INTRODUCTION

Mating often alters female behaviour by triggering physiological changes (e.g., Obara, 1982; Julian & Gronenberg, 2002). Such physiological changes are attributable not only to physical stimuli, such as penis insertion and stretch reception in the bursa copulatrix, but also other factors. During copulation, males transfer not only sperm, but also seminal fluid, spermatophores (including sperm cells), and mating plugs to females through genitalia (Chen, 1984; Eberhard, 1996; Simmons, 2001; Wedell, 2005). It is well-known for several insects that male-derived substances reduce female mating receptivity and whether this extract and spermatophores per se affected the release of sex pheromone by females. The mating receptivity of virgin females injected with an extract of male reproductive organs was significantly lower than that of control females injected with distilled water, but not significantly different from that of females injected with an extract of male thorax (the negative control). The amount of sex pheromone released by females, however, did not differ among the different treatments. When the interval between two subsequent copulations of males is less than 1 h, males do not transfer a spermatophore during the second copulation. It is thus possible to produce artificially mated females with and without a spermatophore. However, the amount of sex pheromone released by mated females with and without a spermatophore did not differ. These results indicate that male-derived substances do not suppress release of sex pheromone by female S. rubrovittatus but, they may reduce their mating receptivity.

Abstract. In insects, male-derived substances transferred during copulation often alter female physiology. Thus these substances may affect female behaviour, including mating receptivity and release of sex pheromone. In the sorghum plant bug Stenotus rubrovittatus (Matsumura) (Hemiptera: Miridae), males transfer a spermatophore into the bursa copulatrix of females during copulation. Mated females of S. rubrovittatus do not mate again for at least 3 days and release lower amounts of sex pheromone than virgin females. A previous study indicates that females that receive a spermatophore are less likely to be sexually receptive to males. Therefore, we tested whether an extract of the male reproductive organ affected female mating receptivity and whether this extract and spermatophores per se affected the release of sex pheromone by females. The mating receptivity of virgin females injected with an extract of male reproductive organs was significantly lower than that of control females injected with distilled water, but not significantly different from that of females injected with an extract of male thorax (the negative control). The amount of sex pheromone released by females, however, did not differ among the different treatments. When the interval between two subsequent copulations of males is less than 1 h, males do not transfer a spermatophore during the second copulation. It is thus possible to produce artificially mated females with and without a spermatophore. However, the amount of sex pheromone released by mated females with and without a spermatophore did not differ. These results indicate that male-derived substances do not suppress release of sex pheromone by female S. rubrovittatus but, they may reduce their mating receptivity.

Do male-derived substances affect female mating receptivity and release of sex pheromone by females of the sorghum plant bug Stenotus rubrovittatus (Hemiptera: Miridae)?

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Key words. Hemiptera, Miridae, Stenotus rubrovittatus, injection, mating receptivity, reproductive organ, sex-pheromone release, spermatophore

INTRODUCTION

Mating often alters female behaviour by triggering physiological changes (e.g., Obara, 1982; Julian & Gronenberg, 2002). Such physiological changes are attributable not only to physical stimuli, such as penis insertion and stretch reception in the bursa copulatrix, but also other factors. During copulation, males transfer not only sperm, but also seminal fluid, spermatophores (including sperm cells), and mating plugs to females through genitalia (Chen, 1984; Eberhard, 1996; Simmons, 2001; Wedell, 2005). It is well-known for several insects that male-derived substances reduce female mating receptivity and whether this extract and spermatophores per se affected the release of sex pheromone by females. In the western tarnished plant bug Lygus hesperus (Hemiptera: Miridae), males transfer a spermatophore into the bursa copulatrix of females during copulation. Mated females of S. rubrovittatus do not mate again for at least 3 days and release lower amounts of sex pheromone than virgin females. A previous study indicates that females that receive a spermatophore are less likely to be sexually receptive to males. Therefore, we tested whether an extract of the male reproductive organ affected female mating receptivity and whether this extract and spermatophores per se affected the release of sex pheromone by females. The mating receptivity of virgin females injected with an extract of male reproductive organs was significantly lower than that of control females injected with distilled water, but not significantly different from that of females injected with an extract of male thorax (the negative control). The amount of sex pheromone released by females, however, did not differ among the different treatments. When the interval between two subsequent copulations of males is less than 1 h, males do not transfer a spermatophore during the second copulation. It is thus possible to produce artificially mated females with and without a spermatophore. However, the amount of sex pheromone released by mated females with and without a spermatophore did not differ. These results indicate that male-derived substances do not suppress release of sex pheromone by female S. rubrovittatus but, they may reduce their mating receptivity.
MATERIAL AND METHODS

Plant bugs

Adults of the sorghum plant bug *S. rubrovittatus* (Hemiptera: Miridae), were collected from grass fields at the National Agricultural Research Center, Tsukuba, Japan (36°01′N, 140°06′E), on 16 July 2010 and were allowed to lay eggs on millet (*Setaria italica*) seedlings kept at 25°C and a photoperiod of 16L : 8D (light phase, 06:00–22:00; hereafter termed “laboratory conditions”). Newly hatched nymphs were transferred onto wheat (*Triticum aestivum*) seedlings and kept under the same laboratory conditions. This cycle was repeated for several generations. Individual fifth-instar nymphs were isolated in glass tubes (30 mm in diameter, 150 mm in height) that were attached to transparent-plastic cups (32 mm in diameter, 38 mm in height) containing five wheat seedlings on one side (hereafter termed “glass tube containing five wheat seedlings”). The other side of each tube was covered with a sheet of gauze to prevent nymphs from escaping and the tubes were kept vertically and in the same laboratory conditions. Adult emergence was checked every morning and, when adults emerged, the wheat seedlings were replaced. The adults were used in the experiments described below.

Effects of an extract of the male reproductive organs on female mating receptivity

To determine whether the extract of male reproductive organs affected the release of sex pheromone by females, 67 virgin females (3 d after emergence) were chilled on ice for a few minutes until immobile and then placed on an agarose medium using fine forceps. Extracts of the male reproductive organs and thorax were dissolved in Milli-Q water as described above and 0.05 µl of the solutions injected into tergites of the female abdomen under the wings (reproductive organs: n = 23; thorax: n = 21). Milli-Q water (0.05 ml) was similarly injected into control females (n = 22). Immediately after the injection, each female was transferred to a glass tube containing five wheat seedlings and kept for 1 d under laboratory conditions before collecting pheromone.

Effect of spermatophores on the release of sex pheromone by females

Males of *S. rubrovittatus* transfer a spermatophore to females during copulation (Sugeno & Watanabe, 2011). However, when the interval between two copulations of males is less than 1 h, males do not transfer spermatophores to females during the second copulation (Oku & Kitsunezuka, 2011). Thus, it is possible to produce mated females with and without a spermatophore. To determine whether the presence of a spermatophore affected the release of sex pheromone by females, 67 virgin females (3 d after adult emergence) were separately placed in glass vials (50 ml) containing three wheat seedlings. Then, an unmated male was introduced into 35 of the glass vials and most copulated with the females. A total of 29 mated females were obtained within 1 h (“females with a spermatophore”). Immediately after copulation, mated males were individually transferred to glass vials containing a virgin female and three wheat seedlings. Copulation was monitored for 1 h and 21 mated females were obtained (“females without a spermatophore”). All mated females were individually transferred into glass tubes containing five wheat seedlings and kept for 1 d under laboratory conditions before collecting pheromone.

Collection, extraction and quantitative analysis of airborne sex pheromone

The sex pheromone of *S. rubrovittatus* females consists of three principal components; hexyl butyrate, (*E*)-hex-2-en-1-yl butyrate, and (*E*)-4-oxohex-2-enal (Yasuda et al., 2008). To collect the compounds released by individual females, they were separately introduced into side-armed glass tubes sealed with black screw caps fitted with Teflon®-faced rubber liners (see Oku & Yasuda, 2010). A stir bar coated with polydimethylsiloxane (Twister™, GERSTEL GmbH & Co. KG, Germany, film thickness 1 mm, length 10 mm) was placed in each glass tube. The polydimethylsiloxane adsorbed the components of the sex pheromone. These females were kept in these tubes for 1 d under laboratory conditions. Results for individuals that died before or during the airborne collection were not included in the analysis.

To extract the three components of the sex pheromone, each stir bar was removed from the glass tubes and placed in a glass vial (2 ml). The stir bars were soaked in 1.3 ml of hexane and stirred for 30 min at room temperature using a magnetic stirrer. Each aliquot of hexane contained heptadecane (5 µg), which served as an internal standard for estimating the relative quantities of each component. The hexane was concentrated using a rotary evaporator before gas chromatography-mass spectrometry (GC-MS).

Effect of the extract of male reproductive organs on the release of sex-pheromone by females

To determine whether the extract of male reproductive organs affected the release of sex pheromone by females, 66 virgin females (3 d after emergence) were chilled on ice for a few minutes until immobile and then placed on an agarose medium using fine forceps. Extracts of the male reproductive organs and thorax were dissolved in Milli-Q water as described above and 0.05 µl of the solutions injected into tergites of the female abdomen under the wings (reproductive organs: n = 23; thorax: n = 21). Milli-Q water (0.05 ml) was similarly injected into control females (n = 22). Immediately after the injection, each female was transferred to a glass tube containing five wheat seedlings and kept for 1 d under laboratory conditions before collecting pheromone.
was performed on an Agilent 6890N GC with a HP-INNOWax column (30 m length × 0.25 mm internal diameter × 0.25 mm film thickness) by splitless injection combined with an Agilent 5975 Network Mass Selective Detector. Mass spectrometric data were acquired by continually alternating between full scanning (range: m/z 35–350) and selected ion monitoring (SIM) modes, using the method described in Oku & Yasuda (2010). Injection temperature was 230°C. Helium was used as the carrier gas and the flow rate was held constant at 1.0 ml/min. The initial GC oven temperature was 50°C (2 min hold), followed by an increase to 240°C at 10°C/min, with a further hold for 5 min. The relative quantities of each component were estimated from standard linear calibration curves obtained using authentic samples of hexyl butyrate, (E)-hex-2-en-1-yl butyrate, and (E)-4-oxohex-2-enal analyzed together with heptadecane (see supplementary material in Oku & Yasuda 2010). Hexyl butyrate (>98.0% chemical purity) and (E)-hex-2-en-1-yl butyrate (>95.0%) were purchased from Tokyo Chemical Industry Co., Ltd., Japan. (E)-4-Oxohex-2-enal (96.9%) was obtained from the Shin-Etsu Chemical Co. Ltd, Japan.

Statistical analysis

Prior to determining the effect of the extract of male reproductive organs on female mating receptivity, the heterogeneity among the replicates of each treatment was determined (Sokal & Rohlf, 1995). Because no heterogeneity was detected (see Results), the data were pooled and the whole data set was analysed using a Chi-square test. When the effect was determined to be significant at the 5% level, a post hoc Fisher exact test with Bonferroni correction was applied to determine the validity of an observed difference between treatments (the Bonferroni-corrected significant level was 0.017). To evaluate the effects of the extract of male reproductive organs and spermatophores on the release of sex pheromone by females, the relative quantities of the three components of the sex pheromone were summed. These values were compared among/between treatments using either a Kruskal-Wallis test or a Mann Whitney U test. Moreover, when females died before or during air collections, the mortality was compared among treatment groups using a Chi-square test. JMP version 8.0.2 (SAS Institute, 2009) was used carry out these analyses.

RESULTS

Effects of an extract of the male reproductive organs on female mating receptivity

There was no heterogeneity in the results of the three replicates (control: P = 0.689; thorax: P = 0.181; male reproductive organs: P = 0.426; see Table 1) and the pooled data clearly indicated that female mating receptivity differed among the treatments (χ² test, χ²(2) = 13.011, P = 0.002; Fig. 1). The mating receptivity of females injected with the extract of male reproductive organs was significantly lower than that of control females. Although the mating receptivity of females injected with the extract of male reproductive organs did not differ significantly from that of females injected with extract of male thorax, there was also no significant difference between control females and females injected with extract of male thorax. No females died during this experiment.

Effect of the extract of male reproductive organs on the release of sex-pheromone

The relative qualities of the three components of the sex pheromone released by females did not differ among the treatments; control females, females injected with extract of male thorax and those injected with extract of male reproductive organs (Kruskal-Wallis test, χ² = 0.518, P = 0.772; Fig. 2). There was no significant difference in the percentage of females dying in the three treatments; five control (22.7%), 5 injected with male thorax extract (23.8%) and 8 injected with male reproductive-organ extract (34.8%) (χ² test, χ² = 1.01, P = 0.604).

Effect of spermatophores on the release of sex pheromone by females

The relative quantities of the three components of the sex pheromone released by females did not differ between the two treatments; mated females with and without a spermatophore (Mann Whitney U test, Z = 0.154, P = 0.878; Fig. 3). No females died before or during the airborne collections of pheromones.

Table 1. Mating receptivity of virgin females injected with distilled water as a control, extract of male thorax as a negative control, or extract of male reproductive organs (MRO) and results of the statistical analysis of heterogeneity. P > 0.05 indicates the frequency distributions of the replicates do not differ significantly from each other.

<table>
<thead>
<tr>
<th>No. of receptive ♀ (no. of trials)</th>
<th>Heterogeneity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G₀, df, P</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Replicate 1</td>
<td>5 (10)</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>5 (15)</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>4 (9)</td>
</tr>
<tr>
<td>Thorax</td>
<td></td>
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<tr>
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<tr>
<td>Replicate 2</td>
<td>3 (14)</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>0 (9)</td>
</tr>
<tr>
<td>MRO</td>
<td></td>
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<tr>
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<tr>
<td>Replicate 2</td>
<td>1 (11)</td>
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<tr>
<td>Replicate 3</td>
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DISCUSSION

The extract of male reproductive organs reduced female mating receptivity, though that of the male thorax also had a little effective in this regard. Perhaps, a protein extract, irrespective of its origin, could have a “negative” physiological effect that makes females unresponsive to mating. A possible alternative explanation is that it is the consequence of the negative effect of the metathoracic-gland defensive secretion, which although unknown in *S. rubrovittatus*, is present in a mirid bug *Lopidea robiniae* (Hemiptera: Miridae) (Staples et al., 2002). The result, however, indicates that active extracts can be obtained from male organs, irrespective of their origin. It is likely that spermatophores reduce female mating receptivity in *S. rubrovittatus* (Oku & Kitsunezuka, 2011). Although the active principle appears to be a component of the secretion of the male accessory glands (Sugeno & Watanabe, 2011) this has not been experimentally confirmed. In the present study, the male reproductive tissue that was extracted included accessory glands, seminal vesicle, testis and ejaculatory ducts. Therefore, the results presented are only preliminary evidence that substances in spermatophores reduce female mating receptivity. Possibly, the presence of a spermatophore in the bursa copulatrix of females acts synergistically with the substances in the spermatophore. The mechanical effect of the presence of a spermatophore is reported for other insects. In *Pieris rapae crucivora* (Lepidoptera: Pieridae), the presence of spermatophores in the bursa copulatrix stimulates the nervous system, which in turn reduces the mating receptivity of the females (Obara et al., 1975; Sugawara, 1979). According to Oku et al. (2010), 90% of intact virgin females (3 d after adult emergence) will mate with unmated males (3 d after adult emergence). In contrast, the mating receptivity of injected virgin females in this study was 41.2%. Furthermore, more than 20% of the females died within two days of being injected, regardless of treatment. This indicates that the injection itself affected female activity and that there is a need to reduce the effect of being injected. These points require further study.

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