Males of the seed bug Togo hemipterus (Heteroptera: Lygaeidae) use accessory gland substances to inhibit remating by females

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ABSTRACT

In species in which females mate repeatedly, males can adopt several strategies to reduce the risk of sperm competition with future males. The refractory period of females significantly increased as the mating duration increased in the seed bug *Togo hemipterus* (Heteroptera: Lygaeidae). To elucidate the mechanisms by which mated females are inhibited from remating, we investigated the effects of male-derived substances on the inhibition of mating receptivity of virgin females by injecting the substances into their abdomens. The length of time from injection to mating in virgin females was significantly longer for females injected with accessory gland B solution than for those injected with seminal vesicle, accessory gland A, or control solutions. This is the first report showing that heteropteran males inhibit female remating by using substances from an accessory gland. We discuss and consider the adoption and evolution of this strategy by *T. hemipterus* males by focusing on female genitalia structures, oviposition habit, and paternity and comparing these traits with those of other heteropterids.

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1. Introduction

Multiple mating by females occurs in many species. Among the many explanations that have been advanced for this behavior are the reduced risk of infertility, the benefits of material contributions from several males, and the genetic benefits for offspring (reviewed in Ridley, 1988; Andersson, 1994; Arnqvist and Nilsson, 2000; Arnqvist and Rowe, 2005). However, multiple mating by females increases both the risk and intensity of sperm competition for males. In species in which females mate repeatedly and sperm competition exists, the first male to mate has gains in reproductive fitness if his mate remains sexually unreceptive temporarily or permanently, thereby averting competition by sperm from other males. Thus, the ability to temporarily or permanently inhibit female receptivity to further mating represents a highly adaptive strategy to reduce the risk of sperm competition (Simmons, 2001). The optimal remating rate differs between males and females, resulting in a conflict of interest between sexes that could lead to sexually antagonistic coevolution (Parker, 1979; Rice, 1996; Chapman et al., 2003a). Ultimately, the level of female remating could affect the relative strengths of pre- and post-copulation sexual selection (Markow, 2002).

Reduced female receptivity in insects has been shown to be caused by several mechanisms, including physical stimulation,

sperm, mating plugs, mate guarding, spermatophores, and substances secreted from accessory glands (reviewed in Simmons, 2001; Chapman and Davies, 2004; Arnqvist and Rowe, 2005). The mechanisms of reduced female receptivity have been studied extensively in *Drosophila melanogaster*. Female copulatory inhibition has been shown to be caused by both the sex peptide 70, a product of the male accessory gland (Chen et al., 1988; Aigaki et al., 1991; Kubli, 2003; Gillott, 2003; Chapman and Davies, 2004), and sperm (Manning, 1962, 1967; Gromko et al., 1984; Scott, 1987). In other dipteran species, sperm and/or seminal fluid substances may inhibit female remating (Miyatake et al., 1999; Mossinson and Yuval, 2003; Radharkrishnan and Taylor, 2007). The mechanisms in other insect taxonomic groups have not been well investigated (but see Gillott, 2003; Wedell, 2005; Yamane et al., 2008), but several studies have reported that mating often reduces female receptivity to subsequent mating (reviewed in Simmons, 2001).

In hemipteran species, Heady (1993) reported that a substance in the ejaculate of planthopper *Prokelisia dolus* (Homoptera) decreased female calling behavior, thus sexually inhibiting females. Although relative few studies of mating inhibition via ejaculate substances have been conducted in hemipterids, none have been performed on heteropterids. However, many studies have reported that males undertake post-copulatory mate guarding of females (after ejaculation) to reduce the risk of sperm competition (e.g., Sillén-Tullberg, 1981; Carroll and Loye, 1990; Hosokawa and Suzuki, 2001). A male strategy to ensure paternity is very important in hemipterids, due to high P_2 values (mean, 44–100%; reviewed by Simmons, 2001). In general, the post-copulatory mate guarding hypothesis predicts that

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males guard mates until oviposition or do not guard at all (Yamamura, 1986). Some oviposition habits affect their decisions whether or not to adopt a strategy of post-copulatory mate guarding (Yamamura, 1986; Alcock, 1994). Thus, oviposition habits can shed light on how the strategy for ensuring paternity evolved.

Our preliminary observations showed that mating pairs of *T. hemipterus* are rarely observed either in the field or in rearing stocks because of the female refractory period and short copulatory duration. Moreover, forced copulation cannot occur in *T. hemipterus* females because of the morphology of female sexual organs. Female genitalia are usually folded against the abdomen. When the female decides to accept a male, she extends her genitalia, which the male then grips with his claspers, and copulation is achieved. Thus, the *T. hemipterus* female is a suitable subject for investigation of the refractory period and other inhibitors of mating.

We hypothesized that *T. hemipterus* males inhibit female remating through their seminal fluid. We investigated the relation between mating duration and refractory period by adjusting mating duration artificially and the effects of male-derived substances on the inhibition of mating receptivity of virgin *T. hemipterus* females by injecting these substances into their abdomens.

It has not been previously studied why males do not use ordinary strategies such as post-copulatory mate guarding but instead using a seminal fluids. To elucidate why *T. hemipterus* males may have adopted this strategy to reduce the risk of sperm competition and how it evolved, we focused on female genital structures, oviposition habits, and paternity and compared these traits with those of other heteropterids.

2. Materials and methods

2.1. Insect

The *T. hemipterus* adults used in this study were originally derived from field-collected females on the campus of Kyoto University, Kyoto, western Japan (35°01′N, 135°46′E). Nymphs were reared in jars (0.43 l, top: 120 mm diameter, bottom: 100 mm diameter, 55 mm height) containing wet sand to maintain moderate humidity; the entrances were covered with nylon mesh. We provided distilled water and fresh brown rice as food every 4–5 days during each developmental stage. The insects were maintained under a 16-h light:8-h dark photoperiod (light period, 07:00–23:00 h) at 25 ± 2 °C. After the imaginal molt and cuticular hardening, we transferred the insects to the jars containing wet cotton and brown rice. Five days later, we segregated them by sex to prevent mating prior to experiments. Individuals of the same sex and age were housed together for 20 days, with no more than 6 individuals per jar. The pre-reproductive period of this species is approximately 25 days at 25 °C; the mating season then continues for about 3 months (Himuro, unpublished data). In all experiments, we used sexually mature virgin individuals.

2.2. Relation between mating duration and refractory period

For the experiments, we put a male and female in a plastic case (50 mm × 50 mm × 25 mm) with a grain of brown rice and wet cotton and recorded their behavior for 4 h (13:00–17:00 h), using a digital video camera (SONY DCR-PC120 NTSC) under constant light at 25 ± 2 °C. If mating occurred, we recorded the mating duration with a stopwatch (CBM HS01). At first, we examined the natural mating duration ($n = 45$). Then, to examine the relation between mating duration and refractory period, we separated mating pairs, using forceps and/or by hand, to stop mating after 10, 20, 30, or 60 min (each $n = 10$), along with matings that were not disturbed ($n = 33$). Thereafter,

the females were removed and reared individually in Petri-dishes (90 mm diameter, 20 mm depth) containing wet cotton and fresh brown rice. The day after mating, we placed the mated female and a male in a plastic case (50 mm × 50 mm × 25 mm) with a grain of brown rice and wet cotton for 2 h (13:00–15:00 h) every day and recorded the refractory period: the period between one mating and the next. We also examined the refractory period for males, using the same methods, the day after mating, we placed the mated male and a virgin female in a plastic case every day and recorded the refractory period ($n = 33$).

2.3. Effects of accessory gland and seminal vesicle

At 20–25 days after the imaginal molt and cuticular hardening, adult virgin males were anesthetized using CO_2 and dissected in saline (7.5 g NaCl, 0.35 g KCl and 0.21 g $CaCl_2$ per 1.0 l distilled water, pH 5.5) under a binocular microscope (Nikon SMZ645). The reproductive tracts, including accessory glands A and B, seminal vesicle, testis, and ejaculatory duct (Fig. 1) were removed with forceps. We put each type of accessory gland and the seminal vesicles excised from five males in separate Eppendorf tubes (1.5 ml) with 20 μl saline. Each extract was homogenized with a forceps and a microtube pestle.

At 20–25 days after the imaginal molt and cuticular hardening, adult virgin females were anesthetized using CO_2 and injected with 100 nl of one of the homogenized solutions or saline between the 5th and the 6th ventral abdomen segments, using a fine glass capillary tube with an extremely thin head connected to an oil pressure injection machine (NANOJECT II Auto-Nanoliter Injector; Drummond Scientific, Broomall, PA) (accessory gland A solution, $n = 35$; accessory gland B solution, $n = 32$; seminal vesicle solution, $n = 40$; control, $n = 41$). The injections were conducted in a laboratory maintained at 25 °C. After injection, females were reared individually. Beginning the next day, we recorded the period between injection and mating, using the methods described above, and observed behaviors of females and males.

To examine the effect of heat on the accessory gland B solution, we put glands from 5 males in an Eppendorf tube (1.5 ml) with 20 μl saline and boiled the mixture at 100 °C in a water bath for 40 min. We then cooled the solution for 1 h and injected it into
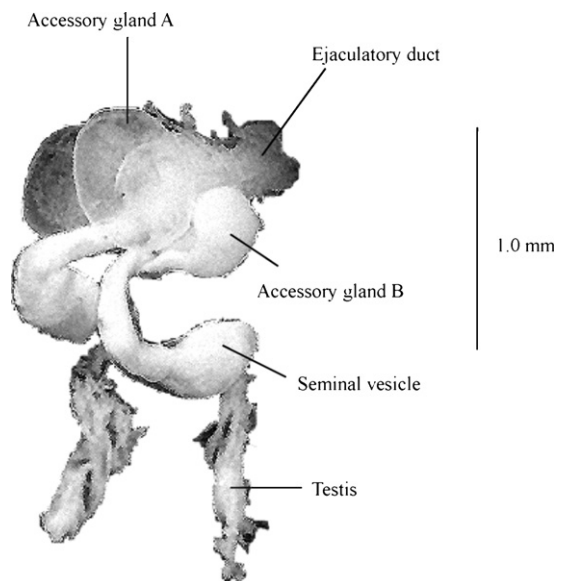

Fig. 1. Male reproductive tract of *Togo hemipterus*.