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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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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.

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 (e.g., Chen et al., 1988; Himuro & Fujisaki, 2008; Yamane et al., 2008). Furthermore, male-derived substances may target the release of sex pheromone by females and so prevent their attracting males. In some insects, female sex pheromone levels decline after mating (e.g., Raina et al., 1986; Babilis & Mazomenos, 1992; Tang et al., 1992; del Mazo-Cancino et al., 2004). In the corn earworm moth *Helicoverpa zea* (Lepidoptera: Noctuidae), male-derived substances cause mated females to cease producing and releasing sex pheromone (Raina, 1989; Kingan et al., 1993, 1995). In the western tarnished plant bug *Lygus hesperus* (Hemiptera: Miridae), there is a male-derived substance in the spermatophore that suppresses the sexual attractiveness of mated females (Brent & Byers, 2011).

In the sorghum plant bug, *Stenotus rubrovittatus* (Matsumura) (Hemiptera: Miridae), mated females do not mate again for at least 3 days (Okutani-Akamatsu et al., 2009). Males of *S. rubrovittatus* transfer a spermatophore to females during copulation (Sugeno & Watanabe, 2011). A previous study indicates that it is likely that spermatophores reduce female sexual receptivity (Oku & Kitsunezuka, 2011). However, it remains unclear whether the spermatophore per se, or a substance in the spermatophore, is responsible for this effect. Therefore, we investigated whether an extract of the male reproductive organs affects female mating receptivity. In *S. rubrovittatus*, females release sex pheromones to attract conspecific males (Okutani-Akamatsu et al., 2007). Mated females release lower amounts of sex pheromone than virgin females (Oku & Yasuda, 2010). Therefore, we also examined whether an extract of male reproductive organs and spermatophores suppress release of sex pheromone by females.

MATERIAL AND METHODS

Plant bugs

Adults of the sorghum plant bug *Stenotus rubrovittatus* (Matsumura) (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 obtain extracts of male reproductive organs, unmated adult males (3–7 d after emergence) were dissected using forceps under a binocular microscope. The males were dissected on a solid pad of agarose medium in a Petri dish (90 mm diameter, 15 mm depth). The reproductive organs were removed from the abdomen. In addition, the thorax was separated from the head, abdomen and legs and used as a negative control. Excised tracts were stored separately in micro test tubes (1.5 ml) at –80°C. The frozen tracts (approximately 200 samples of each tract) were subsequently homogenized using glass rods and sonicated for 30–45 s in 500 µl of Milli-Q water in 15-ml glass test tubes. After centrifugation (12,000 rpm) at 4°C for 10 min, the supernatants were carefully removed. Each pellet was extracted twice with 250 µl of Milli-Q water, and the supernatants were pooled and lyophilized. After drying, the aqueous extracts were stored at –80°C.

To determine whether the extract of male reproductive organs affected female mating receptivity, virgin females (3 d after emergence) were chilled on ice for a few minutes to immobilize them and then placed on an agarose medium using fine forceps. The dried extracts of the male reproductive organs (ca 4.4 mg) and thorax (ca 7.9 mg) were separately dissolved in Milli-Q water to obtain 0.33 mg/µl solutions, and 0.05 µl of the resulting solutions (corresponding to 0.5–0.6 male) were injected into the abdominal tergites under the wings of each of the females using an extremely thin glass capillary connected to an oil-based pressure injection apparatus (Nanoject Auto-Nanoliter Injector, Drummond Scientific Company, PA, USA). Control females were injected with the same volume of Milli-Q water (0.05 µl). Immediately after injection, each female was transferred into a 50-ml glass vial containing five wheat seedlings for 30 min under laboratory conditions. An unmated male (3 d after adult emergence) was then introduced into each vial and the behaviour of the females (i.e., whether they were receptive to mating) was observed for 1 h. This experiment was replicated three times (9–15 replicates per trial) because it was difficult to obtain large numbers of *S. rubrovittatus* at any one time.

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 day 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. Then, the stir bars were removed from each extract and stored at –20°C prior to analysis using gas chromatography-mass spectrometry (GC-MS).

To determine the relative quantity of the three components in the sex pheromone released by each individual, GC-MS analysis

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 date 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 quntities 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 indicate that female mating receptivity differed among the treatments (χ^2 test, $\chi^2_2 = 13.011$, 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.

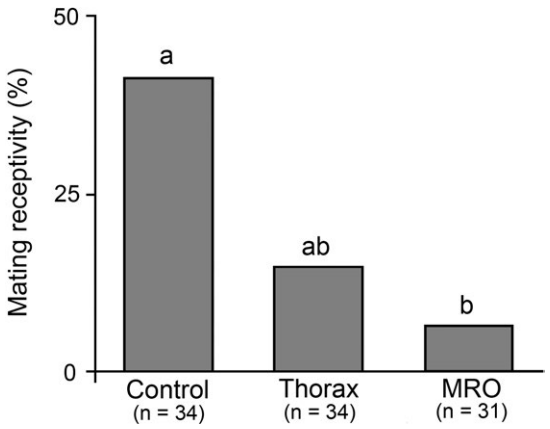

Fig. 1. Comparison of the mating receptivity of virgin females injected with either distilled water as a control, extract of male thorax as a negative control, or extract of male reproductive organs (MRO). Different letters indicate significant differences at P < 0.017 (post hoc Fisher exact test with Bonferroni correction).

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, $\chi^2_2 = 0.518$, 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%) (χ^2 test, $\chi^2_2 = 1.01$, 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.

	No. of receptive ♀ (no. of trials)			Heterogeneity		
	Replicate 1	Replicate 2	Replicate 3	G_H	df	P
Control	5 (10)	5 (15)	4 (9)	0.746	2	0.689
Thorax	2 (11)	3 (14)	0 (9)	3.416	2	0.181
MRO	0 (11)	1 (11)	1 (9)	1.705	2	0.426

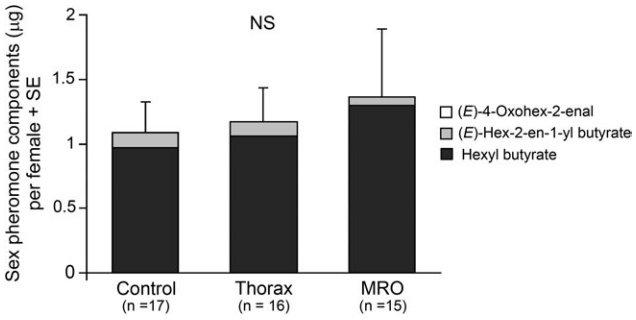

Fig. 2. Comparison of the relative quantities of the three components of the sex pheromone released by virgin females injected with either distilled water as a control, extract of male thorax as a negative control, or extract of male reproductive organs (MRO). NS means "not significant".

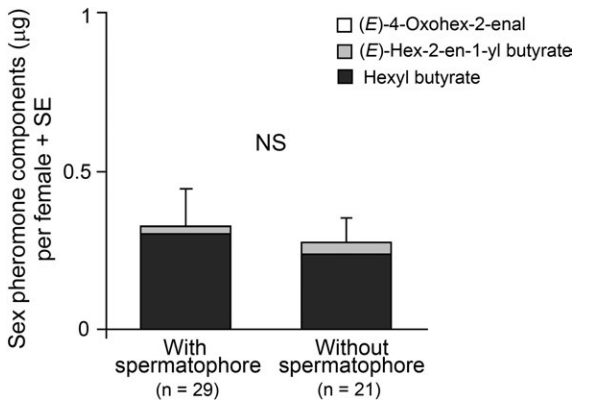

Fig. 3. Comparison of the relative quantities of the three components of the sex pheromone released by mated females with a spermatophore and those without a spermatophore. NS means "not significant".

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 unreceptive 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.

On the other hand, extracts of male organs did not affect the release of sex pheromone by virgin females and spermatophores did not affect that of sex pheromone by mated females. The relative quantities of the three components of the sex pheromone released by mated females (Fig. 3) were obviously lower than those recorded for virgin females (Fig. 2), which is consistent with the result of Oku & Yasuda (2010). Unlike in *L. hesperus* (Brent & Byers, 2011), the male-derived substances did not suppress the release of sex pheromone by *S. rubrovittatus* females. It is likely that the release of sex pheromone by females is controlled by other factors. If so, what might be the nature of such factors? It is possible that the physical stimulus of copulation causes physiological changes in females that suppress sex pheromone release. Furthermore, there is another possibility. In *Lygocoris pabulinus* (Hemiptera: Miridae), it is reported that hexyl butanoate, synthesized by both sexes, inhibits sex pheromone release by females (Groot et al., 2001). Such inhibition may be a strategy employed by males to prevent females from attracting other conspecific males. Males of *S. rubrovittatus* secrete the same compounds that are present in the female sex pheromone, although the proportions of the various compounds in the secretion differ between the sexes (Yasuda & Mochizuki, 2009). The male secretion of *S. rubrovittatus* may serve, as in *L. pabulinus*, to inhibit the release of sex pheromone by females. However, there is no evidence that either *S. rubrovittatus* or *L. pabulinus* males release such compounds during mating. Further studies are required to identify the factors controlling the release of sex pheromone by mated females of *S. rubrovittatus*.

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