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MALE-PRODUCED AGGREGATION PHEROMONE OF *Carpophilus obsoletus* (COLEOPTERA: NITIDULIDAE)[1]

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Abstract—Males of *Carpophilus obsoletus* Erichson produce an aggregation pheromone to which both sexes respond. The pheromone was identified by GC-MS as (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene (**1**), which is also a minor constituent of the pheromone blends of *C. hemipterus* (L.), *C. freemani* Dobson, and *C. lugubris* Murray. The pheromone was synergized in wind-tunnel bioassays by propyl acetate, a "host-type" coattractant. In a dose–response study, 50 pg of **1**, plus propyl acetate, was significantly more attractive than just propyl acetate. Pheromone emission from groups of 65 males, feeding on artificial diet, averaged 2.2 ng/male/day. Emissions from individual males were larger, averaging 72 ng/day and ranging as high as 388 ng/day. Synthetic **1** was tested in a date garden in southern California (500 μg/rubber septum), using fermenting whole-wheat bread dough as the coattractant. The pheromone plus dough attracted significantly more beetles than dough alone (means were 4.2 and 0.0 beetles per week per trap). Captured beetles were 54% females. Field trap catches were highest during the months of July and August.

Key Words—*Carpophilus obsoletus*, sap beetle, Coleoptera, Nitidulidae, aggregation pheromone, hydrocarbon, tetraene, date.

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INTRODUCTION

Carpophilus obsoletus Erichson (Coleoptera: Nitidulidae) is a small (ca. 4 mm) dark brown sap beetle that occurs throughout the tropical, subtropical, and milder temperate regions of the world (Dobson, 1954). This insect can be a serious pest of commodities such as dried fruit (Dobson, 1954). *C. obsoletus* also attacks maize (Rathore and Senger, 1969). *C. obsoletus* is a minor species in the nitidulid complex attacking dates in southern California (R.S.V., personal observation; Bartelt et al., 1992b).

Male-produced aggregation pheromones have been identified in other *Carpophilus* species: *C. antiquus* Melsheimer (Bartelt et al., 1993b), *C. freemani* Dobson (Bartelt et al., 1990a), *C. hemipterus* (L.) (Bartelt et al., 1990b, 1992a), *C. lugubris* Murray (Bartelt et al., 1991), and *C. mutilatus* Erichson (Bartelt et al., 1993a). All pheromones isolated to date have been triene or tetraene hydrocarbons, usually in blends. The fact that *C. obsoletus* responded significantly to the pheromone blend of *C. hemipterus* in field tests (Bartelt et al., 1992b) prompted us to characterize its pheromone. Pheromone isolation was guided by wind-tunnel bioassays. Activity of the pheromone was verified under field conditions.

METHODS AND MATERIALS

Beetles. The *C. obsoletus* culture was started from insects captured in a date garden near Oasis, California. The beetles were reared on the diet reported by Dowd (1987), except that additional brewer's yeast replaced the pinto beans. Insects from the culture were used both for pheromone production and for wind-tunnel bioassays.

Pheromone Collections. Beetles were separated by sex and placed with diet medium in volatile-collection flasks as described earlier for *C. hemipterus* (Bartelt et al., 1990b). Initially, each flask contained ca. 65 beetles. Twice as many collectors were set up for males (six flasks for males versus three flasks for females) because all of the previously studied, related species had male-produced pheromones. Volatiles were also collected from three flasks containing just diet, for chromatographic comparisons. Later in the project, volatile collections were also obtained from three flasks containing individual male beetles on diet because pheromone production from individuals has been reported to be higher than that from groups in another species (*C. antiquus*) (Bartelt et al., 1993b). Filters of Super Q porous polymer (Alltech Associates, Deerfield, Illinois) were used to clean the incoming air and to capture the volatiles from the feeding beetles. The temperature during collections was 27°C, the humidity of incoming air was ca. 30%, and the photoperiod was 14L:10D.

Counts were kept so that amounts of volatiles could be expressed in beetle-

days (the average amount of material collected per beetle per day). The pooled collections over a three-week period amounted to ca. 6930 beetle-days from males and 3465 from females. About 10% of this material was set aside as a standard for bioassay tests, and the remainder was used for chromatographic isolation of the pheromone.

Bioassay. Pheromone isolation was guided by wind-tunnel bioassays. The wind-tunnel bioassays were conducted as described by Bartelt et al. (1990b). The wind tunnel contained ca. 500–1000 beetles, and ca. 100 were in flight at any time during bioassays. Two different treatment preparations to be compared were applied to pieces of filter paper, and these were hung side by side in the upwind end of the wind tunnel. When more than two treatments were to be compared, they were tested in pairs with a balanced incomplete block experimental design. Responses by the beetles to an active preparation included an upwind, casting flight followed by alighting on the filter paper. Each test lasted 3 min, and the numbers of landings were recorded. Propyl acetate (20 μl, 1% solution in mineral oil) was added as a coattractant in all bioassay treatments except where otherwise indicated; previous studies have shown that bioassay count is enhanced by the presence of a "host-type" volatile coattractant (Bartelt et al., 1990a,b). The control consisted of filter paper containing only propyl acetate.

Existence of Male-Specific Pheromone. Seven one-week volatile collections from groups of male beetles were compared with the corresponding volatile collections from female beetles in the wind-tunnel bioassay in order to establish the existence of a male-specific pheromone in *C. obsoletus* (7 beetle-days per test; total of 14 tests).

Chromatography and Pheromone Isolation. High-performance liquid chromatography (HPLC) was initially conducted on pooled male-derived and pooled female-derived collections using a Spectra Physics model SP8700 solvent delivery system equipped with a silica column (Microsorb Si 80-125-C5, Rainin Instruments, Woburn, Massachusetts). Hexane was run for 5 min, at a flow rate of 1 ml/min, followed by a solvent gradient programmed from 100% hexane to 100% diethyl ether in 15 min. Effluent was collected as 1-ml fractions. Each male-derived chromatographic fraction, plus coattractant, was bioassayed four times against a control consisting of only the coattractant. Active male-derived fractions were then compared with the corresponding female-derived fractions and control in the wind-tunnel bioassay (eight replications per treatment). All collected fractions were later analyzed by GC.

The silica fraction containing maximal male-specific activity (3–4 ml after injection) was rechromatographed using a 25-cm × 0.46-cm (ID) Lichrosorb Si60 column (5-μm particle size) (Alltech), coated with $AgNO_3$ as described by Heath and Sonnet (1980). The column was eluted isocratically with a solution of 10% toluene in hexane at a flow rate of 1 ml/min. A Waters Associates model

6000 pump and R401 refractometer detector were used for this analysis. The beetle-derived samples were not concentrated enough to be detected by the refractometer, so effluent was collected as 1-ml fractions, which were also bioassayed (four times per fraction) and later analyzed by GC. The corresponding female-derived chromatographic fraction was processed similarly, then active male-derived fractions were again compared with the corresponding female-derived fractions and control in the wind tunnel bioassay as described above.

Analysis. Volatile collections and chromatographic fractions were analyzed by gas chromatography (GC). GC was performed with a Hewlett-Packard (HP) 5890 Series II instrument equipped with flame ionization detector, splitless injector, a HP 7673 autosampler, and interfaced to a HP 3396A integrator. The oven temperature was programmed from 50°C to 270°C at 10°C/min; the injector temperature was 220°C, and the detector temperature was 270°C. A 15-m × 0.25-mm capillary column (DB-1, with 1.0-μm film thickness, J&W Scientific, Folsom, California) was used with 2-μl sample injections. An internal standard (*n*-nonadecane) was used for pheromone quantitation.

For confirmation of peak identities, positive ion, electron impact mass spectra (70 eV) were obtained with a HP 5970 MSD instrument, with sample introduction through a DB-1 capillary GC column. An oven temperature program similar to that described above was used.

Chemicals. Synthetic (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene (**1**) was available from earlier research (Bartelt et al., 1990a). It was purified to >95% purity by $AgNO_3$-coated silica HPLC using 10% toluene in hexane as the isocratic elution solvent, for use in wind-tunnel bioassay tests. For field bioassay studies, technical grade **1** was used without HPLC purification; this was 72% all-*E* **1**, 19% *Z* isomers, and 9% various uncharacterized minor products (each <1% of total mixture).

Additional Wind-Tunnel Experiments. The importance of the coattractant was verified after the identification of the *C. obsoletus* pheromone (**1**). In this experiment, treatments were: synthetic pheromone at 0.5 ng alone, synthetic pheromone at 0.5 ng with propyl acetate, propyl acetate alone (control), and a blank consisting of only filter paper. All comparisons were done, two at a time, in a balanced incomplete block experiment (six replications per treatment).

Synthetic **1** was compared with beetle-derived preparations of **1** at various stages of purification so that the existence of other attractant compounds might be revealed. These stages included: crude pheromone extracts (before any chromatography), pheromone extracts after only hexane–ether gradient silica HPLC, and pheromone extracts after hexane–ether silica gradient HPLC and HPLC using the silver nitrate-coated column. Each beetle-derived sample (which contained **1**) was tested against an equal amount of synthetic **1** and a control consisting of filter paper plus coattractant. In all cases, the amount of beetle-derived or synthetic pheromone was 0.5 ng/test and the coattractant was propyl acetate

(pairwise tests, balanced incomplete block design, eight replications per treatment). In additional experiments, the silica–HPLC-purified pheromone from beetles was compared to synthetic pheromone at doses of 0.05 ng and 5.0 ng.

Pheromone Production: Group vs. Individual Beetles. Pheromone production by a group of 65 male beetles was compared with pheromone production by individual male beetles, on the basis of the amount of pheromone emitted (nanograms) per beetle per day. Mature beetles (2-week-old adults) were used in this study so that consistent pheromone production could be expected. Pheromone was collected twice a week for a period of two months. The collections from individual beetles were then continued as long as the beetles remained alive.

Field Bioassay. Field tests were conducted in a date garden near Oasis, California. The location and methodology were as described previously for an experiment with *C. hemipterus* (Bartelt et al., 1992b). The wind-directed pipe traps (Dowd et al., 1992) were hung 1 m above the ground, and trap spacing was ca. 20 m. The traps were baited either with fermenting whole-wheat dough only, or a combination of pheromone plus dough. Whole-wheat bread dough is a commonly used nitidulid attractant, and its major volatile emissions have been characterized (Lin and Phelan, 1991). There were two replications of each treatment in a randomized block design. Beetles were collected from the traps weekly from April 21 to September 29, 1992. The pheromone septa (containing 500 μg **1**) were replaced every two weeks, and the dough baits were replaced weekly. Field bioassay results presented in this paper are part of a larger study to be published at a later date.

Statistical Analysis. Wind-tunnel and field data were transformed to the log $(X + 1)$ scale before analysis to stabilize variance. Balanced incomplete block experiments involving comparisons among three or more treatments were analyzed by the method of Yates (1940). Analysis of variance (ANOVA) was used to analyze field trap catch data. ANOVA was also used to compare pheromone production rates from individuals and groups of beetles.

RESULTS AND DISCUSSION

Evidence for Male-Specific Pheromone. Volatile collections from male beetles were significantly more attractive in the wind-tunnel bioassay than those from female beetles, establishing the existence of a male-specific pheromone. Mean bioassay counts (7 beetle-days per test, $N = 14$) for male-derived volatiles and female-derived volatiles were 37.6 and 2.1, respectively ($F = 106$, 1 and 13 *df*, $P < 0.001$). Only male-produced aggregation pheromones have been discovered so far in *Carpophilus* species.

Isolation of Hydrocarbon Pheromone. After HPLC on the silica column,

the most active male-derived fractions (3–5 ml after injection, Table 1) had very low polarity. Although several fractions with higher polarity also had significant activity, attempts to isolate the active compounds were unsuccessful because of low and inconsistent bioassays. However, these polar, male-derived fractions (18–21 ml) were similar in activity to the corresponding female-derived fractions (data not shown); thus, these materials appear not to be male-specific attractants and may emanate from the diet.

Rechromatography of the most active hydrocarbon fraction (3–4 ml after injection) on the $AgNO_3$-coated silica column resulted in a group of active fractions 7–10 ml after injection (lower section of Table 1).

GC analysis of the male-derived and female-derived fractions 7–10 ml after injection revealed the presence of a single peak at a retention time of 13.82 min in the male-derived fractions. Amounts of the peak in each of the active fractions were: 7–8 ml after injection (trace), 8–9 ml after injection (0.12 ng/beetle-day, 11%), and 9–10 ml after injection (0.95 ng/beetle-day, 89%). The 13.82-min peak was not observed in the corresponding female-derived $AgNO_3$-coated silica column fractions.

The activity of the 4 to 5-ml silica column fraction (Table 1) was probably due to the same compound. By GC, the 13.82-min peak was present in the fraction in the amount of 0.03 ng/beetle-day.

Identification of Hydrocarbon Pheromone. The GC retention time and mass spectral fragmentation pattern of the male-specific compound matched that of (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene **1**, shown in Figure 1 (Bartelt et al., 1990b).

This compound was previously reported as a component of the *C. hemipterus* pheromone blend (Bartelt et al., 1990b, 1992a) and the *C. freemani* pheromone blend (Bartelt et al., 1990a). It has recently been identified as a component of the *C. lugubris* pheromone blend (R.J.B., unpublished results). The fact that *C. obsoletus* was caught in field traps, baited with only the *C. hemipterus* pheromone blend, also supports our identification (Bartelt et al., 1992b).

Minor amounts ($<5\%$ as abundant as **1**) of other male-specific compounds were also detected when the silver-coated silica chromatographic fractions were carefully compared by GC. By GC retention (elution before **1**), mass spectrometry (spectra similar to **1**), and experience with other tetraenes (Bartelt et al., 1992a), these other compounds were believed to be *Z* isomers of **1**. Some isomerization/degradation has invariably occurred with all of the tetraenes (Bartelt et al., 1992a), and it is unknown whether the minor isomers are actually in emissions from *C. obsoletus*. These were not analyzed further in this study.

Effect of Coattractant. The synthetic pheromone was active in wind-tunnel tests, and it was synergized by the host-type attractant, propyl acetate. Mean bioassay counts ($N = 8$) for blank filter paper, control (propyl acetate, 20 μl, 1% in mineral oil), pheromone alone (0.5 ng), and pheromone (0.5 ng) with

TABLE 1. MEAN BIOASSAY COUNTS ($N = 4$) FOR CHROMATOGRAPHIC FRACTIONS DERIVED FROM MALE *C. obsoletus*[a]

Volume after injection (ml)	Beetle-derived	Control
A. Initial separation by polarity (hexane–ether gradient on silica column)		
2–3	3.1	5.1
3–4	104.4***[b]	1.7
4–5	50.2***	3.1
5–6	10.7	5.1
6–7	9.2*	4.1
7–8	7.0**	1.5
8–9	10.0***	1.7
9–10	3.8	3.6
10–11	3.0	3.0
11–12	5.2	4.2
12–13	6.3	5.0
13–14	4.8	3.5
14–15	7.8	4.0
15–16	6.5*	2.3
16–17	10.1*	3.9
17–18	3.7	3.2
18–19	30.8***	2.0
19–20	11.2***	2.3
20–21	15.3***	1.4
21–22	7.0*	0.9
22–23	3.7	1.5
23–24	1.6	1.4
24–25	4.8	2.3
25–26	3.4	1.4
B. Rechromatography of the active, 3 to 4-ml fraction from A (above) on silver-nitrate coated silica.		
2–3	1.4	0.7
3–4	2.1	2.0
4–5	1.0	1.4
5–6	1.9	0.5
6–7	1.5	2.2
7–8	10.8***	0.9
8–9	42.3***	0.9
9–10	50.0***	0.0
10–11	8.0*	2.7
11–12	6.0	2.3
12–13	4.4	3.1
13–14	4.6	1.6

[a] Initial chromatographic fractions were used at 30 beetle-days per test and subsequent chromatographic fractions were used at 27 beetle-days per test. Bioassay counts are the numbers of beetles flying upwind to the filter paper baits and alighting during the 3-min tests. Coattractant was propyl acetate (1% in mineral oil, 20 μl/test).

[b] Differences from the control at the 0.05, 0.01, and 0.001 levels denoted by *, **, and ***, respectively [t tests in $\log(X + 1)$ scale, using pooled error].

propyl acetate (1% in mineral oil, 20 μl) were 0.1, 5.1, 5.7, and 26.1, respectively. The combination of pheromone and propyl acetate was significantly more attractive than either alone. Both single attractants were significantly more attractive than the control but did not differ from each other (LSD, 0.05 level). The idea of synergism is supported by the fact that the total activity of the combination of pheromone and coattractant is greater than the sum of the activities of pheromone alone plus coattractant alone.

Beetle-Derived vs. Synthetic Pheromone. The presence of **1** accounted for all of the observed activity of beetle-derived materials at three stages of purification (Table 2). Thus **1** was the dominant attractant in the volatile collections, and there was no evidence for additional important sex-specific attractants having been overlooked. We found no significant differences in the activity of the beetle-derived pheromone vs. the synthetic pheromone. The beetles were very sensitive to **1** in the wind tunnel, responding significantly to a 50-pg dose.

Pheromone Production: Group vs. Individual Beetles. Pheromone production was significantly lower for the group of males than for individual males.

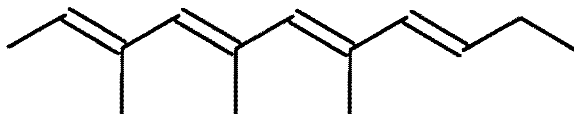

FIG. 1. Male-produced aggregation pheromone of *Carpophilus obsoletus* Erichson: (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene **1**.

TABLE 2. COMPARATIVE ACTIVITY OF SYNTHETIC **1** AND BEETLE-DERIVED **1** AT VARIOUS STAGES OF PURIFICATION

Step of purification[a]	Amount of **1** per test (ng)	Mean bioassay count ($N = 8$)[b]		
		Beetle-derived **1**	Synthetic **1**	Control
Whole collection	0.5	14.5a	14.2a	0.1b
After hexane–ether	0.05	5.3a	4.3a	0.2b
gradient	0.5	8.3a	8.3a	0.0b
	5.0	15.6a	14.6a	0.2b
After silver-coated silica HPLC	0.5	9.4a	9.9a	0.0b

[a]Coattractant was propyl acetate (1% in mineral oil, 20 μl/test).
[b]Bioassay counts are the numbers of beetles flying upwind to the filter paper baits and alighting during the 3-min tests In each row, means followed by the same letter are not significantly different [LSD, 0.05 level, balanced incomplete block analysis, $\log(X + 1)$ scale].

Overall means were 2.2 ng/male/day for the group ($N = 15$ volatile collections) and 72 ng/day for individuals ($N = 45$; 15 volatile collections from each of three beetles); the t statistic for the difference was 6.92 ($P < 0.0001$). However, there was also significant variability among the three individuals tested: Their means (standard deviation) were 28.4 (33.5), 45.3 (27.4), and 141 (90.2) ng/day. For six of the 45 collections, no pheromone was detected, but daily production was as high as 388 ng/day. Except for brief lapses, pheromone production occurred throughout the entire two-month study. The reason for occasional nonproduction of pheromone by mature individual beetles is unknown but may involve environmental conditions such as condition of diet. Group pheromone production never stopped entirely, possibly because beetles vary somewhat in their perception of an unfavorable environment.

Monitoring of pheromone production by the individual males was continued until beetle death; one individual survived 9.5 months and produced pheromone during all but the last month. Over this period, pheromone production decreased gradually, in approximately linear fashion until ceasing at 8.5 months [regression model: daily pheromone production (ng) = 152 − 0.6 × age (days), $R^2 = 0.3586$, $F_{1,50} = 27.96$, $P < 0.001$]. Considerable day-to-day variation in pheromone production was observed; the standard deviation about the regression line was 55 ng/day.

Given a maximum pheromone-production level of 388 ng/beetle-day and the observation that beetles will respond in the wind-tunnel bioassay to a level of only 50 pg of the pheromone (with propyl acetate as a host-type synergist), only one hundredth of one percent of the total maximum daily pheromone emission is required for the biological response of aggregation.

Field Trap Data. Synthetic **1** was active in the test at the date garden. The pheromone, in combination with fermenting whole-wheat bread dough, attracted a total of 200 *C. obsoletus* (54% female); the dough by itself attracted no *C. obsoletus*. Mean trap catches ($N = 48$; two traps, 24 weeks) for pheromone plus fermenting bread dough and dough only were 4.2 and 0, respectively ($F = 316$, $P < 0.001$). Weekly trap catches varied (Figure 2). Seasonal activity peaked during the months of July and August, but substantial catches were also recorded in late April. A similar pattern of trap catch over time was observed for *C. obsoletus* previously (Bartelt et al., 1992b).

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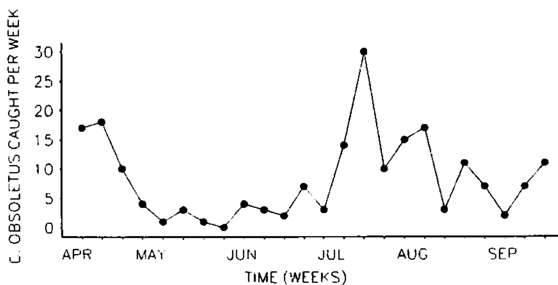

FIG. 2. Trap catch pattern over time for *C. obsoletus* in a date garden near Oasis, California, April 21 to September 29, 1992. Each point is the weekly catch, totaled over two traps baited with a combination of pheromone (500 μg **1**) plus fermenting bread dough. (No *C. obsoletus* were caught in the two control traps baited with dough only.)

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