Research Article

Age-related and Individual Variation in Male *Piezodorus hybneri* (Heteroptera: Pentatomidae) Pheromones

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Males of the *Piezodorus hybneri* stink bug produce a pheromone comprising β -sesquiphellandrene (Sesq), (*R*)-15-hexadecanolide (R15), and methyl (*Z*)-8-hexadecenoate (Z8). We collected airborne volatiles from individual *P. hybneri* males and analyzed them by GC-MS. Daily analysis from 1 to 16 days after adult emergence showed that pheromone emission started around 3 to 6 days after adult emergence and peaked ($\sim 1 \mu\text{g}/\text{male}/\text{day}$) on day 11. The proportion of Sesq tended to increase with age to about 80% on days 12 to 16. On the other hand, the proportion of R15 tended to decrease with age. The proportion of Z8 reached a maximum of about 34% on day 9 but otherwise remained below 20%. The total amount of pheromone emitted by individual males varied considerably: three males emitted more than $10 \mu\text{g}$, whereas another three males emitted little or no pheromone and failed to survive by the end of the experiment. These results suggest that the amount of *P. hybneri* pheromone and its blend ratio could be affected by the male's physical conditions, such as vitality and age.

1. Introduction

The stink bug *Piezodorus hybneri* (Heteroptera: Pentatomidae) is an important soybean pest in southern Japan [1, 2]. Male adults of *P. hybneri* attract conspecific adults of both sexes [3] via a pheromone comprising β -sesquiphellandrene (Sesq), (*R*)-15-hexadecanolide (R15), and methyl (*Z*)-8-hexadecenoate (Z8) [4]. These synthetic chemical mixtures attract conspecific adults, especially females in fields [5]. Our previous study [6] revealed that *P. hybneri* males produce the pheromone simultaneously with their development to sexual maturity, and that diapausing males produce no pheromone components; thus, the pheromone is likely to play a role in sexual communication. The average amount of Sesq in whole-body extracts increased steadily until day 30 after adult emergence, whereas the other two components peaked at day 10 and then decreased somewhat [6]. Consequently, the proportions of the pheromone components, especially

Sesq, changed with age. These findings suggest that the proportions of components in emissions also change with age. However, pheromone production might not coincide with emission. In addition, marked variation in the pheromone component ratio among individuals of the same population was found the southern green stink bug, *Nezara viridula* (Heteroptera: Pentatomidae) [7]. Therefore, it is necessary to monitor the pheromone emission of *P. hybneri* over time from the same males to examine variation in the pheromone blend.

Mating behavior of *P. hybneri* males began on day 4 after adult emergence and showed high mating activity between days 5 and 15 [6]. Development of the ectodermal accessory gland, which is involved in reproduction and an indicator of male sexual maturity, showed that males fully matured by day 10 [6]. Thus, in this study, we collected and analyzed the volatiles from individual *P. hybneri* males from 1 to 16 days after adult emergence.

TABLE 1: Individual variation of pheromone titer and extraction from *Piezodorus hybneri* males.

Male	Days of first detection	Pheromone emission (μg)			Amount extracted at 16 d (μg)
		Daily maximum	15–16 d	Sum (1–16 d)	
1	5–6	0.91	0.09	2.91	12.07
2	4–5	3.20	0.58	14.54	27.49
3	4–5	0.75	0.26	4.22	10.51
4	5–6	1.81	1.51	12.41	48.81
5	5–6	0.31	0.00	0.62	0.00
6	—	0.00	—	0.00	—
7	5–6	0.09	—	0.09	—
8	4–5	0.32	—	1.03	—
9	3–4	1.03	0.29	5.68	5.18
10	3–4	3.47	0.50	18.62	27.02
Average		1.32	0.46	6.01	18.73

2. Materials and Methods

2.1. Insects. Adults of *P. hybneri* were caught in soybean fields of the NARO Kyushu Okinawa Agricultural Research Center (32°52'5" N, 130°44'2" E), Kumamoto, Japan, in 2005. Their progeny were kept in the laboratory ($24 \pm 1^\circ\text{C}$, 16L–8D photoregime) and used for experiments in March 2006. The bugs were reared on a diet of soybean (*Glycine max*) seeds, red clover (*Trifolium pratense*) seeds, and water.

2.2. Collection of Airborne Volatiles with Glass Beakers. Following the method of Yasuda et al. [8] with some modifications, we collected and analyzed the volatiles from individual *P. hybneri* males ($n = 10$) in glass beakers. Collection started 1 day after adult emergence. A single adult male was confined in a 50 mL glass beaker with a few soybean seeds and moist cotton. The beaker was placed upside down and sealed with aluminum foil. The male was kept in the beaker for 24 h under laboratory conditions. After 24 h, the male was removed and the beaker surface was rinsed with 3 mL hexane containing 2 μg octadecane as an internal standard. After this treatment, any pheromone component as well as internal standard was not detected from the hexane rinsing the beaker. The hexane was collected for analysis of volatiles. The male was placed in a new beaker with food and the process was repeated until day 16. After the final collection, the males that survived were extracted with 2 mL hexane containing 2 μg octadecane as an internal standard and then rinsed once with 1 mL hexane. All extracts were stored in glass vials with Teflon-lined screw caps at -20°C until analysis. Extracts were concentrated to ca. 100 μL in an evaporator just before gas chromatography—mass spectrometry (GC-MS) analysis.

2.3. GC-MS Analysis. Quantitative GC-MS analysis was done on an Agilent 6890 N GC with an HP-5 ms column (30 m $\times$ 0.25 mm ID $\times$ 0.25 μm film thickness; Agilent Technologies) and an Agilent 5975i Network Mass Selective Detector using an internal-standard method. Mass spectrometry data by selected ion monitoring (SIM) and full scan (range: 35–350 m/z) were acquired synchronously.

Quantitative (selected) and reference ions for SIM were m/z 254 and 57, respectively, for octadecane, m/z 204 and 69 for ($\pm$)- β -sesquiphellandrene, m/z 210 and 55 for ($\pm$)-15-hexadecanolide, and m/z 268 and 55 for methyl (*Z*)-8-hexadecenoate. Injection was performed in splitless mode with a split/splitless injector using an Agilent 7683 series automatic liquid sampler at 250°C . Helium was used as the carrier gas at a constant flow of 1.0 mL/min. The GC oven temperature was an initial 50°C (2-min hold), increased to 240°C at $15^\circ\text{C}/\text{min}$, and then held for 5 min. To determine the quantity of each component, standard curves obtained using known amounts of authentic chemicals with the internal standard (octadecane) were used.

2.4. Chemicals. ($\pm$)- β -Sesquiphellandrene, ($\pm$)-15-hexadecanolide, and methyl (*Z*)-8-hexadecenoate were synthesized according to a previous report [4].

3. Results

The temporal patterns and the amounts of pheromone emission by males varied greatly among individuals (Figure 1). Nine out of the 10 males emitted pheromone (Table 1). Emission started 3 to 6 days after adult emergence, peaked ($\sim 1 \mu\text{g}/\text{male}/\text{day}$) on day 10 or 11, and remained high until day 16. The maximum average emission was $1.32 \mu\text{g}/\text{day}$, and two bugs exceeded $3 \mu\text{g}/\text{day}$. The total amount of pheromone varied more than 30-fold among surviving individuals (0.62–18.62 μg). Male No. 6 did not emit any pheromone and died on day 11. Males No. 7 and 8 emitted little pheromone and died on days 7 and 15, respectively. There was a strong correlation between the amount of pheromone collected on day 16 and the amount extracted on day 16 ($r^2 = 0.885$, $P = 0.0016$).

The proportions of the three pheromone components, especially Sesq, showed great variability (Figures 1 and 2). The proportion of Sesq tended to increase with age to about 80% on days 12 to 16. On the other hand, the proportion of R15 tended to decrease with age. The proportion of Z8 reached a maximum of about 34% on day 9, but otherwise remained below 20%.

FIGURE 1: Daily changes in pheromone emission and the proportion of β -sesquiphellandrene from individual males of *Piezodorus hybneri*. The bar shows the total amount of pheromone ($\mu\text{g}/\text{male}/\text{day}$). The line chart indicates the proportion of β -sesquiphellandrene (%) among the total pheromone components. Inverted triangle indicates the day when the bugs died. Because male No. 6 did not emit any pheromone, there was no data available in this figure.

4. Discussion

Repeated collection of volatiles with a glass beaker from individual males over a period of 15 consecutive days showed that the pheromone component ratio varied with male age. The proportion of Sesq tended to increase with age and reached about 80% by days 12 to 16. This trend in variable pheromone blend ratios agreed with our previous results obtained from extracts of males [6]. The high correlation between the quantities of pheromone in emissions and those in body extracts, on day 16, indicates that pheromone emission parallels pheromone production in this species.

In some heteropteran species, variations of the pheromone blend ratio among individuals or with physiological

condition have been reported. In *N. viridula*, the pheromone blend ratio varied among individuals within a population [7, 9], although individuals' ratios remained constant [9]. Large variability of pheromone component ratios in the bean bug *Riptortus pedestris* (Heteroptera: Alydidae) was also reported [10]. Recently, Moraes et al. [11] reported that food conditions affected the pheromone ratios in the Neotropical brown stink bug *Euschistus heros* (Heteroptera: Pentatomidae). We report here that large changes in the pheromone blend of *P. hybneri* males occur during the first 2 weeks of adulthood. This is the first study documenting age-related shifts in the pheromone blend of Heteroptera.

The pheromone blend ratio is generally calculated from the amounts of components emitted at a specific age.

FIGURE 2: Proportions of each pheromone component of *Piezodorus hybneri* males in relation to adult age. Sesq: β -sesquiphellandrene; R15: (*R*)-15-hexadecanolate; Z8: methyl (*Z*)-8-hexadecenoate. Vertical lines represent SE.

However, this approach is based on the premise that the ratio remains constant throughout adult life. Leal et al. [4] reported that the pheromone blend ratio of *P. hybneri* was Sesq:R15:Z8 = 10:4:1. However, our results show that the ratio was not constant and was affected by male age. This may be the case in other species, and thus it should be taken into consideration when pheromone blend ratios are determined.

Pheromone quantity could be also affected by the male physical conditions. Pheromone production of *P. hybneri* is paralleled to the development of male sexual maturity [6], and young *P. hybneri* males cannot produce or emit a large amount of pheromone. In addition, our results show a large variation in pheromone quantity among individuals, and the poor pheromone emitters died early. These results suggest that only vital and sexually mature males can produce sufficient pheromone.

The biological function or attractiveness of each pheromone components or its blends for *P. hybneri* is largely unknown. In fields, each *P. hybneri* pheromone component alone lacked the attractiveness, while three-component mixture was attractive to *P. hybneri* [5]. Leal et al. [4] showed that the full three-component mixture was attractive more than the any binary mixtures in laboratory conditions, whereas the attractiveness of the different blend ratios has never been investigated. In order to clarify the biological functions of *P. hybneri* pheromone blend or its components, it is necessary to compare the attractiveness among different pheromone blends or male ages.

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