Individual variation in the amounts of pheromone components in the male bean bug, *Riptortus pedestris*1 (Heteroptera: Alydidae) and its attractiveness to the same species

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Abstract

The individual variation of *R. pedestris* males and females in their attractiveness to the same species and the amounts of five pheromone components were examined in field experiments and GC-MS analysis, respectively. The number of adults attracted by a single male varied greatly among the individuals tested. The number of adults attracted was 0.92 ± 0.12 (mean ± SE/day). Of 64 males examined individually, four attracted three or more adults per day, while 12 males attracted only 0.5 adults or fewer per day and 14 individuals attracted no adults. The five pheromone components also differed greatly among those males. As for tetradecyl isobutyrate (14:iBu), which is thought to be an essential component of the pheromone, it was apparently detected from 46 males while eight had only a low amount of 14:iBu and ten had no 14:iBu. Some of the males had 14:iBu without having (E)-2-hexenyl (E)-2-hexenoate (E2-6:E2Hx) and (E)-2-hexenyl (Z)-3-hexenoate (E2-6:Z3Hx), which are synergistic components of the pheromone, and the rest had E2-6:E2Hx and/or E2-6:Z3Hx without having 14:iBu. The amount of 14:iBu or octadecyl isobutyrate (the fourth pheromone component) showed a positive and significant co-relationship with the number of attracted adults. Males with a well-developed fat-body tended to have more 14:iBu and to attract more adults than those with an undeveloped fat-body. Most of the females tested individually did not attract conspecific adults, and had only (E)-2-hexenyl hexanoate, the fifth component of the pheromone.

Key words: *Riptortus pedestris*; aggregation pheromone; tetradecyl isobutyrate; soybean

INTRODUCTION

Adult males of the bean bug, *Riptortus pedestris* (*R. clavatus*) (Heteroptera: Alydidae), which is one of the most important pests of soybean production in Japan, attract both adult sexes (Numata et al., 1990) and conspecific nymphs (Leal et al., 1995). A three-component mixture of tetradecyl isobutyrate (14:iBu), (E)-2-hexenyl (E)-2-hexenoate (E2-6:E2Hx) and (E)-2-hexenyl (Z)-3-hexenoate (E2-6:Z3Hx), has been identified as an aggregation pheromone of this bug (Leal et al., 1995). The synthetic mixture of the components (14:iBu : E2-6:E2Hx : E2-6:Z3Hx=1 : 5 : 1) is commercially available as an attractant in traps for monitoring *R. pedestris* (Fuji Flavor Co., Ltd.). Octadecyl isobutyrate (18:iBu) and (E)-2-hexenyl hexanoate (E2-6:Hx) are additional pheromone components of *R. pedestris* (Yasuda et al., 2007a, b).

The pheromone component E2-6:Z3Hx attracts the egg parasitoid, *Ooencyrtus nezarae*, which is one of the most important natural enemies of *R. pedestris* (Mizutani et al., 1997) in field experiments; however, the attractiveness of male adults of *R. pedestris* to *O. nezarae* is not clear (Mizutani, 2001). The synthetic mixture of the three components and E2-6:E2Hx attracts *Piezodorus hybneri*, which is another important pest bug of soybeans (Endo et al., 2003), but *R. pedestris* males do not attract *P. hybneri* (Mizutani et al., unpublished data). The study of *R. pedestris* pheromone was mainly conducted using a synthetic mixture and/or one of its components because the pheromone components had been identified (Leal et al., 1995);
however, there has been only one report about the attractiveness of intact male adults (Numata et al., 1990). The attractiveness of male adults should be reexamined in detail to clarify the reason for the difference in the attractiveness between synthetic chemicals and male adults.

In this study, we observed individual variation in the attractiveness and pheromone composition of male adults of *R. pedestris*. We discuss here the relationship between the attractiveness of males and their amount of pheromone components and/or the conditions of their internal organs.

**MATERIALS AND METHODS**

**Insects.** *R. pedestris* adults were collected from fields in Tsukuba and reared in the laboratory on soybean seeds, red clover (*Trifolium pratense*) seeds, and water, and maintained at 25°C with a photoperiod of 16L8D. Newly emerged adult males and females were separated into different cages, and virgin adults (>7 days old in preliminary field experiment, 12–14 days old in field experiments 1 and 2) were used as attractants in each experiment.

**Preliminary field experiment.** Preliminary field experiments to check the individual attractiveness of males were conducted in a grassy field with rows of coniferous trees at the National Agricultural Research Center (NARC) in Tsukuba, Japan. A male was put into a steel-mesh cage (25×20×30 cm) with dried soybean seeds and water. Five cages were set on the ground linearly at about 10-m intervals from August 5th to 10th (Experiment P-1) and from August 10th to 15th (Experiment P-2) in 2004. The cages were set at 0900, and observations were started at 1300. The numbers of wild female and male adults observed on the cage were counted at two-hour intervals each day from 0500 to 2100 in Experiment P-1 and from 0700 to 1900 in Experiment P-2. The cages were set at 0900, and observations were started at 1300. The numbers of wild female and male adults observed on the cage were counted at two-hour intervals each day from 0500 to 2100 in Experiment P-1 and from 0700 to 1900 in Experiment P-2. The cages were rotated in turn at 1100 each day. Observations were finished at 1000 on August 10th (Experiment P-1) and 0900 on August 15th (Experiment P-2). Ten males were tested in this experiment.

**Field experiment 1.** Field experiments to clarify individual differences of attractiveness and the amount of pheromone components contained in males were conducted in the field used in the preliminary field experiment. A male was put into a stainless steel-mesh cage (10 cm outside diameter×8 cm height, Fig. 1B) with a dried soybean seed and water. The soybean seed was partly covered with aluminum foil to prevent sucking by individuals outside the cage. The cage was fixed in front of a plastic plate (18×25.5 cm) at a height of ca. 1 m from the ground (Fig. 1A). Ten or twelve traps were set at about 10-m intervals in a grassy field. The traps were set at 1800, and the observations were started at 0900 the next day. The numbers of female and male adults observed on the cage and the plate were counted, and the adults were collected at 0900, 1300, and 1700 each day. After the experiments, males used as attractants were separately extracted with hexane and analyzed as described below. This experiment was conducted from August 2nd to 4th, from August 15th to 17th, from September 7th to 9th, and from October 5th to 7th in 2005. Forty-four males were tested in this experiment.

**Field experiment 2.** Field experiments to confirm the evidence of attraction and the amount of pheromone components contained in female individuals were conducted in the grassy field in the same way as in field experiment 1. In this experiment, male adults were used as a positive control to check the attractiveness of females. A female or male was put into the cage, which was fixed to the plastic plate in the same way as in field experiment 1. A total of 8–12 traps (4–6 males and 4–6 females) were set randomly at about 10-m intervals in the field. The numbers of female and male adults observed on the cage and the trap were counted.
and collected. The females and males used as attractants were individually extracted and analyzed in the same way as in field experiment 1. This experiment was conducted from August 9th to 11th, from August 31st to September 2nd, from September 13th to 15th, and from September 28th to 30th in 2005. One female died during the experimental period and was excluded from the analysis. Nineteen females and 20 males were tested in this experiment.

**Analyses of pheromone components from whole-body extracts of R. pedestris.** Intact male or female R. pedestris used as attractants in field experiments 1 and 2 were separately dipped in 2 ml of hexane for 1 h at room temperature. The hexane solution of n-hexadecane (2 μg) was added as an internal standard in each sample. The extract was decanted from the male or female body into a glass vial. The residual body was rinsed with 1 ml of hexane, and the rinse was added to the extract. The extracts were stored at −20°C until gas chromatography–mass spectrometry (GC-MS) analysis. The extracts of hexane solution were concentrated to ca. 100-μl volumes just before GC-MS analysis.

Quantitative GC-MS analyses were done on an Agilent 6890N GC with an HP-INNOWax column (30 m×0.25 mm inside diameter×0.25 μm film thickness) by on-column injection combined with an Agilent 5973Network Mass Selective Detector using an internal-standard method. Injection was made directly onto the capillary column through the cool-column injector, and the injector temperature was programmed to oven temperature plus 3°C. Helium was used as the carrier gas and the flow rate was 1.0 ml/min in the constant flow mode. The initial GC oven temperature was 50°C (1 min hold), increased to 180°C at 10°C min⁻¹, increased again to 240°C at 20°C min⁻¹, and was then held for 5 min.

**Authentic chemicals for quantitative GC-MS analyses.** Tetradecyl isobutyrate (14:iBu) and octadecyl isobutyrate (18:iBu) were obtained from 1-tetradecanol and 1-octadecanol, respectively, reacted with isobutyric anhydride and pyridine. (E)-2-Hexenyl (E)-2-hexenoate (E2-6:E2Hx) and (E)-2-hexenyl (Z)-3-hexenoate (E2-6:Z3Hx) were obtained from the Fuji Flavor Co., Ltd., and, (E)-2-hexenyl hexanoate (E2-6:Hx) and n-hexadecane were purchased from the Tokyo Chemical Industry Co., Ltd., Japan.

**Conditions of internal organs of R. pedestris males used as attractants.** After extraction, the conditions of internal organs such as food in stomach, fat-body development, and erection fluid reservoir (EFR) development were observed by dissection of the R. pedestris males used as attractants. The conditions of internal organs were classified into four stages (Fig. 2).

## RESULTS

### Attractiveness of male individuals

The number of R. pedestris adults observed on the male-baited traps varied greatly among the traps (Fig. 3, preliminary field experiment). In traps No. 4 and 6, some adults were continuously

<table>
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<tr>
<th>Organs</th>
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<td>Fat-body Development</td>
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<td>EFR Development</td>
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Fig. 2. Classification of conditions of several internal organs of Riptortus pedestris males. The characteristics of the organs were observed by dissection. *EFR: erection fluid reservoir.*
observed during the experimental period. On the other hand, in traps No. 8 and 10, an adult was observed only once or several times during the experimental period.

The mean number of adults attracted per day was 0.97 ± 0.15 (mean ± SE), and there was a great difference in the numbers attracted among males in field experiment 1 (Fig. 4). Four of the males (No. M-18, M-21, M-31, and M-36) attracted ca. 3 adults or more per day on average, while 8 of the...
males (No. M-7, M-8, M-20, M-27, M-38, M-39, M-40, and M-42) attracted only 0.5 adults or fewer per day on average and 9 of the males (No. M-2, M-4, M-6, M-10, M-25, M-26, M-28, M-29, and M-30) attracted no adults.

**Attractiveness of female individuals**

The mean number of adults attracted per day was 0.80 ± 0.16 (mean ± SE) in males and 0.12 ± 0.06 in females in field experiment 2. Four females attracted 7 adults (5 males and 1 female, 1 unidentified). One female attracted only males (3 males).

**Amount of pheromone components from the whole-body extract**

The hexane extracts of 64 males used as attractants in field experiments 1 and 2 were individually analyzed with GC-MS. The amount of pheromone components (14:iBu, E2-6:E2Hx, E2-6:Z3Hx, 18:iBu, and E2-6:Hx) varied greatly among the extracts derived from each male (Fig. 5). In 46 extracts, 14:iBu, which is the essential attractive component of the pheromone, was detected. In 8 extracts (No. M-4, M-35, M-37, M-38, M-39, M-40, M-57, and M-64), 14:iBu was detected only slightly (0.1 µg or less). In 10 extracts (No. M-2, M-26, M-28, M-29, M-45, M-50, M-51, M-52, M-53, and M-59), 14:iBu was not detected. In some extracts (No. M-26, M-28, M-29, M-50, M-51, M-53 and M-59), only the synergistic components of the pheromone (E2-6:E2Hx, E2-6:Z3Hx, 18:iBu and E2-6:Hx) were detected. In No. M-2 and M-52 extracts, pheromone components were hardly detected, and in No. M-45, they were not detected at all. There was no relationship of the amounts among components, and the ratio of pheromone components varied greatly among extracts.

The hexane extracts of 19 females used as attractants in field experiment 2 were separately analyzed with GC-MS. In 17 extracts, only E2-6:Hx was detected, and the amount of E2-6:Hx varied greatly among extracts. In 2 extracts, no pheromone components were detected.

**Relationship between the amounts of pheromone components and the number of captured adults**

The amounts of pheromone components in whole-body extracts of each male were compared with mean numbers of captured adults in the trap per day (n=64). The relationship between the amount of 14:iBu or 18:iBu and mean numbers of attracted adults (Fig. 6A, B) was positively correlated ($r^2=0.266$ and 0.216, respectively, $p<0.0001$); however, there were no significant regressions between E2-6:E2Hx, E2-6:Z3Hx, or E2-6:Hx and the mean numbers of attracted adults (Fig. 6C, D, E). Furthermore, E2-6:E2Hx was removed from the set of predictor variables of the equation in stepwise regression analysis (14:iBu was locked into the model, 0.05 for the $P$-to-enter value and 0.05 for the $P$-to-remove) using JMP version 6.02J (SAS Institute Japan Inc.).
Conditions of internal organs of males used as attractant

Among the males used as attractants in field experiments 1 and 2, ca. 60% of males had food in their stomach. About 85% and 95% of males had a well-developed fat-body and EFR, respectively. There was no relationship between the amount of 14:iBu in the extracts or the mean number of attracted adults per day and the amount of food in the stomach of the males (Fig. 7). On the other hand, males with a well-developed fat-body showed a tendency to have more 14:iBu than those with an undeveloped fat-body. Furthermore, males with a well-developed fat-body showed a tendency to attract more adults than those with an undeveloped fat-body (Fig. 7). Males with well-developed EFR showed the same tendency as the fat-body (Fig. 7); however, there was no relationship between the amount of 14:iBu in the extracts and the degree of EFR development of the males.

DISCUSSION

Our results in the field experiments indicate that R. pedestris males have great individual variation in attractiveness, and the amounts of pheromone components and their ratios varied greatly among the males tested. There was a correlation between the degree of attractiveness and the amount of 14:iBu, which is an essential component of the pheromone. In field experiments using one of the synthetic pheromone components as an attractant, the number of bugs attracted to 14:iBu increased in proportion to the 14:iBu dose (Huh et al., 2005). These results strongly suggest that the individual variation in the attractiveness of R. pedestris males was caused by individual variation in the amount of 14:iBu; however, it cannot explain all of the individual variation in R. pedestris males, as shown in Fig. 6. Further experiments are necessary to clarify the relation between the amount and/or the

Fig. 6. Relationship between the amounts of pheromone components (A: 14:iBu, B: 18:iBu, C: E2-6:Hx, D: E2-6:Z3Hx, E: E2-6:E2Hx) of whole-body extracts of caged R. pedestris males used as attractants in field experiments 1 and 2 and the number of attracted adults per day to males in Tsukuba from August to October 2005.
ratio of the pheromone components and individual variation in the attractiveness of males.

In the present study, the amount of 14:iBu in the extracts or the mean number of attracted adults per day depended upon the degree of fat-body development in males. In *R. pedestris*, males provided with food only released three pheromone components (14:iBu, E2-6:E2Hx and E2-6:Z3Hx) (Leal et al., 1995) and such males attracted adults and nymphs (Morishima et al., 2005). Morishima et al. (2005) suggested that pheromone release in males was affected by their fat-body development. These results suggest that the production of 14:iBu is closely related to fat-body development.

In some predatory stink bugs, feeding is the key to the production of the attractant pheromone in males (*Oplomus dichrous*, Aldrich and Lusby, 1986 etc.). Also, in the brown-winged green bug *Plautia stali* (=*P. crossota stali*) Scott (Heteroptera: Pentatomidae), attraction of male and female adults by the pheromone is considered to be effective in the exploitation of food resources (Shiga and Moriya, 1989). A similar strategy was proposed in the pheromone system of *Leptoglossus phyllopus* (Heteroptera: Coreidae) (Aldrich et al., 1976) and spined soldier bug *Podisus maculiventris* (Heteroptera: Pentatomidae) (Aldrich et al., 1984; Sant’Ana et al., 1997; Shetty and Hough-Goldstein, 1998). In *R. pedestris*, it is considered that the attraction of male and female adults by pheromone is related to food exploitation (Yasuda et al., 2007b). The relationship between the amount of 14:iBu and the degree of fat-body development of males in the present study supported the hypothesis that the release of the pheromone might play a key role in food exploitation.

Some of the males had only E2-6:E2Hx and/or E2-6:Z3Hx without having 14:iBu, which is an essential component of the pheromone in relation to its attractiveness (Endo et al., 2005; Huh et al., 2005). In field experiments that used synthetic pheromone components as attractants, the number of bugs attracted to 14:iBu was positively proportional to the 14:iBu dose (Huh et al., 2005). On the other hand, the addition of E2-6:E2Hx and/or E2-6:Z3Hx resulted in significantly higher attractiveness than with 14:iBu alone (Endo et al., 2005; Huh et al., 2005); however, the amount of E2-6:E2Hx or E2-6:Z3Hx added to 14:iBu did not enhance the attraction (Endo et al., 2005; Mizutani et al., unpublished data). These results suggest that E2-6:E2Hx and E2-6:Z3Hx have a different function from 14:iBu in the attraction of conspecific individuals of *R. pedestris*. In the southern green stink bug, *Nezara viridula* (Heteroptera: Pentatomidae), individual variation was observed in the ratio of bisabolene epoxide isomers in the male-produced pheromone and it is concluded that further behavioral investigations are needed to ascertain the functional significance to the mating system of the components that make up the pheromone blend (Ryan et al., 1995). In *R. pedestris*, further observations are also necessary to understand the real roles of E2-6:E2Hx and E2-
6:Z3Hx in the attractiveness of males.

In the present study, 75% of tested males (15/20) attracted 42 adults (16 males, 21 females, 5 unidentified) and 6 nymphs, while ca. 20% of tested females (4/19) attracted 7 adults (5 males, 1 female, 1 unidentified) and no nymphs. Females had only E2-6:Hx, one of the male pheromone components. Jung and Im (2003) reported that virgin females of *R. pedestris* have a specific sex attractant; however, since our results indicated that the attractiveness of *R. pedestris* females is very weak compared with that of males, the sex attractant of females, if there is one, must be different from the aggregation pheromone released by males.

**REFERENCES**


