Possible function of *Piezodorus hybneri* (Heteroptera: Pentatomidae) male pheromone: Effects of adult age and diapause on sexual maturity and pheromone production

Nobuyuki Endo,¹,* Tetsuya Yasuda,² Keiichiro Matsukura,¹ Takashi Wada,¹ Shin-etsu Muto³ and Rikiya Sasaki³

¹ National Agricultural Research Center for Kyushu Okinawa Region; Koshi, Kumamoto 861–1192, Japan
² National Agricultural Research Center; Tsukuba, Ibaraki 305–8666, Japan
³ Fuji Flavor Co., Ltd.; Hamura, Tokyo 205–8503, Japan

(Received 13 April 2007; Accepted 18 June 2007)

Abstract

To estimate the biological function of the male-produced pheromone of *Piezodorus hybneri*, we investigated the relationship between sexual maturity and pheromone production. Copulation was first observed at day 4 after emergence of males. The males showed high mating activity between days 5 and 15. Development of the ectodermal accessory gland (EAG) was also investigated as an indicator of male sexual maturity. The EAG, which was entirely immature at adult emergence, developed gradually between days 3 and 10. Males started to produce pheromones at days 3 to 5 after adult emergence, and production became maximal at days 5 to 10, remaining at a high level until day 30. No pheromone components were detected in any diapause males, which showed neither mating behavior nor EAG development. These results indicate that *P. hybneri* males produce their pheromone simultaneously with the development of sexual maturity, and thus the function of this pheromone seems to be related to sexual behavior.

Key words: *Piezodorus hybneri*; pheromone; sexual maturity; diapause; ectodermal accessory gland

INTRODUCTION

The stink bug, *Piezodorus hybneri* (Gmelin) is one of the major soybean pests in southern Japan (Kono, 1991; Wada et al., 2006). Male adults of *P. hybneri* attract conspecific adults of both sexes, indicating the presence of attractive pheromones (Higuchi, 1999). The pheromone system of the species has been reported to be a mixture of β-sesquiphellandrene, (R)-15-hexadecanolide, and methyl (Z)-8-hexadecenoate, and is defined as a sex pheromone because males show pre-copulatory behavior in response to filter paper impregnated with each of these components as well as the full mixture (Leal et al., 1998). However, the fact that the pheromone attracts both males and females is puzzling, and furthermore, it sexually stimulates males rather than females; thus, the biological function of this pheromone remains unclear.

In some heteropteran species, diapause and non-diapause males can be discriminated by the degree of development of the ectodermal accessory gland (Kotaki and Yagi, 1989; Numata and Kobayashi, 1989). Male accessory glands are involved in reproduction in many insect species (Chen, 1984; Gillott, 2003) and the secretion produced by male accessory glands is transferred to the female during copulation. Thus, by evaluating the development of this organ, it may be possible to determine the sexual maturity of individual males.

In this study, we investigated the effect of adult age on sexual maturity including mating ability, development of the ectodermal accessory gland (EAG), and pheromone production in males of *P. hybneri*. Based on these data, we discuss the possible role of this male-produced pheromone.

MATERIALS AND METHODS

Insects. Adults of *P. hybneri* were caught in soy-
bean fields of the National Agricultural Research Center for Kyushu Okinawa Region (32°52′5″N, 130°44′2″E), Kumamoto, Japan, in 2005. Their progeny were kept in the laboratory (24±1°C, 16L:8D photoregime) and used for the experiments. They were reared on a diet of soybean (Glycine max) seeds, red clover (Trifolium pratense) seeds, and water. After molting to the adult stage, the bugs used for experiments were transferred and kept individually in 60 ml plastic cups to prevent sexual contact. In experiments to investigate the effect of male diapause, bugs were reared from hatching under short-day conditions (24±1°C, 12L:12D photoregime). Two types of adult males appeared under these rearing conditions: one type with a red or pink band on the pronotum, and another with a white band. We defined males with a red or pink band as males in reproductive diapause (see Discussion).

Mating experiment. The mating ability of males at particular ages (0, 1, 2, 3, 4, 5, 10, 15, and 30 days after adult emergence) reared under long-day conditions was evaluated by observation of their mating behavior. A male of each age was transferred individually to a plastic cup (60 ml), into which a 10–12-day-old virgin female had been introduced (10 couples of each age). As copulation usually continues for more than one hour, we observed their mating behavior every hour for 6 h and males that succeeded in mating were checked. The experiments were conducted during the light period.

Preparation of body extract and evaluation of EAG development. Males at particular ages (0, 3, 5, 10, 15, and 30 days after adult emergence) were extracted individually with hexane. A whole-body extract from the male was prepared by immersing one individual in 2 ml hexane containing 2 μg octadecane (C18) as an internal standard for 10 min. The bug was then rinsed once with 1 ml of hexane. The extracts were stored for a few weeks in glass vials with Teflon-lined screw caps at −20°C until gas chromatography–mass spectrometry (GC-MS) analyses. The extracted male was then dissected under a stereoscopic microscope to check for the development of EAG. EAG development was classified into four grades: immature (<0.5 mm), small (0.5–1.4 mm), developed (1.5–2.4 mm) and fully developed (>2.4 mm). Males reared under the short-day photoregime were also extracted and dissected in the same way.

GC-MS analyses. Hexane body extracts were concentrated to ca. 100 μl using an evaporator just before GC-MS analysis, using an Agilent 6890N GC with an HP-5ms columns (30 m×0.25 mm ID×0.25 μm film thickness, Agilent Technologies) combined with an Agilent 5975i Network Mass Selective Detector. Mass spectrometry data for selected ion monitoring (SIM) and full scan (range: 35–350 m/z) were acquired synchronously. Quantitative (selected) and reference ions for SIM were 254 and 57 for C18, 204 and 69 for (±)-β-sesquiphellandrene, 210 and 55 for (±)-15-hexadecanolide, and 268 and 55 for methyl (Z)-8-hexadecenoate, respectively. Injection was performed in the splitless mode of a split/splitless injector using an Agilent 7683 series automatic liquid sampler, and injector temperature was 250°C. Helium was used as the carrier gas under constant flow mode (1.0 ml/min). The initial GC oven temperature was 50°C (2-min hold), increased to 240°C at a rate of 15°C/min, and was then held for 5 min.

Chemicals. (±)-β-Sesquiphellandrene, (±)-15-hexadecanolide, and methyl (Z)-8-hexadecenoate were synthesized as described previously (Leal et al., 1998).

RESULTS

Under long-day conditions, mating of males was first observed on day 4 after adult emergence, when 40% of males completed copulation (Fig. 1). The proportion increased to 60% on day 5 and remained at this level until day 15. Although some 30-day-old males did copulate, the proportion that

![Fig. 1. Effect of adult age on copulation of Piezodorus hybneri males (n=10).](NII-Electronic Library Service)
succeeded in mating was only 40%.

Males possessed immature EAGs at emergence (Fig. 2). At day 3 after adult emergence, many of the males (58.3%) began to develop EAGs and some (15.8%) attained fully developed EAGs at day 5 after emergence. The frequency of males having developed (fully developed and developed) EAGs almost peaked at day 10 (84.2%), and remained at this level throughout the observation period (until day 30). On the other hand, all diapause males had immature EAGs (not shown in figures).

No pheromone components were found in males at day 0 under long-day conditions (Table 1). The components were first detected in some males at day 3 (16.7%). The proportion of males possessing the pheromone increased to about 85% at day 5 and remained at this level until day 30. The total amount of pheromone components increased to about 10 µg/male at day 10, and remained at this level throughout the observation period (until day 30). The pheromone components were not detected in extracts from any of the diapause males on day 10 (Table 1).

The relationship between the total pheromone amount and EAG development is shown in Fig. 3.

![Fig. 2. Development of the ectodermal accessory gland (EAG) of *Piezodon sp. hynberi* males in relation to adult age. Immature: <0.5 mm in length, Small: 0.5–1.4 mm, Developed: 1.5–2.4 mm, Fully developed: >2.4 mm. Different letters above bars indicate significant differences (p<0.01) between the frequency of males having developed (fully developed and developed) EAGs at different ages using multiple comparison test for proportions (Zar, 1996).](image)

![Fig. 3. Total amount of pheromone components of *Piezodon sp. hynberi* males in relation to development of the EAG. Data were taken from the same individuals as in Fig. 2 and Table 1. Immature: <0.5 mm in length, Small: 0.5–1.4 mm, Developed: 1.5–2.4 mm, Fully developed: >2.4 mm. Vertical lines above bars indicate standard errors of means. Different letters above bars indicate significant differences (p<0.01) by Steel-Dwass test for non-parametric multiple comparisons.](image)

### Table 1. Production of pheromone components in *Piezodon sp. hynberi* males in relation to adult age and reproductive diapause

<table>
<thead>
<tr>
<th>Condition</th>
<th>Age of male (days)</th>
<th>No. of males extracted</th>
<th>Amount extracted (µg/male)*</th>
<th>% of males having pheromoneb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sesq</td>
<td>R15</td>
</tr>
<tr>
<td>Non-diapause</td>
<td>0</td>
<td>11</td>
<td>0.00 a</td>
<td>0.00 a</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>12</td>
<td>0.01 a</td>
<td>0.06 ab</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>19</td>
<td>1.26 b</td>
<td>1.67 c</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>27</td>
<td>2.91 bc</td>
<td>3.68 c</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>23</td>
<td>4.68 bc</td>
<td>1.98 bc</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>24</td>
<td>5.22 c</td>
<td>2.82 c</td>
</tr>
<tr>
<td>Diapause</td>
<td>10</td>
<td>13</td>
<td>0.00 a</td>
<td>0.00 a</td>
</tr>
</tbody>
</table>

Sesq: β-sesquiphellandrene, R15: (R)-15-hexadecanolide, Z8: methyl (Z)-8-hexadecenoate.

* Different letters in the same column indicate significant differences (p<0.05) by Steel-Dwass test for non-parametric multiple comparisons.

b Different letters in the same column indicate significant differences (p<0.05) by multiple comparison test for proportions (Zar, 1996).
Pheromone components were not detected in males with immature EAGs. There was a clear relationship between the amount of the pheromone in males and the stage of EAG development. The percentage of males without pheromone among those with developed and fully developed EAGs was 10.5% (4/38) and 8.1% (3/37), respectively.

DISCUSSION

We found significant differences in mating behavior and the developmental level of a reproductive organ, the EAG, in *P. hybneri* males as males matured. Mating behavior began on day 4, and the proportion of males completing copulation peaked between days 5 and 10 after eclosion (Fig. 1). Almost in synchrony with mating behavior, the EAG began to develop on day 3, and was fully developed by days 5 to 10 after eclosion (Fig. 2). As the developmental level of the EAG paralleled the frequency of mating behavior, the EAG seems to be a good indicator of sexual maturation in *P. hybneri* males.

When the bugs were rear from hatching under short-day conditions (12L-12D photoregime), nearly half of the males appeared to have a red or pink band across their pronotum, the other half having a white band. On the other hand, only males with white bands were observed when reared under long-day conditions (16L-8D photoregime). This phenomenon could represent one form of the seasonal color polyphenism found in several hemipteran species (Kotaki and Yagi, 1987; Kobayashi and Numata, 1995; Musolin and Saulich, 1999). Such color changes are often associated with diapause induction (Harris et al., 1984; Musolin and Numata, 2003). In the present study, red- or pink-banded males did not show mating behavior, and therefore must have been in reproductive diapause. Since we did not check diapause in white-banded males reared under short-day conditions, the precise relationship between band colors and diapause of males is unknown at present.

The present study clarified that the male pheromone was produced simultaneously with the occurrence of mating behavior and EAG development. Pheromone production began at days 3 to 5, peaked on day 10, and thereafter remained at almost the same level until day 30 (Table 1). This trend paralleled the occurrence of mating behavior and the progress of EAG development. In contrast, no pheromone components were detected in males in diapause, which showed neither mating behavior nor development of EAGs, suggesting that the male pheromone in *P. hybneri* plays a role in sexual behavior.

Interestingly, the proportion of different pheromone components in extracts from *P. hybneri* males changed with the adult age. The average amount of \( \beta \)-sesquiphellandrene increased steadily until day 30, while the other two components peaked at day 10 and then somewhat decreased (Table 1). As a result, the proportion of \( \beta \)-sesquiphellandrene increased from ca. 5% (day 3) to 50% (days 15 to 30). The other two components tended to be proportionally reduced as males became older. These findings suggest that the pheromone component ratio emitted by males may differ according to age; however, the amount of pheromone present in the extract is not directly comparable to the actual amount of pheromone released by live males. In addition, marked variation in the pheromone component ratio among individuals of the same population has been reported for the southern green stink bug, *Nezara viridula* (Ryan et al., 1995); therefore, we need to investigate the actual emission of pheromone from males to clarify whether the pheromone component ratio changes with age.

Recently, we found that both adults and nymphs of *P. hybneri* were attracted to (E)-2-hexenyl (E)-2-hexenoate, one of the pheromone components of the bean bug, *Riptortus pedestris* (= *R. clavatus*; see Kikuhara, 2005) (Heteroptera: Alydidae) (Endo et al., 2003, 2006b). This attraction of *P. hybneri* to the pheromone of its competitor bug in soybean fields seems to be related to food searching rather than sexual behavior (Endo et al., 2006a, b). On the other hand, in the present study, we revealed that the *P. hybneri* pheromone is related to sexual behavior. In order to clarify the biological functions of the *P. hybneri* pheromone or the difference in these attractants, it will be necessary to compare the attractiveness of (E)-2-hexenyl (E)-2-hexenoate and the sex-related pheromone of *P. hybneri* in the field, including the response of nymphs or the sex ratio and physiological conditions of attracted adults.

ACKNOWLEDGEMENTS

We thank Dr. T. Kotaki (National Institute of Agrobiological
Sciences) and Dr. N. Mizutani (National Agricultural Research Center) for instruction in the dissection technique for *P. hybneri*. We also thank Dr. J. R. Aldrich (USDA ARS) for reviewing our draft. We are also grateful to Mrs. K. Nagata for her assistance in rearing the bugs.

REFERENCES


