INTRODUCTION

Adult males of the bean bug *Riptortus pedestris* (= *R. clavatus*, Kikuhara, 2005) (Heteroptera: Alydidae) are one of the most important soybean pests in Japan. The adult male releases a pheromone that attracts both sexes of conspecific adults and nymphs (Numata et al., 1990). Five components, tetradecyl isobutyrate (14:iBu), octadecyl isobutyrate (18:iBu), (E)-2-hexenyl (E)-2-hexenoate (E2-6:E2Hx), (E)-2-hexenyl (Z)-3-hexenoate (E2-6:Z3Hx) and (E)-2-hexenyl hexanoate (E2-6:Hx) have been identified as composing the pheromone system of this bug (Leal et al., 1995; Endo et al., 2005; Yasuda et al., 2007a, b). This pheromone system has often been referred to as an aggregation pheromone because the pheromone attracts not only the opposite sex (female adults), but also male adults and nymphs (Leal et al., 1995; Endo et al., 2005; Yasuda et al., 2007a, b).

The functions of the pheromones emitted by male adults of heteropterans, including *R. pedestris*, remain unclear. In the southern green stink bug, *Nezara viridula* (Heteroptera: Pentatomidae), the male-produced pheromone was considered to be a sex attractant pheromone (Brennan et al., 1977; Baker et al., 1987; Borges et al., 1987; Aldrich et al., 1993), because only sexually mature non-diapausing males were attractive and females in reproductive diapause were unresponsive to the males (Brennan et al., 1977). However, in the brown-winged green bug *Plautia crosota stali* (Heteroptera: Pentatomidae) (Shiga and Moriya, 2007), this pheromone system has not been tested.
1989) and the predatory spined soldier bug *Podisus maculiventris* (Heteroptera: Pentatomidae) (Aldrich et al., 1984; Sant’Ana et al., 1997), the pheromone was considered to play a key role in food exploitation because adults with depleted nutritional reserves were attracted to males in *P. crossota stali* (Shiga and Moriya, 1989), and only starved females and males showed positive anemotaxis toward a synthetic pheromone in *P. maculiventris* (Shetty and Hough-Goldstein, 1998).

In *R. pedestris*, the pheromone system has been considered to have a function in food exploitation rather than a sex-related function (Yasuda et al., 2007b). Morishima et al. (2005) showed the males allowed to take food attracted a significantly larger number of conspecific adults than those not allowed to take food. In addition, according to Wada et al. (1997), males reared in a short photoperiod entered diapause without exhibiting sexual behavior, but attracted significant numbers of adults in the field. However, no pheromone components were detected in diapausing males, which were attracted by the male bait in autumn, while two pheromone components (E2-6:E2Hx and E2-6:Z3Hx) were found in non-diapausing males attracted in summer (Mizutani et al., 2008). This suggested some influence of photoperiod on pheromone production of *R. pedestris*.

In this study, we examined the attractiveness to field adults and the pheromone production of males reared in different photoperiods. The field experiments were conducted in two different locations, where previous field studies (Wada et al., 1997) on the attractiveness of males reared in short photoperiods had been carried out. In addition, we examined pheromone production and the development of internal organs in relation to the ages of males kept under different photoperiods in laboratory conditions. Finally, we discuss the possible functions of the pheromone system of *R. pedestris*.

**MATERIALS AND METHODS**

**Insects.** *Riptortus pedestris* adults were collected as the initial population of a stock culture in soybean fields at Tsukuba in 2001 and reared in a laboratory on soybean seeds, red clover (*Trifolium pretense*) seeds, and water at 25°C under 16L8D photoperiod. Several times a year, field-collected males and females were added to the stock culture. For the experiments, eggs were collected from the stock culture and hatched nymphs were reared on the same diet at 25°C under two different photoperiods: 16L8D (long photoperiod) and 10L14D (short photoperiod). The newly emerged adult males were separated into plastic cups (12 cm outside diameter × 5 cm height) and kept until use in field experiments.

### Attractiveness of males reared in long and short photoperiods in fields.

Field experiments to check the attractiveness of males reared in different photoperiods were conducted twice at two different locations. The first experiment was carried out at the National Agricultural Research Center (NARC) in Tsukuba (36°01′N, 140°05′E), Japan in mid-October, 2006. Virgin males (7–12 days old) were used as bait (attractants to field insects) in this experiment. A male was confined in a stainless steel-mesh cage (10 cm outside diameter × 8 cm height) with a soybean seed and water (male trap). The soybean seed was partly covered with aluminum foil to prevent feeding by field *R. pedestris* outside of the cage, which would be attracted to the trap. The cage was placed in front of a plastic plate (18×25.5 cm) at a height of ca. 1 m above the ground (see Mizutani et al., 2007). Twelve traps baited with males reared in the long photoperiod (6 traps) and short photoperiod (6 traps) were set randomly at about 10-m intervals in a grassy field. In another grassy field, ten traps (5 traps baited with males reared in the long photoperiod and 5 with males reared in the short photoperiod) were set in the same way. The traps were set at 1130 on 10 October, 2006. Thereafter, we observed field adults attracted to the trap were counted at 1600 on the first day and from the second day at 0930, 1300, and 1730 until 1300 on 13 October.

The second experiment was carried out in two grassy fields of the National Agricultural Research Center for Kyushu Okinawa Region (KONARC) in Kumamoto (32°52′N, 130°44′E), Japan in late October, 2006. Ten traps (5 traps baited with males reared in the long photoperiod and 5 with males reared in the short photoperiod) were set randomly in line at about 10-m intervals in each of the grassy fields. The traps were set at 1630 on 17 October. The numbers of female and male adults observed on each trap were counted at 0900, 1430, 1630 and 1730 on 18 Octo-
ber, 0930, 1200 and 1330 on 19 October, and 1330 and 1600 on 20 October, 2006.

After the field experiments, the contents of the pheromone components and status of the internal organs of bait males were examined in both the first and second experiments, as described below.

Contents of pheromone components and status of internal organs in bait males in field experiments. Intact males used as attractants were separately dipped in 2 mL of hexane for 1 h at room temperature. The hexane solution of hexadecane (1 µg) was added as an internal standard in each sample. The extract was decanted from the male 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 the gas chromatography-mass spectrometry (GC-MS) described below.

After extraction, we dissected the males to observe the conditions of the internal organs, examining the food residue in the stomach, fat-body development, and erection fluid reservoir (EFR) development. The conditions of the internal organs were classified into four categories (see Mizutani et al., 2007). According to Numata and Kobayashi (1989), the size of the EFR can be used as an index for the diapause status of the male.

Contents of pheromone components and development of internal organs in relation to the ages of males reared under different photoperiods in the laboratory. The contents of five pheromone components (14:iBu, 18:iBu, E2-6:E2Hx, E2-6:Z3Hx and E2-6:Hx) were compared among males reared in short and long photoperiods in the laboratory. Extracts of unmated males at 0, 3, 6, 9 and 12 days after emergence were analyzed in the same way as in field experiments. After extraction, the conditions of the internal organs were observed in the same way as in the field experiments.

Chemical analyses of pheromone components from whole-body extracts of R. pedestris males. The extracts of hexane solution were concentrated to ca. 100-µL volumes under reduced pressure below 30°C just before GC-MS analysis. Quantitative GC-MS analyses were performed on an Agilent 6890N GC with an HP-INNOWax column (30 m×0.25 mm inside diameter×0.25 µm film thickness) by splitless injection combined with an Agilent 5975i Network Mass Selective Detector using an internal-standard method. Injection was made in the splitless mode in the split/splitless injector using an Agilent 7688 series automatic liquid sampler, and the injector temperature was 230°C. Helium was used as the carrier gas and the flow rate was 1.0 mL/min in constant flow mode. The initial GC oven temperature was 50°C (1 min hold), and was then increased to 180°C at 10°C min⁻¹, increased again to 240°C at 20°C min⁻¹, and then held for 5 min.

Authentic chemicals for quantitative GC-MS analyses. 14:iBu and 18:iBu were obtained from 1-tetradecanol and 1-octadecanol, respectively, reacted with isobutyric anhydride and pyridine, and purified by column chromatography. Column chromatography on silica gel (ca. 5 g SiO₂, particle size: 75–150 µm, Wako gel C-200, Wako Pure Chem. Ind., Ltd., Osaka, Japan) was conducted. The components were successively eluted with hexane and 5% ether in hexane. These esters were eluted with 5% ether in hexane and 95% pure by GC-MS analysis. E2-6:E2Hx (91.7% pure) and E2-6:Z3Hx (88.6% pure) were obtained from Fuji Flavor Co. Ltd., and E2-6:Hx (95% pure) and hexadecane (98% pure) were purchased from Tokyo Chemical Industry Co., Ltd., Japan.

RESULTS

Attractiveness of males reared under long and short photoperiods in field experiments

About half of the males reared under the long photoperiod (5 of 11 males in the experiment conducted in NARC and 4 of 10 in KONARC) attracted field adults of R. pedestris. The number of adults attracted (day/trap) was 0.14±0.06 in NARC and 0.22±0.12 in KONARC (mean±SE, Table 1). On the other hand, no R. pedestris adults were attracted by traps baited with males reared under the short photoperiod (Table 1).

Content of pheromone components and status of internal organs in male adults used as attractants in field experiments

In the extracts of most males reared under the long photoperiod, 14:iBu, which is an essential component of the pheromone, was detected (Table 2). The mean content of 14:iBu was 0.43 µg in NARC and 1.58 µg in KONARC. More than 50% of males in which 14:iBu was detected (5 of 8 males in NARC and 4 of 7 males in KONARC)
attracted *R. pedestris* adults. Even among males reared under the long photoperiod, no field *R. pedestris* adult was attracted by males in which 14:iBu were not detected (3 males in NARC and KONARC, respectively). In almost all males (8/8 in NARC, 6/7 in KONARC) that had 14:iBu, 18:iBu, which is the synergistic component of the pheromone, was detected. E2-6:E2Hx, E2-6:Z3Hx and E2-6:Hx were detected in the extracts of almost all males reared under the long photoperiod. Males reared in the long photoperiod had well-swollen or swollen EFRs (Table 3).

On the other hand, 4 pheromone components (14:iBu, 18:iBu, E2-6:E2Hx and E2-6:Z3Hx) were not detected at all in extracts from males reared under the short photoperiod with the exception of a male in NARC in which E2-6:E2Hx was slightly detected (Table 2). E2-6:Hx was detected in extracts from all males. The males reared under the short photoperiod had immature (slightly swollen or unchanged) EFRs, which indicated that the males were under reproductive diapause (Table 3).

With regard to the amount of food residue in the stomach and degree of fat-body development, there were no clear differences between males reared under the two different photoperiods (Table 3).

### Contents of pheromone components and development of some internal organs in relation to age of males reared under different photoperiods in the laboratory

Pheromone components were frequently detected in extracts from males reared under the long photoperiod (Table 4). The number of males in which three components (14:iBu, 18:iBu and E2-6:Z3Hx) were detected increased with increasing adult age. E2-6:Hx was detected in almost all extracts from males (19/20) at any adult age. In males reared under the long photoperiod, EFRs developed by approximately 6 days after adult emergence (Table 5). All 12-day-old males had well-swollen or swollen EFRs.

### Table 1. Attractiveness of *Riptortus pedestris* males reared under long and short photoperiods in field experiments conducted in Tsukuba (NARC) and Kumamoto (KONARC) in 2006

<table>
<thead>
<tr>
<th>Place</th>
<th>Photoperioda</th>
<th>No. of male traps</th>
<th>No. of traps attracting adultsb</th>
<th>Daily no. of adults attracted (mean±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NARC</td>
<td>Long</td>
<td>11</td>
<td>5</td>
<td>0.14±0.06*</td>
</tr>
<tr>
<td></td>
<td>Short</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>KONARC</td>
<td>Long</td>
<td>10</td>
<td>4</td>
<td>0.22±0.12*</td>
</tr>
<tr>
<td></td>
<td>Short</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*a Males were reared at 25°C under two different photoperiods (Long: 16L8D, Short: 10L14D).

b Number of traps that attracted at least one field adult of *R. pedestris*.

* Significant difference at 5% level between the two rearing photoperiods by Mann-Whitney U-test.

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### Table 2. Content of pheromone components from extracts of *Riptortus pedestris* bait males* after field trap experiments conducted in Tsukuba (NARC) and Kumamoto (KONARC) in 2006

<table>
<thead>
<tr>
<th>Place</th>
<th>Photoperioda</th>
<th>No. of males examined</th>
<th>Content of pheromone componentsb,c (µg/male, mean±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>14:iBu 18:iBu E2-6:E2Hx E2-6:Z3Hx E2-6:Hx</td>
</tr>
<tr>
<td>NARC</td>
<td>Long</td>
<td>11</td>
<td>0.43±0.16 (8) 0.10±0.03 (8) 9.01±1.14 (11) 3.57±0.70 (11) 3.06±0.74 (11)</td>
</tr>
<tr>
<td></td>
<td>Short</td>
<td>11</td>
<td>0 (0) 0 (0) 0.01 (1) 0 (0) 0.86±0.13 (11)</td>
</tr>
<tr>
<td>KONARC</td>
<td>Long</td>
<td>10</td>
<td>1.58±0.67 (7) 0.60±0.22 (6) 8.20±1.90 (10) 2.78±0.66 (9) 1.75±0.38 (10)</td>
</tr>
<tr>
<td></td>
<td>Short</td>
<td>10</td>
<td>0 (0) 0 (0) 0 (0) 0 (0) 0.85±0.10 (10)</td>
</tr>
</tbody>
</table>

*a Males were reared at 25°C under two different photoperiods (Long: 16L8D, Short: 10L14D).

b 14:iBu, tetradecyl isobutyrate; 18:iBu, octadecyl isobutyrate; E2-6:E2Hx, (E)-2-hexenyl (E)-2-hexenoate; E2-6:Z3Hx, (E)-2-hexenyl (Z)-3-hexenoate; E2-6:Hx, (E)-2-hexenyl hexanoate.

c Numbers in parentheses indicate the numbers of males in which each pheromone component was detected.
On the other hand, in the extracts of males reared in the short photoperiod, 14:iBu, 18:iBu, and E2-6:Z3Hx were not detected at all, while E2-6:E2Hx was found in only slight amounts (less than 0.04 μg) in three 0-day males (Table 4). E2-6:Hx was detected in all extracts. Males reared under the short photoperiod had immature EFRs throughout the experiment (Table 5).

With regard to the amount of food residue in the stomach and degree of fat-body development, there were no clear differences between males reared under the two different photoperiods (Table 5).

**DISCUSSION**

The sexual maturation of male adults greatly influenced their pheromone emission in *R. pedestris*. Males reared in the short photoperiod did not attract conspecific individuals, while males reared in the long photoperiod were attractive. In addition, 14:iBu, an essential component of the *R. pedestris* pheromone, was not detected in males reared in the short photoperiod. The status of the reproductive organ also showed that males reared in the short photoperiod entered reproductive diapause and had small, immature EFRs. These results indicate the strong relationship between pheromone release and sexual maturation, and also shown that diapausing...
males under a short photoperiod do not have their pheromone in *R. pedestris*. These results also imply that the pheromone of *R. pedestris* has a function related to sexual behavior. A similar phenomenon in which the photoperiod influences the emission of male pheromone has been reported in several true bug species. In *Eysarcoris lewisi* (Heteroptera: Pentatomidae), males reared in the short photoperiod (8L16D) did not attract conspecific adults and nymphs (Takita, 2007). In *Piezodorus hybneri* (Heteroptera: Pentatomidae), no pheromone components were detected in any diapause males reared in the short photoperiod (12L12D) (Endo et al., 2007). In the southern green stink bug *N. viridula*, only sexually mature non-diapausing males attracted conspecifics, and diapausing females lacked the response to the male pheromone (Brennan et al., 1977).

Our results, however, disagree with those of an earlier examination reported by Wada et al. (1997), in which field adults were caught in traps baited with *R. pedestris* males reared in a short photoperiod. Although we are not able to clarify the reason for this disagreement, we suspect contamination of the pheromone or the possibility that high temperature affects the termination of reproductive diapause (affects pheromone emission) in *R. pedestris* males.

The pheromone of *R. pedestris* seems to be multi-functional. Field females with immature ovaries and males with immature EFRs were attracted to males reared in a long photoperiod or synthetic pheromone bait in autumn (from mid-September to December) (Tabuchi et al., 2005, 2006; Mizutani et al., 2008 and the present study). The attracted adults were apparently under reproductive diapause, judging from the status of their internal organs, suggesting that the *R. pedestris* pheromone has a function other than as a sex attractant. In *P. maculiventris*, it was hypothesized that adults used the male pheromone as a cue to indicate the presence of prey, in addition to a cue for mating (Shetty and Hough-Goldstein, 1998). The *R. pedestris* pheromone was also considered to act as a sex attractant, at least for males which release the pheromone, and as a cue to indicate the presence of food sources, at least for immature adults and nymphs attracted to male pheromone. However, the adaptive significance of the pheromone system for *R. pedestris* in hypothetical functions, particularly in the latter case, remains to be elucidated. To fully understand the functions of the *R. pedestris* pheromone, it is necessary to clarify the benefits of males that release the pheromone in
Attractiveness of Diapausing Males of *R. pedestris*

the mating process as well as in food exploitation for adults and nymphs attracted by the pheromone.

E2-6:Hx was the only one of the four synergistic components to be detected in males reared in long and short photoperiods, while the other three synergistic components (18:iBu, E2-6:E2Hx, and E2-6:Z3Hx) were detected only in males reared in the long photoperiod. In addition, E2-6:Hx was detected in the extracts of not only attracted males, but also the attracted females (Mizutani et al., 2008). Furthermore, this component was reported to be an alarm pheromone in earlier literature (Leal and Kadosawa, 1992). This suggested that the function of E2-6:Hx is different from that of the other three synergistic components.

Reproductive diapause in male insects has been characterized by many different criteria and has not been adequately defined compared to female reproductive diapause, which is clearly defined by the absence of oocyte development and of oviposition (Pener, 1992). In some bug species, including *R. pedestris* (Numata and Kobayashi, 1989), *P. crosotta stali (=P. stali)* (Kotaki and Yagi, 1989) and *Leptocorisa chinensis* (Heteroptera: Alydidae) (Tachibana and Watanabe, 2007), the size of the EFR can be used as an index for the diapause status of the male. The present study clarified that diapausing males of *R. pedestris* with immature EFRs did not have pheromone components at all, while non-diapausing males with mature EFRs had pheromone components. A similar phenomenon in which diapausing males did not attract conspecific individuals and/or did not have pheromone components has been reported in *P. hybneri* (Endo et al., 2007) and *E. lewisi* (Takita, 2007), indicating that the profile of pheromone contents could be used as a criterion to define the status of male reproductive diapause in these bugs.

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REFERENCES


