

# MULTIFUNCTIONAL COMMUNICATION IN *Riptortus clavatus* (Heteroptera: Alydidae): CONSPECIFIC NYMPHS AND EGG PARASITOID *Ooencyrtus nezarae* USE THE SAME ADULT ATTRACTANT PHEROMONE AS CHEMICAL CUE

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**Abstract**—The bean bug, *Riptortus clavatus* lays scattered eggs (as opposed to the egg masses of pentatomids) on host as well as nonhost plants. Therefore, the first feeding stage (second-instar) nymphs emerging from eggs laid on nonhost plants need a signal that enables them to locate a food source at the lowest energy cost. Male-released (*E*)-2-hexenyl (*E*)-2-hexenoate, (*E*)-2-hexenyl (*Z*)-3-hexenoate, and myristyl isobutyrate play the double role of attractant pheromone for adults as well as aggregation pheromone, which enables the second-instar nymphs to find the host food plant. These male-specific semiochemicals are released only when foodstuff is available. On the other hand, females of *Ooencyrtus nezarae*, the most effective parasitoid of the host in Kumamoto, Japan (where the field experiments were conducted), utilize these semiochemicals as kairomones in order to locate the potential host community. Field experiments revealed that the synthetic pheromone rivaled 10 live males in the attraction of adults and second-instar nymphs. Captures of the egg parasitoid *O. nezarae* females in cylindrical sticky traps were signif-

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icantly higher in traps baited with the synthetic semiochemicals than in control traps. The number of females captured was significantly higher than the number of males, although the captures in the sticky suction trap system revealed that the populations of male and female were not significantly different.

**Key Words**—*Riptortus clavatus*, *Ooencyrtus nezarae*, Alydidae, Encyrtidae, attractant pheromone, aggregation pheromone, kairomone, (*E*)-2-hexenyl (*E*)-2-hexenoate, (*E*)-hexenyl (*Z*)-3-hexenoate, myristyl isobutyrate.

## INTRODUCTION

The bean bug, *Riptortus clavatus* (Thunberg), is an economically important soybean pest in Japan. Females lay single eggs on nonhost as well as host plants. The selective pressure of egg parasitoids probably contributes to this habit of scattering eggs. It is important to the first feeding stage (second-instar) nymphs to have a signal to locate a food source plant. Surveys conducted inside soybean fields indicated that the population of second-instar nymphs is much higher than both the number of eggs and the population of first-instar nymphs because second-instar nymphs moved from nonhost to host plants (Nakamori et al., 1994).

It has been demonstrated by field experiments that males induced aggregation of adult bugs (Kadosawa, unpublished data) and that this aggregation was semiochemically mediated (Numata et al., 1986). In the course of a study of the attractant pheromone of the bean bug, we found that the same semiochemicals are utilized by the second-instar nymphs to locate their host plants. In addition, the signal is used as a long-range kairomone by an egg parasitoid of the bean bug, *Ooencyrtus nezarae*.

## METHODS AND MATERIALS

### *Chromatographic, Mass, and Infrared Spectral (MS, IR) Analyses*

Gas chromatographic (GC) analyses were performed on a Hewlett-Packard 5890 equipped with one of the following capillary columns: DB-23 (30 m  $\times$  0.25 mm; 0.25  $\mu$ m), DB-wax (30 m  $\times$  0.25 mm; 0.25  $\mu$ m), or an HP-1 (12 m  $\times$  0.2 mm; 0.33  $\mu$ m), operated in splitless mode either at 50°C for 1 min programmed at 4°C/min to 180°C, held at this temperature for 1 min, programmed again at 10°C/min to 230°C and held at this temperature for 20 min [in short, 50(1)-180(1)/4-230(20)/10 or 50(1)-210(10)/10]. Mass spectra were recorded on a Hewlett-Packard 5891 mass selective detector using the same type of capillary columns under the same conditions as described for the GC. This was operated either in the electron impact (EI) or chemical ionization (CI) mode

using ammonia. Gas chromatography-Fourier transform infrared (GC-FTIR) was done on a Hewlett-Packard 5965B equipped with a DB-23 capillary column operated at 50(1)-210(10)/8. The light pipe and the transfer line were operated at 250°C.

#### *Gas Chromatography-Electroantennographic Detector (GC-EAD)*

The responses of *R. clavatus* antennae were recorded with a GC-EAD system with silver wire electrodes (Struble and Arn, 1984). Separations were achieved on a DB-23, DB-wax, or HP-1 capillary column operated at 50(1)-210(10)/10, 50(1)-180(1)/4-230(20)/10, and 70(1)-170(1)/3-250(10)/10, respectively.

#### *Extracts*

The airborne volatiles of adult bugs confined in an all-glass aeration apparatus and fed on soybean seeds (or deprived of food) were collected on a Tenax TA column, eluted with hexane, and concentrated. Whole-body extracts of adults and nymphs were obtained by immersing at least five individuals in hexane for 3 min. Hexane extracts of soybean and horse bean seeds were prepared by macerating the seeds before immersing in hexane.

#### *Isolation of Pheromone*

Crude airborne extracts of male bugs were separated on a silica gel column (Wako C-200) by successively eluting with hexane-ether (100:0, 95:5, 90:10, 80:20, 50:50, and 0:100) mixtures. Further purification was done by collecting small fractions from a silica gel-silver nitrate (10%) column eluted with hexane-ether (95:5 and 90:10).

#### *Chemical Derivatizations*

Dimethyl disulfide (DMDS) adducts of the active unsaturated esters were obtained by reaction with dimethyl disulfide (DMDS, 100  $\mu$ l) catalyzed by iodine. The mixture was stirred for 50-60 min at 65°C in a 1-ml conical vial containing a magnetic spin vane and closed with a Teflon-lined cap. After the mixture was cooled to room temperature, hexane (200  $\mu$ l) was added, and iodine was removed by washing with sodium thiosulfate. The organic phase was dried over anhydrous sodium sulfate, concentrated to 50  $\mu$ l, and analyzed by EI-MS using either a DB-wax or an HP-1 capillary column operated at 100(1)-230(20)/10 and 100(1)-230(30)/5, respectively.

Hydrolysis was done with potassium hydroxide in methanol at 60°C for 2 hr. The solvent was distilled off, the residue redissolved in ether, washed with

hydrochloric acid 2 M, dried over anhydrous sodium sulfate, concentrated, and analyzed by EI-MS.

Hydrogenation (catalyzed by  $\text{PtO}_2$ ) was carried out in a 1-ml conical vial containing a magnetic spin vane and equipped with a Claisen head fitted with a rubber balloon and Teflon-lined cap.

### Synthesis

(*E*)-2-Hexenyl (*E*)-2-hexenoate. To a solution of (*E*)-2-hexenoic acid (34 g) in dry benzene (90 ml), oxalyl chloride (75 g) was added dropwise at 45–50°C. After stirring at 65°C for 2 hr, the solvent was evaporated and the crude material was distilled to yield (*E*)-2-hexenyl chloride (32.5 g; bp 62–63°C/20 mm Hg). This acid chloride (30.3 g) was added dropwise to an ice-cooled mixture of (*E*)-2-hexenol (20 g) and pyridine (23.7 g) in dry chloroform (100 ml). The reaction mixture was refluxed for 1 hr, cooled to room temperature, water was added, and the organic layer was washed with sodium carbonate, dried over anhydrous sodium sulfate, evaporated, and distilled to give (*E*)-2-hexenyl (*E*)-2-hexenoate (28 g, bp 79–80°C/0.7 mm Hg).

(*E*)-2-Hexenyl (*Z*)-3-hexenoate. (*Z*)-3-Hexenyl chloride (bp 50–51°C/19 mm Hg) was prepared by the reaction of oxalyl chloride with the corresponding acid. The reaction of this acyl chloride with (*E*)-2-hexenol yielded (*E*)-2-hexenyl (*Z*)-3-hexenoate (bp 82–83°C/1.7 mm Hg).

Myristyl Isobutyrate. Reaction of myristyl alcohol with isobutyryl chloride gave myristyl isobutyrate (bp 127–128°C/0.7 mm Hg). The other esters were prepared in small scale by the reaction of acid chlorides and alcohols without solvent, followed by distillation of the products.

### Insects

*R. clavatus* adults were collected in soybean fields and kept in cages (15 × 12 × 10 cm) in the laboratory (25°C, 70% relative humidity, and 16:8 LD photoperiod) and fed on a mixture of soybean and horse bean. Adults were transferred to other cages after a reasonable number of eggs were laid. Nymphs obtained from these eggs were kept together, but the emerged adults were segregated by sex.

### Field Experiments

Preliminary field tests were conducted in Tsukuba (latitude 36°05'N, longitude 140°10'E) in the fall of 1992. Further evaluation of the synthetic attractant pheromone was carried out in Kumamoto (latitude 32°53'N, longitude 130°45'E) from September 7 to November 11, 1993.

The chemical lure (100 mg), incorporated into plastic pellets (4–5 mm in diameter) made of polyethylene–vinyl acetate, was placed in plastic cages (13 × 9.5 × 8 cm), set at 3 cm above the water surface of a hand-made, blue water-pan trap (16 cm × 35 cm ID). Insect baits were prepared with males (10 individuals) placed in plastic cages supplied with water and dry soybean seeds. Control and baited traps were randomly distributed around a soybean field inside Kyushu National Agricultural Experiment Station (Kyushu Noshi).

Kairomonal response of egg parasitoids to the synthetic attractant pheromone was investigated with cylindrical sticky traps made from two sticky plates (30 × 30 cm; Shin-Etsu Chemical Co., Tokyo) folded to form a cylinder (ca. 15 cm ID). Plastic pellets incorporated with the chemical lure (50 mg) were placed inside small plastic bottles (Fuji Flavor Co., Japan) and set on the top of the sticky cylinder traps. These traps were set on plastic plates placed randomly in the ground around a soybean field inside Kyushu Noshi. Number and sex of the egg parasitoids, *O. nezarae*, captured were determined under a binocular microscope. The population of the egg parasitoid was monitored in a nearby soybean field using a sticky suction trap system (Tokyo A&S Co., Tokyo).

### Statistical Analyses

Field capture data were transformed to log ( $x + 1$ ) before differences between means were tested for significance by the Tukey-Kramer honestly significant difference test at a 5% level. Throughout this paper, treatments labeled with the same letters are not significantly different.

## RESULTS AND DISCUSSION

### Identification of Sex Pheromone

GC-EAD analyses of the airborne volatiles collected from *R. clavatus* males (using female or male antenna as a sensing element) showed the occurrence of four EAD-active peaks, which appeared not only on the polar columns, DB-23 (Figure 1) and DB-wax, but also on a nonpolar HP-1 column. After separation on a silica gel column, the four compounds were recovered in the hexane–ether (95:5) fraction. Peak I has been previously identified as (*E*)-2-hexenyl hexanoate, the alarm pheromone of the bean bug (Leal and Kadosawa, 1992) and was found in the airborne volatiles of male and female bugs. The other three EAD-active peaks (II, III, and IV) were detected in the (airborne or whole-body) extracts of male bugs, but they were not present in the female extracts or in the foodstuff. Collection of volatiles from the same group of males for a whole day with foodstuff followed by one-day collection without foodstuff (and vice versa) revealed that the three compounds were released only by males provided with

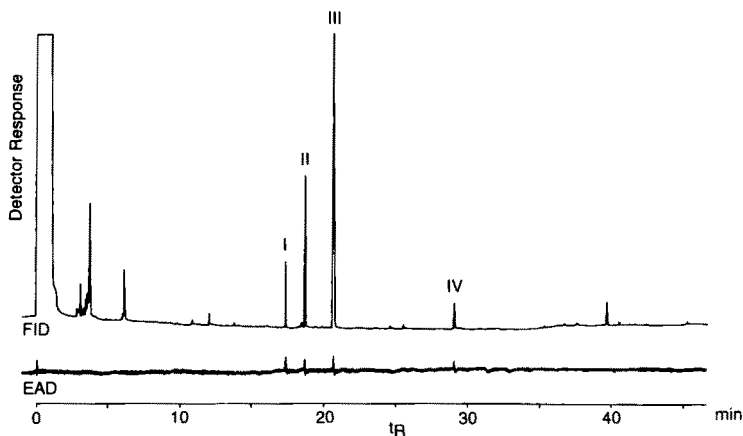


FIG. 1. Coupled GC-EAD response of female *R. clavatus* antenna to the airborne volatiles of male bugs separated on a DB-23 capillary column. The same response was detected with male antenna as a sensing element.

foodstuff. It is unknown, however, whether the bugs release the semiochemicals only when they are feeding. Compounds I, II, and III were not detected either by FID or EAD in the airborne extracts from starved males. These data suggested that compounds II, III, and IV were constituents of the attractant pheromone of *R. clavatus*.

EI-MS analyses of the male-specific, EAD-active compounds II, III, and IV gave their base peaks at  $m/z$  55, 97, and 89, respectively (Figure 2). Although the molecular ion of compound II was missed in EI measurements, it was confirmed to be 196 from the quasi-molecular ion obtained at  $m/z$  214 by chemical ionization with ammonia. Likewise, the molecular ions of compounds III and IV were corroborated to be  $m/z$  196 and 284, respectively.

Hydrogenation of II and III gave hexyl hexanoate, suggesting that these two compounds were isomers. Vapor-phase IR (Figure 3) of III gave a profile of a conjugated ester ( $\nu\text{C}=\text{O}$ , 1740 and  $\nu\text{C}=\text{C}$ , 1655  $\text{cm}^{-1}$ ). The profiles of II ( $\nu\text{C}=\text{O}$ , 1755  $\text{cm}^{-1}$ ) and IV ( $\nu\text{C}=\text{O}$ , 1751  $\text{cm}^{-1}$ ) indicate that these compounds were nonconjugated esters. The fact that the C—H stretching band of IV was predominant over the carbonyl stretching band suggested that this ester had at least one long carbon chain.

Alkaline hydrolysis of II gave 2-hexenol and 3-hexenoic acid, whereas the products of the hydrolysis of III were 2-hexenol and 2-hexenoic acid.

EI-MS of the DMDS adduct of II displayed a base peak at  $m/z$  89 along with the molecular ion  $\text{M}^+$  290, indicating that the unsaturation of the

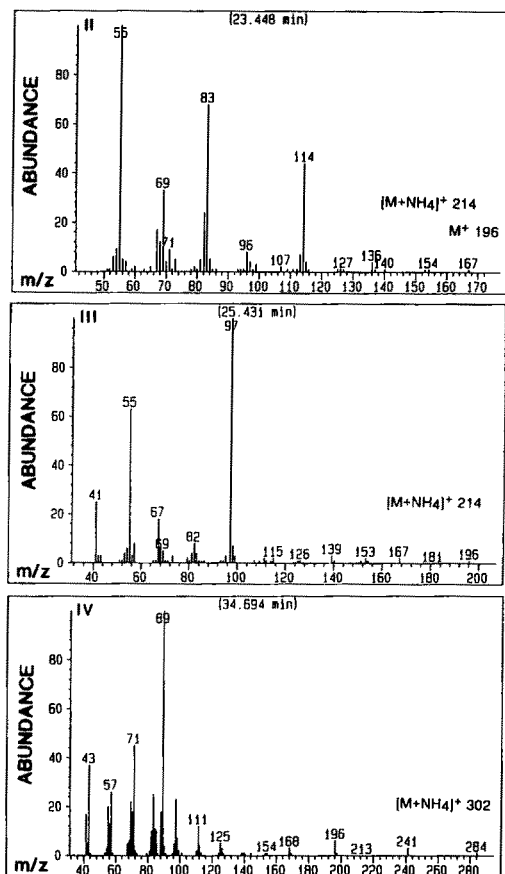


FIG. 2. Electron impact-mass spectra of the three constituents (II, III, and IV) of *R. clavatus* attractant pheromone. The quasi-molecular ions  $[M+NH_4]^+$  obtained by chemical ionization with ammonia are also displayed.

acid moiety of II was in position 3 [ $CH_3CH_2CHSMe^+$ ,  $m/z$  89;  $CH_3CH_2CH(SMe)CH(SMe)CH_2CO^+$ , 191(5%)]. It remained to be determined what the configuration of the two double bonds was. The IR spectrum had a band characteristic of the *E* configuration, which suggested that at least one of the double bonds would be in that configuration. Synthetic (*E*)-2-hexenyl (*Z*)-3-hexenoate gave the same retention times as the natural product on three different capillary columns and showed identical MS. On the other hand, (*E*)-2-hexenyl (*E*)-3-hexanoate had longer retention times on polar capillary

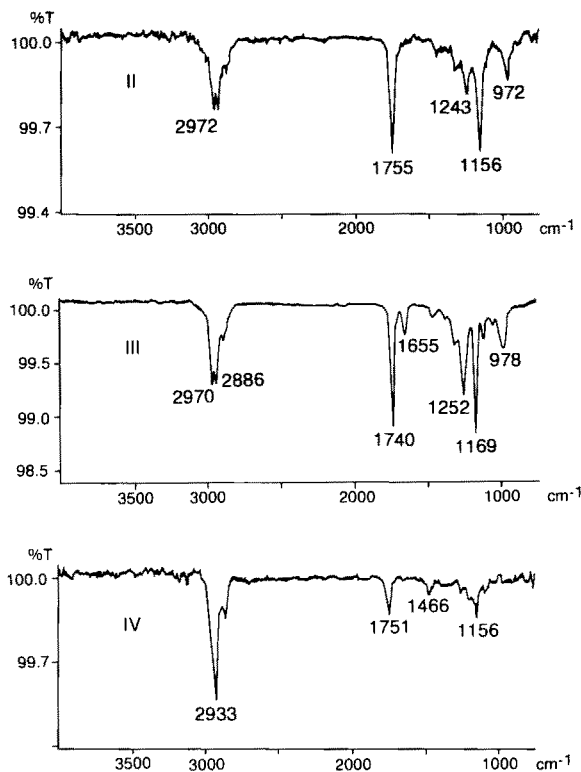


FIG. 3. Vapor-phase IR data for the three components (II, III, and IV) of the attractant pheromone system of *R. clavatus*.

columns and gave an MS with the base peak at  $m/z$  55, but having a different profile: 114 (72%), 83 (55), and 69 (50). Therefore, compound II was identified as (*E*)-2-hexenyl (*Z*)-3-hexenoate.

The partial DMDS derivative of peak III gave the base peak at  $m/z$  97 ( $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}=\text{CHCO}^+$ ), the second peak (75%) at  $m/z$  103 ( $\text{CH}_3\text{CH}_2\text{CH}_2\text{CHSMe}^+$ ) and the molecular ion at  $M^+$  290. These data and the occurrence of an *E* configuration band ( $978\text{ cm}^{-1}$ ) in the IR spectrum (Figure 3), suggested that compound III was (*E*)-2-hexenyl (*E*)-2-hexenoate. The synthetic ester was identical to the natural product in the retention times on three capillary columns and spectral data. Although (*Z*)-2-hexenyl (*E*)-2-hexenoate showed an MS profile very close to that of the natural product, it had a shorter retention time on polar capillary columns. Thus, compound III was identified as (*E*)-2-hexenyl (*E*)-2-hexenoate.



The facts that hydrolysis of IV gave myristyl alcohol, that IV did not undergo hydrogenation, and that the MS of IV had a base peak at  $m/z$  89 ( $M^+$  284), suggested that this compound was either myristyl *n*-butyrate or myristyl isobutyrate. These two esters were almost indistinguishable from their MS, but myristyl *n*-butyrate showed much longer retention times on polar capillary columns, whereas myristyl isobutyrate was identical to the natural product. Therefore, the attractant pheromone of *R. clavatus* was identified as a mixture of (*E*)-2-hexenyl (*Z*)-3-hexenoate, (*E*)-2-hexenyl (*E*)-2-hexenoate, and myristyl isobutyrate, in short E2HZ3H, E2HE2H, and MI. These three compounds were found in an average ratio of 1:5:1.

#### Field Evaluation of Attractant Pheromone

Preliminary experiments in Tsukuba indicated that the major component (E2HE2H) alone was inactive and that there was no significant difference between catches of *R. clavatus* in traps baited with males and those in traps using the synthetic attractant pheromone mixture (100 mg) as a lure. Further experiments in Kumamoto corroborated that the attractancy of the synthetic attractant pheromone (E2HZ3H, E2HE2H, and MI: 1:5:1; 100 mg) to *R. clavatus* was not significantly different from that of 10 caged males (Figure 4). In addition, the field results with synthetic chemicals showed that there was a significant difference in catches of male and female *R. clavatus*.

Despite the fact that in the field male and female adults are attracted to the male-released pheromone of *Nezara viridula* (Harris and Todd, 1980; Aldrich et al., 1987), the semiochemicals involved are often referred to as "sex pheromones," probably because a correlation between attraction and mating was

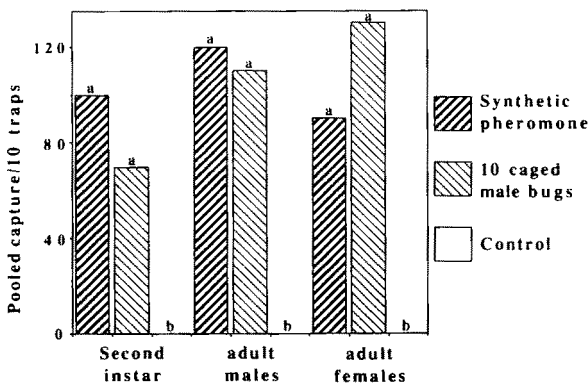


FIG. 4. Captures of second instars and male and female adults of *R. clavatus* in traps baited with synthetic attractant pheromone and 10 caged males (control = no lure).

found in the laboratory (Borges, 1987). Recently, it has been reported that the male-released volatiles act as a sex pheromone mediating mate finding and that aggregation observed in the field may be mediated by other factors (Brézot et al., 1993). The male-released pheromone system of *R. clavatus*, however, was confirmed with synthetic chemicals (as opposed to live insects or volatiles collected from them) to be an attractant pheromone.

### *Ecological Significance of Semiochemicals to Second Instars*

Field tests showed that traps loaded with the synthetic mixture (E2HZ3H, E2HE2H, and MI; 1:5:1; 100 mg) were equally effective in attracting second-instar nymphs of the bean bug as male-baited traps. The significance of these chemicals to the ecology of *R. clavatus* is not limited to their involvement in the aggregation of male and female bugs, which is relevant for reproduction of the bean bug.

As this species lays eggs on host and nonhost plants, it is important to the first feeding stage (second-instar) nymphs to have a signal that enables them to locate a food source at the lowest energy cost. The facts that the attractant pheromone is only released by feeding males and specifically attracts the second instars (the other stages are not attracted) substantiate its involvement in food source location. Whether the later stages (third, fourth, and fifth instars) lose the ability to respond to these semiochemicals is not known, but nymphs in these stages may not need the information as they would have already reached the host food plant, or they would have starved to death.

GC-MS analyses revealed that none of these semiochemicals is present in the whole-body extracts of nymphs. In the extracts of first, second, and third instars only 2-(*E*)-octenal was detected, whereas fourth and fifth instars had a second component tentatively identified as 4-oxo-2-hexenal. These chemicals may be involved in the alarm and defensive systems of *R. clavatus* nymphs.

The use by the second instars of semiochemicals emitted by male bugs to locate host plants is an important feature of the biology of the bean bug. This is novel in the chemical ecology of hemipterans and seems to have evolved in response to selective pressure from the parasitoid. Due to the lack of terminology, we use the term "aggregation pheromone" to refer to the use of these semiochemicals in this unique context.

### *Kairomonal Activity of Attractant Pheromone*

The possible attraction of egg parasitoids to the host's attractant pheromone was investigated in the field. In order to rule out the possibility that stimuli from bugs initially captured were used by egg parasitoids as cues for host location, we used cylindrical sticky traps in which the bean bug could not be captured. This type of trap, however, could catch the most effective egg parasitoid of the

bean bug in Kumamoto (Japan), *O. nezarae* Ishii (Hymenoptera: Encyrtidae). Experiments conducted from September 21 to October 17, 1993, revealed (Figure 5) that, although the numbers of male and female *O. nezarae* captured in sticky suction trap were not significantly different, significantly more female parasitoids were caught in the traps baited with the synthetic attractant pheromone (E2HZ3H, E2HE2H, and MI; 1:5:1; 50 mg) than in control traps. Catches of female *O. nezarae* were also significantly higher (5.5-fold) in the traps baited with the attractant pheromone than in the sticky suction trapping system. The synthetic bait was not attractive to male *O. nezarae*.

These findings confirm that the attractant pheromone of *R. clavatus* is utilized by females of the egg parasitoid *O. nezarae* as a long-range attractant kairomone. It is also likely that the bean bug has developed the strategy to lay eggs on nonhost plants to escape egg parasitoids. *O. nezarae* has evolved the ability to respond to semiochemicals produced by males that allow second instars to locate host food plants. Because the semiochemicals are released only by fed (or feeding) males, eggs laid on nonhost plants are probably less prone to attack by *O. nezarae* than those laid on host plants, unless the parasitoid can also detect other stimuli associated with females, hosts, or eggs.

Parasitoids have been reported to respond (as reviewed in Whitmann, 1988) to the host's body odor; the sex, epideitic, and aggregation pheromone; salivary constituents; excretory products; webbing; honeydew; body scales; and eggs. Recently, it has been reported that the egg parasitoid *Trissolcus basal* utilizes a defensive substance produced by its host bug, *Nezara viridula*, as a long-range attractant kairomone (Mattiacci et al., 1993). The experimental evidence,

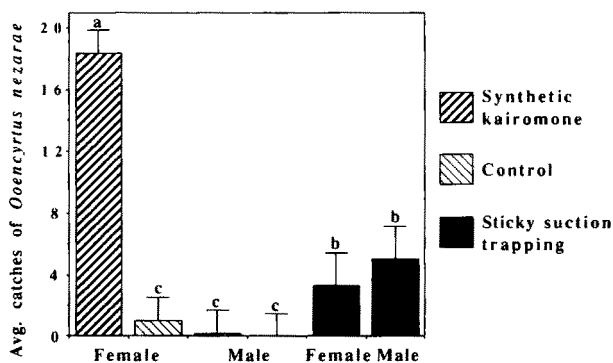


FIG. 5. Response of the egg parasitoid *O. nezarae* to the attractant pheromone of the host *R. clavatus* compared to catches of the egg parasitoid in a sticky suction trapping system.

however, for the long-range attraction is based only on the fact that female *T. basalis* spend significantly more time in the arm of an Y-olfactometer baited with the defensive substance. Attraction of parasitoids to the pheromone of host bugs is not novel. In fact, it has been previously reported that an egg parasitoid, *Telonomus* sp., was attracted to the male-released pheromone of the predatory spined soldier bug, *Podisus maculiventris* (Aldrich et al., 1984).

Field observations demonstrated that significantly more eggs of *Piezodorus hybneri* have been parasitized by *Telonomus triptus* when egg masses were placed close to caged adult bugs than egg masses alone (Higuchi, unpublished), leading to the conclusion that egg parasitoids are attracted to the vicinity of the eggs in response to a cue emanating from the host. Nevertheless, egg parasitoids usually appear later in the field than their hosts, and thus the host's first-laid eggs usually escape parasitoid attack (Higuchi, 1993). Therefore, the use of kairomones to synchronize the parasitoid population at the beginning of the host flight season may pave the way for the development of invaluable tools in integrated pest management programs.

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