb decoys treated with female extract (0.01 FE) at different time intervals prior to testing. Means with the same letter are not significantly different (Student-Newman-Keuls test; $\alpha = 0.05$).

This difference in the activity of the second extracts was most visible in the number of males making copulation attempts: 12 for head/thorax-treated decoys vs. 1 for abdomen-treated decoys. Maceration and further extraction of the two body regions yielded extracts with relatively low levels of activity that did not differ significantly.

Live spermatozoa were found in the spermathecae of 12 of 18 females that had been housed with males for three to five days following eclosion in gelatin capsules. Extracts of these females, of 3- to 5-day-old virgin females from single-sex flasks, and of females naturally emerged from infested bolts elicited roughly similar

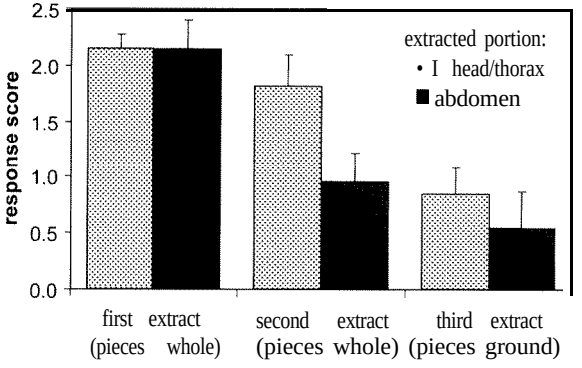

FIG. 6. Mean response scores (+SE) of individual male *R. xylophagorum* ($N = 20$-28) reacting to glass bulb decoys treated with one of three sequential extractions of either the head/thorax or abdomen of females (0.1 FE).

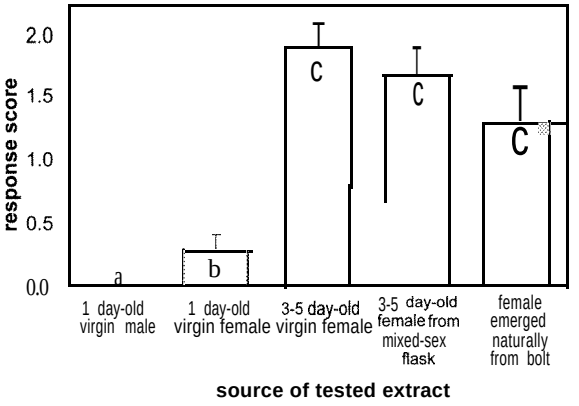

FIG. 7. Mean response scores (+SE) of individual male *R. xylophagorum* (N = 32) reacting to glass bulb decoys treated with the acetone extracts (0.05 FE) of I-day-old virgin males, I -day-old virgin females, 3- to S-day-old virgin females, 3- to S-day-old females that had been housed in mixed sex flasks, or females that had emerged naturally from host-infested pine bolts and were presumably mated. Insects in all treatments except the last had been isolated as pupae from host-infested bolts. Means denoted with the same letter are not significantly different (Student-Newman-Keuls test; $\alpha = 0.05$).

levels of male responses; simultaneously, these three treatments had significantly greater activity than extracts of either 1 -day-old virgin males or females (Figure 7). Extracts of l-day-old virgin females showed significantly greater activity than extracts from 1 -day-old virgin males. Male response scores elicited by cadavers of females that had emerged from bolts either three to five days earlier (2.50 ± 0.23) or 14-16 days earlier (2.53 ± 0.15) did not differ significantly.

Mean response to extract-treated decoys was significantly lower for males removed from mixed-sex flasks immediately before bioassay than for males isolated from females 1-2, 5-9, or 18-36 hr prior to bioassay (Figure 8). Males isolated l-2 hr prior to bioassay were intermediate in responsiveness between males with either longer or shorter periods of isolation.

Most of the activity present in female extract was recovered with the first hexane fraction on silica gel (Figure 9). Male responses did not differ significantly between decoys treated either with all six fractions or with the first fraction alone, indicating the absence of synergism between any of the fractions.

Males remained in motion within a 7-cm-diam. zone on a glass plale for significantly longer durations when females had previously been confined within the zone than when they had not (Figure 10; $P < 0.001$, Mann-Whitney rank-sum test). Half the males placed onto female-exposed plates exhibited wing-fanning behavior, and many such males exhibited accelerated, sinuous movements in and around female-contacted surfaces.

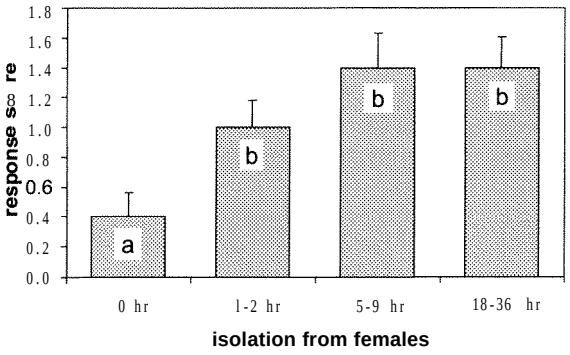

FIG. 8. Mean response scores (+SE) of individual male *R. xylophagorum* ($N = 32$) that had been removed from mixed-sex flasks at variable intervals prior to testing with glass decoys treated with female extract (0.1 FE). Means denoted with the same letter are not significantly different (Student-Newman-Keuls test; $\alpha = 0.05$).

Chemical Analyses. The 68 largest GC peaks in female extract (i.e., all peaks >0.2% the integration area of the largest peak) were quantified for the examined decoy baits, and identifications were made for 28 peaks exhibiting a strong degree of association (Spearman rank-order correlation coefficient >0.75) between their concentrations and male response scores to the individual baits (Table 1). Structures

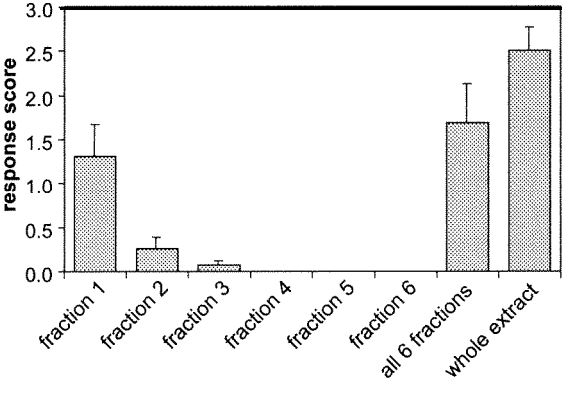

FIG. 9. Mean response scores (+SE) of individual male *R. xylophagorum* ($N = 16$) reacting to glass bulb decoys treated with a hexane extract of females, each of six LC fractions of the extract, or all six fractions simultaneously. Extract fractions were eluted sequentially with hexane (fraction 1), 5% ether in hexane (fraction 2), 30% ether in hexane (fraction 3), ether (fraction 4), acetone (fraction 5), and methanol (fraction 6). Extract and fractions were applied at 0. I FE per decoy.

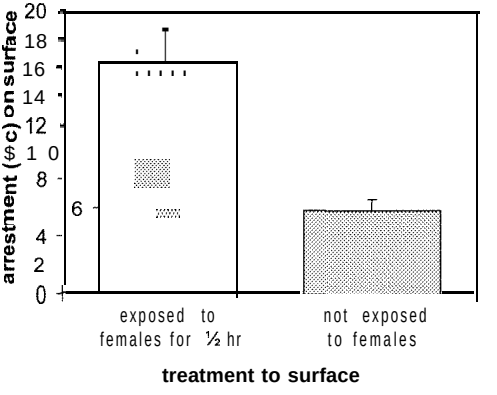

FIG. 10. Mean time (+SE) spent by male *R. xylophagorum* ($N = 29$-40) in motion within a 7-cm-diam. zone of a glass surface that had or had not been contacted by walking females. Male arrestment time differed significantly between the two treatments (Mann-Whitney test; $P < 0.001$).

were determined for all 28 of the correlating peaks based on retention times and interpretation of mass spectra (Blomquist et al., 1987; Carlson et al., 1998). These compounds consisted entirely of alkanes with a 27- to 33-carbon backbone and no, one, or two methyl branches.

DISCUSSION

Female *R. xylophagorum* apparently possess a sex pheromone capable of stimulating courtship behavior and mating in males. Removal of chemical cues from female cadavers by solvent extraction largely eliminated sexual behavior directed toward them, while restoration of chemical cues through the reapplication of female extracts restored male responses. Male response to the pheromone was minimally altered by the absence of visual and tactile cues normally associated with a female. Males responded with the complete courtship sequence (including body alignment and head nods) to pheromone-treated glass decoys, although these decoys differed substantially in color, shape, and texture from female parasitoids. However, the courtship sequence was often terminated at various stages prior to attempted copulation, and this likely resulted from the absence of receptivity signals or other cues from the female **surrogate** (Van Den Assem, 1986). Pheromone-based stimulation of male courtship behaviors (including wing fanning and copulation attempts) in the absence of normal visual/tactile **cues** associated with females has been reported in other parasitic Hymenoptera (Obara and Kitano, 1974; Kitano, 1975; Robacker et al., 1976; Vinson, 1978; Yoshida, 1978; Takahashi and Sugai, 1982; Shu and Jones, 1993).

TABLE 1. CONSTITUENTS OF FEMALE EXTRACT WITH STRONG ASSOCIATION BETWEEN THEIR CONCENTRATIONS AND RESPONSE SCORES OF MALE *R. xylophagorum* (SPEARMAN'S CORRELATION COEFFICIENT >0.75)

Kovats index	Compound name	Correlation coefficient[a]	% of cuticular hydrocarbons[b]	Gender association[c]
2700	*n*-Heptacosane	0.957	3.5	F > M
2743	7-Methylheptacosane	0.861	0.6	—
2752	5-Methylheptacosane	0.829	1.3	F
2774	3-Methylheptacosane	0.787	1.6	F
2800	n-Octacosane	0.875	0.5	F
2811	3, 7-Dimethylheptacosane	0.814	1.0	F
2833	11-, 12-, 13-, and 14-Methyloctacosane	0.771	0.3	—
2846	6-Methyloctacosane	0.814	0.2	—
2859	4-Methyloctacosane	0.874	0.4	—
2893	4,16-Dimethyloctacosane	0.954	0.3	—
2900	*n*-Nonacosane	0.875	6.0	F > M
2911	3, 7-Dimethyloctacosane	0.902	0.5	
2930	11-, 13-, and 15-Methylnonacosane	0.906	10.0	F>M
2942	7-Methylnonacosane	0.814	2.8	—
2952	5-Methylnonacosane	0.814	1.9	F
2961	11,15-Dimethylnonacosane	0.902	2.1	
2967	9,15-Dimethylnonacosane	0.803	1.8	—
2976	3-Methylnonacosane	0.814	11.0	F > M
3015	3, 7-Dimethylnonacosane	0.814	21.0	F>M
3031	13-, 14-, and 15-Methyltriacontane	0.944	0.3	—
3060	2-Methyltriacontane	0.874	0.6	—
3132	13- and 15-Methylhentriacontane	0.850	5.1	F&M
3158	13,17-Dimethylhentriacontane	0.814	7.2	F
3182	5,17-Dimethylhentriacontane	0.902	2.1	—
3209	3,9-Dimethylhentriacontane	0.902	3.7	—
3329	17-Methyltritriacontane	0.874	0.2	—
3355	15,19-Dimethyltritriacontane	0.902	1.2	—
3381	5,17-Dimethyltritriacontane	0.787	1.1	—

Spearman's rank-order correlation coefficient between average male response score and quantity of compound identified in tested bait. Baits subjected to analyses included extracts of 1-day-old virgin males, 1- and 3- to 5-day-old virgin females, mated females, and six silica gel fractions of this latter extract.

[b] Percentage of total cuticular hydrocarbon composition identified in female *R. xylophagorum*. Only those hydrocarbons with correlation coefficient >0.75 are shown (88.3% of total).

[c] Gender association of individual compounds identified in the cuticular hydrocarbons of North American populations of *R. xylophagorum* (Espelie et al., 1996). Treatment abbreviations are F = only in females, F > M = female concentrations greater than four times those in males, F&M = present in roughly similar concentrations in both sexes, — = not reported in Espelie et al. (1996).

The courtship-stimulating pheromone may also mediate mate-finding in *R. xylophagorum*. Males coming into contact with residue deposited on a glass surface by walking females confined their movements to its vicinity for up to 45 sec while apparently searching for females. The residue likewise induced male wing fanning, a behavior, which, in addition to triggering receptivity in females (Kitano, 1975; Van Den Assem, 1986), may also enhance orientation toward sources of female odors (Vinson, 1972, 1978). Males of other parasitoid species exhibit similar, mate-finding-related behaviors (e.g., arrestment, accelerated movement, increased turning rate) on substrates previously contacted by females (Kamano et al., 1989; Pompanon et al., 1997; Fauvergue et al., 1998). Substrate-borne pheromone residues may concentrate searching by males in areas frequented by females, increasing the probability of sexual encounters. Although lacking the ability to attract males over long distances, substrate-borne sex pheromones can be adequate for ensuring a high rate of insemination for female parasitoids (Fauvergue et al., 1995).

The gradual recovery in male responsiveness to sex pheromone following removal from mixed-sex flasks indicated that male courtship behaviors can be temporarily inhibited by continuous contact with females or their environment. The inhibition in male responsiveness was possibly caused by habituation to the female's sex pheromone (Robacker et al., 1976; Askari, 1979) or may have been a reaction to unsuccessful courtship attempts with unreceptive females (Van Den Assem, 1986).

Sex pheromones in hymenopterous parasitoids originate in the abdomen (Tagawa, 1977; Vinson, 1978; Yoshida, 1978; Askari and Alishah, 1979), the head and/or thorax (Takahashi and Sugai, 1982; Kainoh and Oishi, 1993), or both regions (Vinson, 1972), and pheromone-producing glands have been identified in both the abdomen and head (Tagawa, 1977; Weseloh, 1980; Syvertsen et al., 1995; Jones, 1996). Parasitoid pheromones may have their origin in a localized gland but spread across the insect's entire body either passively or as a result of grooming (Tagawa, 1977). The results presented here suggest that the sex pheromone of *R. xylophagorum* was uniformly present across the body surface of females, but do not clearly identify a single body region as the site of pheromone production.

Mating by female parasitoids can cause an abrupt termination of male responses through an apparently copulation-induced change in the release rate or composition of the female pheromone (Fauvergue et al., 1995; McNeil and Brodeur, 1995; Pompanon et al., 1997; Schwörer et al., 1999). *Roptrocerus xylophagorum* males did not respond differently to extracts from virgin females than to extracts from females that had been housed with males, suggesting that mating does not alter pheromone presence on females. However, dissections of spermathecae indicated that some of the females from mixed-sex flasks had probably never mated, and their unintentional inclusion in the tests may have partially obscured changes in male responses.

Concentrations of pheromone on female *R. xylophagorum* apparently did not diminish with age, since males responded equally to cadavers of females that were either younger (3-5 days old) or older (14-16 days old) than the average life span of six to eight days (Samson, 1984; Matthews and Stephen, 1997). The life-long presence of active sex pheromone on female *R. xylophagorum* is not a unique phenomenon within the parasitic Hymenoptera (Vinson, 1978; Swedenborg and Jones, 1992b). There is little evidence to suggest that persistence of sexual attractiveness enhances the fitness of female *R. xylophagorum.* This species is reported to mate immediately after emergence and only once (Bushing, 1967), and the extreme infrequency with which I have observed apparently receptive *R. xylophagorum* females seems to corroborate this report. The persistence of pheromone on female *R. xylophagorum* might be an incidental consequence of the low volatility and high stability of semiochemicals produced earlier in life, and little or no active synthesis or release of pheromone may be necessary for the pheromone's activity to persist throughout the female's life span.

The only extract constituents possessing a strong quantitative association with male responsiveness were apparently cuticular hydrocarbons. All were long-chain alkanes removed from whole insects with relatively brief steeping in hexane and had been previously identified in the cuticular hydrocarbons of other insects (Blomquist et al., 1987; Carlson et al., 1998). Espelie et al. (1996) reported numerous qualitative and quantitative differences in the cuticular hydrocarbon composition of male and female *R. xylophagorum* and suggested that these differences might play a role in close-range mate recognition. Several of the compounds correlating quantitatively with male responsiveness in my study were previously identified by Espelie et al. (1996) as being associated primarily or uniquely with female *R. xylophagorum* (Table 1).

The bioassay results support the hypothesis that the sex pheromone is composed of cuticular hydrocarbons, which function as close-range sex pheromones in several orders of insects (Howard, 1993). As expected for cuticular hydrocarbons, the pheromone was apparently distributed over the entire body surface. It was highly nonpolar, eluting mainly with the first, hexane fraction on silica gel, and it exhibited significantly lower solubility in methanol relative to hexane and acetone. Likewise, the long persistence of the pheromone on objects was consistent with the typical stability and extremely low vapor pressure of long-chain, saturated hydrocarbons. Bioassays with synthetic hydrocarbons will be necessary to confirm the role of these compounds in mediating sexual behavior in *R. xylophagorum.*

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