

MATING BEHAVIOR AND SEXUAL RESPONSE TO  
AGGREGATION PHEROMONE OF *RHYNCHOPHORUS*  
*CRUENTATUS* (COLEOPTERA: CURCULIONIDAE)

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ABSTRACT

Mating behavior of *Rhynchophorus cruentatus* (Fabricius) was investigated in the laboratory. The sequence of behaviors was consistent for all weevils that mated. Males exhibited rostral rubbing and antennal tapping before copulation, guarding females afterwards. Mating frequency and celerity were compared between sequestered male-female pairs and for focal males in simulated aggregations. Field-collected and virgin males in groups were significantly quicker to initiate mating behavior and attempted to mate more often per session than males in sequestered pairs. Increased sexual stimulation in weevil aggregations appears to be semiochemically-mediated. Visual and tactile cues were eliminated as contributing stimuli in simulated aggregation arenas. Males were significantly more stimulated to mate in the presence of synthetic aggregation pheromone, 5-methyl-4-octanol (cruentol) than in its absence.

Key words: aggregation, mating behavior, pheromone, *Rhynchophorus cruentatus*, 5-methyl-4-octanol

RESUMEN

El comportamiento de apareo de *Rhynchophorus cruentatus* (Fabricius) fue investigado en el laboratorio. La secuencia de comportamientos fue la misma para todos los gorgojos que se aparearon. Los machos exhibieron frotamiento con el rostro y golpecitos con las antenas antes de la copulación, la que fue seguida por la guardia de las hembras. La frecuencia y rapidez del apareo fueron comparados entre parejas aisladas de macho y hembra y machos focales en agregaciones artificiales. Se observó que los machos colectados en el campo y los machos vírgenes en agregaciones iniciaban el comportamiento de apareo significativamente más rápido y que intentaban aparearse más seguido en cada sesión que los machos de parejas aisladas. El incremento de la estimulación sexual en agregaciones de gorgojos parece estar mediado por un compuesto semioquímico. Fueron eliminados los estímulos visuales y táctiles como contribuyentes al apareo dentro de arenas con agregaciones artificiales de gorgojos. Los machos fueron significativamente más estimulados para aparearse en la presencia de la feromona sintética de agregación 5-metil-4-octanol (cruentol) que en su ausencia.

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The palmetto weevil, *Rhynchophorus cruentatus* (F.), is the largest weevil (24-33 mm long) in the continental United States (Woodruff 1967). This weevil is sympatric with the native cabbage palmetto, *Sabal palmetto* (Walter) (Woodruff 1967) from the Florida Keys through the coastal regions of South Carolina and Texas (Wattanapongsiri 1966). In most cases, *R. cruentatus* is not considered to be a primary pest of cabbage palmetto, but a secondary pest that attacks transplanted or otherwise stressed

palms in the landscape (Giblin-Davis & Howard 1989). Recent research suggests that it can be a serious pest of field-grown *Phoenix canariensis* (L.) (unpublished data). The weevil is attracted to the fermenting plant volatiles from chopped palm tissue and chopped sugarcane, *Saccharum officinarum* (Weissling et al. 1993). There is a synergistic effect when these plant volatiles are combined with *S,S*-5-methyl-4-octanol (cruentol), the aggregation pheromone produced by *R. cruentatus* males (Weissling et al. 1994a; Perez et al. 1994). Large aggregations of male and female *R. cruentatus* are attracted to this combination, presumably for mating and oviposition purposes (Giblin-Davis et al. 1994; Weissling et al. 1993).

Many species of insects and other animals are known to aggregate for mating purposes (Landolt 1997). Evolutionary biologists have investigated advantages for males calling in conspecifics (which invites competition from other males) (Thornhill & Alcock 1984) and the advantages of multiple mating for females (Sakurai 1996; Lewin 1988). The production of aggregation pheromones may serve to recruit widely distributed conspecifics to rare resources (i.e. stressed hosts). Therefore, it may be advantageous to males to call females as a way to reduce the amount of time spent searching for widely dispersed potential mates. This may also be an adaptation that enables *R. cruentatus* to cope with the seasonal emergence patterns characteristic of this species (Weissling et al. 1994b), and to overwhelm the defenses of a potential palm host (Giblin-Davis et al. 1996).

*Rhynchophorus cruentatus* males are morphologically well adapted to mating in aggregations. On the tibiae of the forelegs, males possess a row of setae, commonly referred to as a "sex comb". A similar structure is used by males of some other species of insects to facilitate grasping and control of females (Spieth 1952). This structure may also prevent aggregated males from dislodging copulating males. Another possible function for the tibial hairs may be to distribute pheromone. Males of *R. palmarum* (L.) apparently produce pheromone in the prothoracic glands and pass the molecules forward to the rostrum where it is distributed by setae (Sanchez et al. 1996). *Rhynchophorus cruentatus* is the only species of *Rhynchophorus* that lacks these rostral setae but instead possesses rostral ferrugae (Wattanapongsiri 1966). Possibly they wipe the rostrum with the tibial hairs to help distribute pheromone. Because little is known about the mating behavior of the agriculturally important Rhynchophorinae we endeavored to characterize the mating behavior of *R. cruentatus* in the laboratory.

#### MATERIALS AND METHODS

Adult weevils were collected in Hendry and Dade Counties, Florida during the peak trapping seasons of 4/96-7/96 and 4/97-7/97. In Hendry County, 120 weevils were collected in four live traps set in a field with cabbage palmettos from which the buds had been removed by "palm heart" collectors. In Dade County, weevils were trapped from a grove of *P. canariensis* that had sustained severe damage from *R. cruentatus*. Fifty-five virgin weevils (harvested directly from cocoons) and 140 adults with unknown mating histories were collected. The trap design used was previously described by Weissling et al. (1992). Each trap was baited with sugarcane (400 g), ethyl acetate (6 ml) in open-topped vials and one controlled-release dispersal unit containing synthetic racemic cruentol; 5-methyl-4-octanol (96% pure). Cruentol was obtained from Dr. A. C. Oehlschlager (ChemTica, San Jose, Costa Rica) (release rate @ 25°C = 3 mg/d). Once collected, weevils were maintained in covered disposable polyethylene containers with sugarcane and separated by sex for no less than 18 h prior to each mating session. Adult insects for bioassay were sexed using dimorphic rostral and tibial char-

acteristics (Wattanapongsiri 1966). *Rhynchophorus cruentatus* is a relatively rare insect (Weissling et al. 1994a) and it was necessary to reuse the same weevils in repeated trials, except in the trials involving virgins. Insects were selected arbitrarily from containers for use. Weevils smaller than 29 mm were not used for male-female pairs and size assortive mating was not examined.

Mating was defined as the attempted insertion of the aedeagus of one male weevil while in contact with another female or male weevil. No assumptions were made about spermatophore transfer. Mating behaviors were observed by placing weevils in polystyrene arenas (Tri-State Plastics, Dixon, KY) (21 cm diam × 8 cm high) for 30 min sessions. Experiments were conducted under ambient fluorescent lighting (0.74 klux) at 22-26°C and 44-60% RH. In all experiments, two sexual stimulation indicators for males were measured: 1) the frequency of mating per 30 min session and 2) the time from placement into the arena until mating was initiated.

#### Experiment 1. Field-collected Weevils: Description of the Mating Sequence of Sequestered Pairs; Observation of Behaviors in Simulated Aggregations and Comparison of Sexual Stimulation Factors for Sequestered Versus Artificially Aggregated Males

*A. Ethogram.* Preliminary examination of weevil pairs allowed for the preparation of an ethogram. This preparatory ethogram was quantified using observations of the complete mating sequence of the 20 sequestered males in experiment 1C.

*B. Observation of behavior in aggregations.* Initially, 10 trials of scan sampling were conducted and some general observations were made about mating tendencies in aggregations. An ethogram of behaviors in the group environment was not prepared because the pace of activity in these arenas was so frenetic that quantification of any single behavior was impossible.

*C. Comparison of the two sexual stimulation indicators for sequestered versus artificially aggregated males.* Comparisons were made for the frequency of mating and the time to begin mating between 20 sequestered males and 20 focal males in an artificial mating aggregation. The artificial aggregation was assembled by simultaneously placing one randomly selected male weevil that was marked with a small amount of metallic marker (Sanford silver coat, Bellwood, IL) on the pronotum, with nine other males and 10 females into an arena. Male sexual stimulation indicators were then compared between sequestered and artificially aggregated males as previously described.

#### Experiment 2. Virgin Weevils: Comparison of Sexual Stimulation Indicators for Sequestered Versus Artificially Aggregated Males

Experiment 1C was repeated with virgin weevils. Twenty replications with sequestered virgin male-female pairs were compared to marked focal virgin males in artificial aggregations, 15 replicates.

In order to isolate the factors responsible for male sexual stimulation in groups, experiments 3, 4, 5 and 6 were conducted using the same containers in a different arrangement. A mating arena consisted of two containers stacked one on top of the other, open sides together, with a barrier separating them (Fig. 1). The lower chamber contained the treatment and the mating pair under observation was placed in the upper chamber, moving about on the barrier. This created a fractional aggregation. Barrier components and upper chambers were washed with soap and water and dried after each repetition. Arena placement was randomized for each replicate of the following experiments.

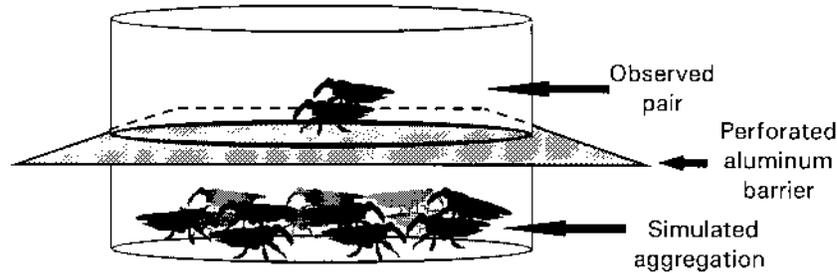


Fig. 1. Schematic diagram of the fractionally aggregated mating arena used in experiments 3 and 4. In experiment 5 only, glass barriers replaced the perforated aluminum. In experiment 6, synthetic aggregation pheromone replaced the weevils in the lower chamber.

#### Experiment 3. Comparison of Sexual Stimulation Indicators for Sequestered Versus Fractionally Aggregated Males

This experiment involved two treatments and tested whether semiochemical, tactile or visual cues influenced the frequency and celerity of mating for upper-chamber males. A barrier assembly consisted of a sheet of pierced aluminum (1.5 mm thick, 2 mm diam perforations, 12 holes/cm<sup>2</sup>) overlaid with black polyester open-weave stretch fabric. The design of this arena allowed for the diffusion of volatiles into the observation chamber, while preventing the mating pair from seeing or touching weevils below. Chambers were allowed to equilibrate for 30 min immediately prior to each trial. The fractionally aggregated arena (treatment 1) held nine females and nine males in the lower chamber, with the mating pair under observation in the upper chamber, simulating the original group of 20 total weevils in experiment 1C. The lower chamber of the control arena (treatment 2) was left unoccupied. There were 20 replications of each treatment.

#### Experiment 4. Comparison of Sexual Stimulation Indicators for Fractionally Aggregated Males by Sex of Semiochemical Source

Three fractional aggregation arenas were arranged as described for experiment 3 to test whether semiochemicals from males, females or both were important in stimulating the mating behavior of the male in the chamber above. The treatments were: nine males plus nine females, 18 females, and 18 males. There were 20 replications of each of the three treatments.

#### Experiment 5. Comparison of Sexual Stimulation Indicators for Fractionally Aggregated Males by Sight and Sound of Other Weevils

To test whether the sight of or vibrations from other weevils were important stimuli, sheets of glass (3.5 mm thick) were used as barriers in place of the fabric and aluminum used in experiments 3 and 4. The glass prevented volatiles from the treatment chamber below from entering the upper chamber. Treatment 1 consisted of a clear glass barrier with nine male plus nine female weevils below creating a situation

where the pair could see and feel vibrations from the lower group but not sense semi-chemicals produced by the group. Treatment 2 utilized clear glass with a vacant lower chamber exposing the weevil pair to no group-produced stimuli. Treatment 3 used the 18-weevil arrangement of treatment 1, but with painted glass as the barrier, allowing only vibrational cues to be received. This glass sheet was sprayed with matte black enamel (Rust-oleum Corp., Vernon Hills, IL) on the side facing the lower chamber 3 wks prior to use to allow the surface to fully dry and ventilate.

#### Experiment 6. Comparison of Sexual Stimulation Indicators for Pheromone in Fractionally Aggregated Chambers

The effects of synthetic aggregation pheromone on mating behavior of weevils were examined. There were three treatments: two doses of ( $\pm$ ) cruentol and a control (no cruentol). The aluminum-fabric barriers were used in each mating arena. One- $\mu$ l capillary glass tubes (Drummond Scientific Co., Broomall, PA) were filled with cruentol and then secured upright in Seal-ease tube sealer and holder (Becton Dickenson & Co., Rutherford, NJ). An equal amount of the Seal-ease was placed in the lower chamber of the control arena. The cruentol-containing arenas held either one or five microcaps (release rate =  $3.02 \pm 0.92$  SD ng/h/ $\mu$ cap). The release rate was calculated by placing ten 1  $\mu$ l microcapillary tubes in Seal-ease in the lower chamber of an arena with an aluminum-fabric barrier and upper chamber cover. After 24 h, the amount of cruentol lost from each tube was measured to determine the mean release rate. Cruentol was handled with disposable gloves and stored in a  $-10^{\circ}\text{C}$  freezer when not in use. Weevils and cruentol were introduced into the testing room in separate, tightly closed containers. Arenas were randomized and placed 2 m apart. Cruentol was introduced into the lower chambers of arenas and was allowed to equilibrate for 3 min immediately prior to introduction of weevils into the upper chamber. After each replicate, lower chambers were quickly covered with lids to minimize diffusion of cruentol into the room. There were 20 replicates of each experiment.

#### Statistics

A preliminary scatterplot of the time to begin mating in experiment 1 suggested that mating in the first 250 sec was optimal for measuring sexual stimulation between sequestered and aggregated males. Therefore, the number of males mating within the first 250 sec was used for analysis by the Kruskal-Wallis (chi-square approximation) test (SAS Institute 1985). The number of apparent matings per 30 min observation period were square root transformed ( $x + 0.5$ ) and analyzed by analysis of variance using PROC ANOVA (SAS Institute 1985). Least significant difference tests (SAS Institute 1985) were used for means separation where significant differences occurred.

### RESULTS AND DISCUSSION

#### Experiment 1. Field-collected Weevils: Description of the Mating Sequence for Sequestered Pairs; Observation of Behaviors in Artificial Aggregations and Comparison of Sexual Stimulation Factors for Sequestered Versus Artificially Aggregated Males

*A. Ethogram.* Of the 20 sequestered pairs studied for sequence of mating events, 14 mated as previously defined. All males and females made numerous physical contacts with one another whether or not they later mated. A summary of the sequence

of mating events is presented in the ethogram in Fig. 2. All males engaged in rostral rubbing, defined as touching the female's elytra with the distal tip of the rostrum and moving it in a serpentine pattern from the pygidium forward. A similar behavior has been observed in *Ips* beetles by Birch (1978) who suggested that this is a placating gesture. Another possible explanation for this behavior is that the weevils are discriminating heterospecific cuticular hydrocarbons (Takahasi & Gassa 1995). Five of the 20 male weevils did not engage in any behaviors other than the general physical contact and rostral rubbing (Fig. 2). The sixth male that did not mate moved directly from rostral rubbing to guarding, described below. Of the males that did mate, all followed a stereotyped sequence of behaviors. Both during and subsequent to the rostral rubbing the male tapped his antennae on the elytra and pronotum of the female as he began to mount her (Fig. 2). This antennal tapping and the actual mounting of the female was the first indication that a copulatory event was about to take place. There were not any other discernible behaviors that could be identified as "courtship". The antennal tapping was immediately followed by attempts to insert the aedeagus (Fig. 2). In all cases, the female remained very still during mating (Fig. 2). After a period of time (approximately 2 min) she initiated the termination of mating by moving her legs and walking across the arena floor. All of the males that mated maintained close physical proximity to the female, referred to here as guarding (Fig. 2). In most cases, the male was able to grasp the female with all six of his legs, remaining on top and riding on her back as she moved about the arena. If he grasped with only two or four of his legs he was dragged around with his pygidium scraping the arena floor. The guarding behavior might serve to deter mating attempts by other males in an aggregation, to stimulate the female to oviposit more quickly or to reduce the likelihood that the female will accept another partner (Eberhard 1996). Ten of the 14 males that mated proceeded from guarding the female to a second copulatory event (Fig. 2). There was not always the same level of female cooperation in subsequent attempts. Of the 10 males which went on to attempt a second mating, six attempted a third time and two of these attempted a fourth time. The sequence of virgin mating behaviors observed in experiment 2 was as illustrated in the ethogram (Fig. 2), except that some of the virgins did not guard their female partners or they separated immediately after mating, returning after several seconds to guard her.

*B. Observation of behavior in aggregations.* Male weevils seemed to be stimulated to mate while in a mixed-gender group. This phenomenon was originally noticed when weevils were being transported from collection sites, before they had been separated according to sex. These original observations led us to hypothesize that males were more highly stimulated to mate in aggregations.

In artificial aggregations, weevils engaged in frequent and apparently deliberate physical contact irrespective of gender. Males scrambled to obtain females quickly and there was a frenetic quality to the activities. Both males and females multiply

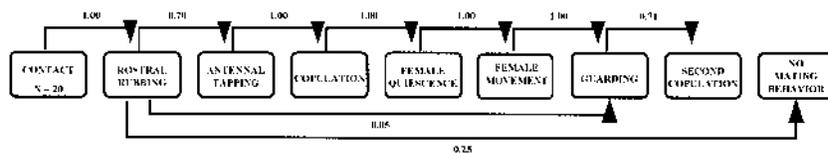


Fig. 2. Ethogram of *Rhynchophorus cruentatus* mating behavior from experiment 1A. Values represent percentage of mating pairs that proceeded to the next activity, as indicated by arrows.

mated. Males attempted to mate with other males in scan sampling and 13.9% of all mating attempts ( $n = 84$ ) were homosexual in experiment 1C. Clusters of two to five males were observed attempting to mate with one female while other females were ignored. Males readily diverted their attentions from one female to others in the vicinity. Rival males attempted to supplant mating males (often successfully) by inserting their rostrums between the bodies of the male and female. The challenge was occasionally followed by venter to venter "wrestling" between males, but most males simply moved on in search of other females.

*C. Comparison of the two sexual stimulation indicators between sequestered and artificially aggregated male weevils.* Artificially aggregated *R. cruentatus* males were more highly stimulated to mate than males in sequestered pairs. The frequency of mating ( $P = 0.0001$ ) and the number of males that mated in 250 secs or less ( $P = 0.0049$ ) (Table 1) were significantly greater for the aggregated males. The heightened sexual stimulation of male weevils in groups is probably an adaptation to the increased competition for females in mating aggregations.

#### Experiment 2. Virgin Weevils: Comparison of Sexual Stimulation Indicators for Sequestered Versus Artificially Aggregated Males

The frequency of mating was significantly greater for artificially aggregated virgin males than for sequestered virgins ( $P = 0.001$ ). The number of males that mated in the first 250 secs was also significantly greater for the aggregated virgins ( $P = 0.0132$ ) (Table 1).

#### Experiment 3. Comparison of Sexual Stimulation Indicators for Sequestered Versus Fractionally Aggregated Males

In this experiment, the ability to see and to physically interact with the 18 other weevils below him was removed from the focal male's experience, although he was still able to receive olfactory and potential acoustical feedback from them. The mean frequency of mating was significantly higher for the males above the occupied chamber than for males above the vacant chamber ( $P = 0.0001$ ) (Table 1). This suggested that vibrational and/or semiochemical stimuli were inducing the increased sexual activity. There was no difference between the sequestered and the fractionally aggregated males for the number of males which mated within 250 secs ( $P = 0.0803$ ).

#### Experiment 4. Comparison of Sexual Stimulation Indicators for Fractionally Aggregated Males by Sex of Semiochemical Source

Males did not mate more often when placed above only males, only females or a combination of both ( $P = 0.1567$ ). There was no significant difference in the number of males which mated in less than 250 secs ( $P = 0.0806$ ) (Table 1).

#### Experiment 5. Comparison of Sexual Stimulation Indicators for Fractionally Aggregated Males by Sight and Sound of Other Weevils

There were no differences in the number of mating events or the time to begin mating for any of the treatments using glass as a barrier to prevent semiochemical stimulation of males. No males began mating in less than 250 secs, although the weevils appeared to experience some difficulty gaining traction on the glass barrier surfaces that might have contributed to their slower starts. Overall, the frequency of mating

TABLE 1. MATING RESPONSE OF *RHYNCHOPHORUS CRUENTATUS* MALES TO ARTIFICIAL AGGREGATIONS AND TO TACTILE, VISUAL AND SEMIOCHEMICAL CUES.

Experiment	Barrier Type	Treatment Chamber	No. Males Mating <sup>1</sup> in $\leq 250$ sec ( $\pm$ SE)	No. of Matings <sup>2</sup> per 30 min ( $\pm$ SE)
1C	No barrier	<u>Field-collected</u>		
		Artificially aggregated	$0.50 \pm 0.1^*$	$4.50 \pm 0.7a$
2	No barrier	Sequestered	$0.10 \pm 0.1$	$1.75 \pm 0.3b$
		<u>Virgin</u>		
3	Perforated aluminum	Artificially aggregated	$0.30 \pm 0.1^*$	$4.87 \pm 0.9a$
		Sequestered	0	$0.65 \pm 0.2b$
4	Perforated aluminum	9 males + 9 females	$0.25 \pm 0.1$	$4.10 \pm 0.7a$
		Vacant	$0.05 \pm 0.1$	$0.95 \pm 0.2b$
5	Unpainted glass	18 males	$0.25 \pm 0.1$	$5.30 \pm 0.6a$
		9 males + 9 females	$0.60 \pm 0.1$	$4.70 \pm 0.6a$
		18 females	$0.40 \pm 0.1$	$3.60 \pm 0.4a$
6	Perforated aluminum	9 males + 9 females	0	$1.15 \pm 0.4a$
		Unpainted glass	0	$0.95 \pm 0.4a$
		Painted glass	0	$0.75 \pm 0.2a$
6	Perforated aluminum	Cruentol: 30.20 ng/h <sup>3</sup>	$0.35 \pm 0.1^*$	$2.95 \pm 0.7a$
		Cruentol: 6.04 ng/h	$0.05 \pm 0.05$	$1.70 \pm 0.4ab$
		Vacant	$0.05 \pm 0.1$	$1.15 \pm 0.4b$

<sup>1</sup>Probability of obtaining chi square (Kruskal-Wallis test, chi square approximation)  $< 0.05$ , as indicated by asterisk.

<sup>2</sup>Data transformed by  $(x+0.5)^{0.5}$  to approximate homogeneity but are presented nontransformed. For each experiment, means within a column followed by the same letter are not significantly different according to ANOVA ( $P > 0.05$ ; LSD).

<sup>3</sup> $\pm$  cruentol release rates were estimated as described in materials and methods.

per session was consistent with that of the weevils in arenas with vacant treatment chambers in experiments 3 and 6, suggesting that volatiles emanating from below may influence mating behaviors. The lack of difference in response among weevils in this experiment indicates that sexual stimulation of male weevils is not substantially influenced by sight alone of other mating weevils (although males often approach mating pairs in an artificial aggregation).

#### Experiment 6. Comparison of Sexual Stimulation Indicators for Fractionally Aggregated Males by Exposure to Synthetic Aggregation Pheromone

The frequency of mating response was significantly greater for the higher-dose group than for the group exposed to lower levels or to no cruentol ( $P = 0.0438$ ). More males mated in a shorter time period when exposed to the higher level of cruentol than when exposed to the lower cruentol level or to no cruentol ( $P = 0.0077$ ) (Table 1). The aggregation pheromone cruentol may serve a secondary function for *R. cruentatus* males: its presence appeared to stimulate them to mate more quickly and more frequently. This would most likely be an evolutionary adaptation to sexual selection pressures posed by the aggregation mating system. A male is cued by cruentol to the presence of other males and he increases his level of sexual activity in order to remain competitive for access to females. Further study is necessary to establish whether other semiochemicals are important in affecting the mating behavior of males of *R. cruentatus* in aggregations.

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