SEXUAL CONFLICT OVER REMATING IN HOUSE CRICKETS: NO EVIDENCE OF AN ANTI-APHRODISIAC IN MALES' EJACULATES

by

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Summary

We tested the hypothesis that male house crickets (*Acheta domesticus* L.) transfer substances in their ejaculates that inhibit female receptivity by experimentally manipulating the amount of ejaculate that females received and recording their propensity to remate. In both virgin and non-virgin females, the length of time over which the spermatophore remained attached after an initial mating had no discernable effect on female latency to remating. This was true regardless of whether females were given the opportunity to remate immediately after an initial mating or prevented from remating until 24-h later. We conclude, therefore, that male *A. domesticus* do not transfer substances in their ejaculates that inhibit the sexual receptivity of females, at least over the short term. However, there was a marked difference in the remating propensity of once-mated and multiply-mated females, with multiply-mated females taking significantly longer to remate. These results suggest that female sexual receptivity changes in response to the gradual accumulation of sperm or ejaculatory products in the female's spermatheca over multiple matings.

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Introduction

Sexual conflict arises when the reproductive interests of males and females do not coincide, and leads to the evolution of adaptations that enhance the fitness of individuals of one sex at the expense of the other (Parker, 1979; Rice & Holland, 1997; Stockley, 1997; Partridge & Hurst, 1998). Male and female reproductive interests may differ with respect to the occurrence and frequency of copulations, paternity of offspring, and the extent of parental care (review in Brown *et al*., 1997). One issue over which the reproductive interests of males and females often diverge concerns the subsequent mating activity of the female. Females often benefit by remating with other males because this may result in the replenishment of depleted sperm stores, the acquisition of material benefits from the male, or the acquisition of indirect genetic benefits (review in Thornhill & Alcock, 1983; Jennions & Petrie, 2000). Females may also obtain direct benefits via polyandry, such as increased fertility and offspring production (Arnqvist & Nilsson, 2000).

Female polyandry is, however, invariably detrimental to male reproductive interests because of sperm competition, which selects for two kinds of adaptations in males: a) those that allow males to incapacitate the sperm of rival males, and b) those that function to reduce the female's propensity to remate (Parker, 1970). In recent studies, substances in males' ejaculates that stimulate oviposition and reduce female receptivity to future matings have been identified in several species (review in Cordero, 1995). Such adaptations are beneficial to males because they increase their genetic representation in the offspring produced by their mates, but they may also impose fitness costs on females (Stockley, 1997).

Females responding to receptivity-inhibiting substances contained in males' ejaculates necessarily forego some of the benefits of multiple matings. Similarly, costs may be incurred through the receipt of substances in the male's ejaculate that promote oviposition, as the female may not be physically ready to allocate nutrients to egg maturation and oviposition at a time that is optimal from the male's standpoint. These costs may lead to a sexual conflict over the timing of remating and/or oviposition, and may favour the evolution of female resistance to male-derived anti-aphrodisiacs or other hormonal substances (Holland & Rice, 1998).

Sexual conflicts over substances transferred in the male's ejaculate are apparent in many insect species. In *Drosophila melanogaster*, accessory-gland

products in the male's ejaculate cause a transient increase in the female's egg laying rate (Xue & Noll, 2000; Heifetz *et al*., 2001), and also decrease her lifespan (Fowler & Partridge, 1989; Chapman *et al*., 1995). Receptivity in female *D. melanogaster* is also temporarily inhibited by accessory gland products transferred with the ejaculate (Xue & Noll, 2000). Similarly, in the pierid butterfly (*Pieris napi*), methyl-salicylate is transferred to females during copulation, a substance that has been shown to reduce both female receptivity and the number of males willing to court the female (Andersson *et al*., 2000).

In crickets, a similar conflict of interest between the sexes is evident. Females typically mate with multiple males, behaviour that is detrimental to each of her mate's reproductive interests. Females also control the fate of their mates' ejaculates. In crickets, copulation is completed with the transfer of a spermatophore, which remains attached outside the female's body after mating. It can take up to an hour for the complete evacuation of the spermatophore, and females frequently remove and eat the spermatophore before sperm transfer has been completed (Sakaluk, 1984, 2000; Simmons, 1986). In addition to sperm, males may transfer chemical substances in the spermatophore, such as prostaglandin synthetase (Destephano & Brady, 1977). This substance is important in activating prostaglandin-synthesizing enzymes (PGE and $PGF_{2\alpha}$) in the female, which promote short-term oviposition behaviour (Murtaugh & Denlinger, 1982, 1987). Females have been found to respond to prostaglandin synthetase in a dosage-dependent manner: the longer the spermatophore remains attached, the more stimulus they receive and the more eggs they lay on the days following copulation (Destephano *et al*., 1982; Murtaugh & Denlinger, 1985). Female crickets (*Acheta domesticus*, *Gryllus bimaculatus* (De Geer), *Gryllus integer*, and *Teleogryllus commodus*) have also been observed to show a reduction in phonotaxis after mating, suggesting that substances transferred in the male's ejaculate reduce female receptivity to future matings (Cade, 1979; Loher, 1981; Koudele *et al*., 1987; Loher et al., 1993; Lickman et al., 1998).

Here we test the hypothesis that male house crickets (*Acheta domesticus* L.) transfer substances in their ejaculates that reduce females' propensity to remate, by experimentally manipulating the duration of spermatophore attachment and hence, the extent to which females are inseminated. If substances contained in males' ejaculates cause females to be less receptive to remating, we would expect to find a positive relationship between the

duration of spermatophore attachment of an initial mating and the time to remating. However, our results show no discernable effect of varying spermatophore attachment duration on the female's remating propensity, although females that were virgin prior to their initial mating showed a greater propensity to remate than did sexually experienced females.

Methods

Crickets were obtained from Fluker Farms® (Baton Rouge, LA) and housed in ventilated 55-litre plastic containers. Late-instar females were held in a separate terrarium to ensure their virginity upon the adult moult. All crickets were provided with egg cartons for shelter, and provisioned with food (Fluker's® cricket chow) and water (supplied in small plastic vials plugged with cotton wicks) *ad libitum*.

Experiment 1: Do ejaculatory products lead to the immediate inhibition of female receptivity?

To test the hypothesis that substances in males' ejaculates immediately lead to a reduction in female receptivity, four experimental treatments were established in which the spermatophore was experimentally removed at varying times after mating: 0, 5, 15, and 60 minutes. These treatments correspond to the full range of ejaculate transfer, as it typically takes approximately 60 minutes for the complete evacuation of the spermatophore (Sakaluk, 2000). Thirty females were randomly assigned to each of the 4 treatments, 15 of whom were initially virgin and 15 of whom were sexually experienced (total $N = 120$). Sexually experienced females were held continuously with males from days 7 to 9 of their adult life prior to the initial experimental mating on day 10. We ensured that the females gained sexual experience during this period using time-lapse video recording to quantify the number of matings that typically occur. When held in mating chambers with *ad libitum* food and water and constant access to two males, females mated, on the average, 3.1 times ($N = 7$) during the 48-hour period.

Matings were staged in specially constructed, Plexiglas viewing chambers ($7.7 \times 10.6 \times 3.4$ cm) observed under red-light illumination. Ten-day-old adult females were randomly assigned to experimental treatments, and paired with a randomly selected, sexually experienced male of similar age ($\pm$ 2 days). Each pair was observed for four hours or until copulation had occurred, after which the spermatophore was removed with fine forceps according to the prescribed treatment. Females that failed to copulate within the four-hour observation period were discarded. For the zero-minute-attachment treatment, the spermatophore was removed immediately after the female dismounted the male. For the 5, 15, and 60-minute-attachment treatments, females were prevented from removing the spermatophore before the prescribed time had expired by confining them in narrow test tubes. Following their initial copulation, females were paired immediately with a different male, and given four hours in which to complete a second mating. For each female, we recorded the time taken to complete the second mating in relation to the time at which the male first exhibited stereotypic courtship behaviour (Alexander & Otte, 1967).

If substances contained in males' ejaculates reduce female sexual receptivity, we would expect to see a positive relationship between spermatophore attachment duration and the

time to remating. If singly-mated females have a higher sexual motivation to remate than multiply-mated females owing to inadequate sperm stores, we might expect the effect of early spermatophore removal to have less influence on latency to remating on females that are initially virgin, than those with prior mating experience.

Experiment 2: Do ejaculatory products have a time-delayed effect on female receptivity?

This experiment was designed to test if substances in the ejaculate have a time-delayed effect on female receptivity. The experimental protocol was similar to that of the first experiment with four key alterations: 1) females were immediately isolated after their initial mating for a 24-h period, 2) they were re-paired with the same male the following day, 3) if the female failed to remate during the 4-h observation period, she was re-paired the following day (and each subsequent day to a maximum of three days) until she remated, and 4) the sample size was increased, with eighty females randomly assigned to each of the 4 treatments, 40 of whom were initially virgin and 40 of whom were sexually experienced (total $N = 320$).The same males were used for rematings because unlike experiment 1, males had ample time to replenish their spermatophores. For each female, we recorded the time taken to complete the second mating in relation to the time at which the male first initiated courtship.

Statistical analyses

To determine the effect of spermatophore attachment on remating propensity, we used PROC LIFETEST in SAS (version 8.02), which permits the inclusion of right-censored data (*i.e.*, observations in which females had not remated by the end of the observation period) (Allison, 1995). Within each mating status (virgin and non-virgin) and in comparisons between virgin and non-virgin females, differences in remating propensity were assessed using the log-rank test. In the experiment in which females were denied the opportunity to remate until 24-h after the initial mating, there was a significant correlation between the latency to their initial mating and their latency to remate ($F_{1,318} = 21.62$, $p < 0.0001$). Thus, the analysis for these females was performed on the adjusted residual output from a regression of initial mating latency on remating latency. This correlation did not arise for females given an immediate opportunity to remate, and thus no comparable adjustment was made for these females ($F_{1,118} = 1.41$, NS).

Results

Manipulation of the duration of spermatophore attachment had no discernable effect on the time to remating, regardless of mating status or the time over which females were given an opportunity to remate (Table 1, Fig. 1). However, non-virgin females took significantly longer to remate than did virgin females, both when females were given an immediate opportunity to remate (Log-rank $\chi^2 = 8.57$, $p = 0.0034$), and when females were prevented from remating until 24-h after the initial copulation (Log-rank $\chi^2 = 90.35$, $p < 0.0001$) (Table 1, Fig. 1).

TABLE 1. *Log-rank tests for differences in female remating propensity arising from varying spermatophore attachment durations*

Mating status	Immediate remating opportunity		Subsequent day(s) remating opportunity	
	Virgin	Non-virgin	Virgin	Non-virgin
Log rank χ^2 (p) within-mating	1.61 (0.657)	0.93 (0.8182)	1.32 (0.7254)	0.81 (0.8471)
Log rank χ^2 (p) between-status	8.57 (0.0034*)		90.35 (<0.0001*)	

If the effect of any receptivity-inhibiting substances in males' ejaculates was all-or-none rather than dosage-dependent, we might expect to see a difference in the remating time of those females whose spermatophores were removed immediately and those that received at least some portion of the male's ejaculate, but we would not expect any differences to emerge between those females experiencing varying degrees of spermatophore attachment. To test for this possibility, we performed a post-hoc comparison between the remating times of females that received no ejaculate (spermatophore removed immediately) with females that were designated to receive at least some ejaculate (5, 15, and 60-minute spermatophore attachment durations). There was no significant difference in latency to remating between the two groups irrespective of mating status (virgin or non-virgin) and regardless of whether females were given the opportunity to remate immediately or 24-h later (Log-rank χ^2, all $p > 0.4$).

Discussion

In both virgin and non-virgin females, the length of time over which the spermatophore remained attached after an initial mating had no discernable effect on female latency to remating. This was true regardless of whether females were given the opportunity to remate immediately or prevented from remating until 24-h later. We conclude, therefore, that male *A. domesticus* do not transfer substances in their ejaculates that inhibit the sexual receptivity of females, at least in the short term. This conclusion is consistent with work conducted on another cricket species, *G. bimaculatus*: Orshan & Pener (1991) found no difference in the sexual receptivity of females whose spermatophores were experimentally removed from 0-10 min after mating, and

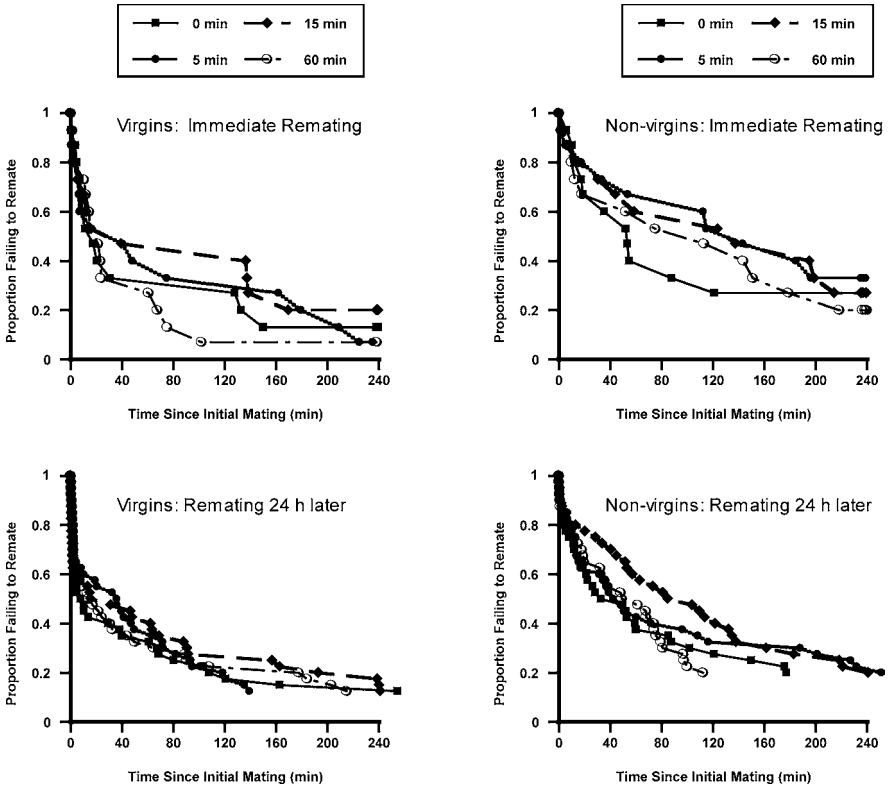

Fig. 1. Survival distribution for latency to remate as a function of the spermatophore attachment duration in the previous mating. Trajectories shown for females given an opportunity to remate 24 h later (Experiment 2) have been truncated at 240 min to allow ease of direct comparison with those given an immediate opportunity to remate (Experiment 1). Although varying spermatophore attachment duration had no effect on female remating propensity, females that were virgin upon their initial mating remated significantly sooner than sexually experienced females in both experiments.

those permitted to retain the spermatophore before voluntarily removing it. It could be, however, that male-derived ejaculatory substances diminish a female's long-distance phonotactic response to male calling song, even if they do not influence her decision to mate. While we cannot rule out this possibility, it seems unlikely that any such substance could reduce a female's response to male calling song without also diminishing her response to the courtship song that is required to induce females to mount males (Alexander & Otte, 1967). In fact, experimental work has shown that female *A. domesticus* exhibiting a willingness to mount males almost invariably show a

positive phonotactic response to male calling song (Stout *et al*., 1976). Moreover, house crickets in nature often occur in dense aggregations (Bate, 1969), which would tend to reduce the effectiveness of any inhibitory substance designed solely to eliminate female phonotaxis.

In *A. domesticus*, the efficacy of oviposition-promoting substances contained in male's ejaculate becomes evident even when the spermatophore is removed 2-3 minutes after its initial attachment (Murtaugh & Denlinger, 1985). If putative receptivity-inhibiting substances were transferred as quickly as oviposition stimulants and if their effect on female receptivity was not dosage dependent, this would obscure any differences between the treatments in which female received at least a portion of the male's ejaculate (*i.e.* the 5, 15, and 60-min spermatophore attachment treatments). However, a post-hoc comparison between the remating times of females that received no ejaculate (spermatophore duration = 0 min) and those that received at least some ejaculate showed no difference between the two groups, so that the absence of a treatment effect cannot be attributed to the lack of a dosage-dependent effect.

The results of our study are in apparent contradiction of an earlier report of the effect of varying spermatophore attachment duration on female receptivity in *A. domesticus.* Sakaluk & Cade (1980) recorded the daily mating activity of 15-20 individually marked females placed with the same number of males in a mating arena, larger than the one used in the present study (78 × 49 × 10 cm), and monitored continuously over a 2-h observation period after which the males were removed. They compared the initial spermatophore attachment durations of females that took varying amounts of time to complete their second matings, and found that females that remated within the same observation period or one day later, had significantly shorter spermatophore attachment durations than females that took a greater number of observation periods to complete their second matings. However, Sakaluk & Cade's (1980) study was strictly correlational, and if the proximate factor promoting rapid mating by females was the same one inducing early spermatophore removal, then the correlation could be regarded as spurious. In the present study, spermatophore attachment durations were imposed on females irrespective of the time at which the females would have chosen to remove the spermatophore, so we regard the results of our study as definitive in ruling out any male-derived receptivity-inhibiting substances in the spermatophore.

In contrast to work on crickets, several studies of the closely-related katydids (Orthoptera: Tettigoniidae) have reported a significant correlation between the length of the female's refractory period after an initial copulation and the duration of spermatophore attachment (Gwynne, 1986; Wedell & Arak, 1989; Simmons & Gwynne, 1991). In at least two of these species, it is suspected that induction of the female refractory period is chemically mediated because the reestablishment of receptivity was not associated with depletion of sperm stored in the female's reproductive tract (Gwynne, 1986; Simmons & Gwynne, 1991). However, although a non-sperm factor in the ejaculate is suspected to induce the non-receptivity, a specific component has yet to be isolated or identified. Why katydids and crickets should differ with respect to receptivity-inhibiting substances contained in males' ejaculates remains unclear, but it may be related to another pervasive feature of katydid mating systems: in a number of species, males provision females with an enlarged, nutrient-rich spermatophore, the consumption of which greatly enhances female reproductive output (Gwynne, 1984; Simmons, 1990; Reinhold, 1999). Insofar as the provision of such nuptial gifts severely constrains male mating success (*e.g.* Gwynne, 1990; Simmons, 1994; Jia *et al*., 2000), selection may have more strongly favoured the evolution of receptivity-inhibiting substances to prevent a male's nutritional investment from being diverted to the offspring of a female's subsequent mating partners. Alternatively, the manipulation of female receptivity via substances contained in males' ejaculates may have been a necessary antecedent to the evolution of such costly gifts.

Although varying spermatophore attachment duration had no effect on the time taken by females to remate, there was a marked difference in the remating propensity of once-mated and multiply-mated females, with multiply-mated females taking significantly longer to remate whether given the opportunity to remate immediately after an initial mating or 24 h later. This result is consistent with results of previous studies showing that both the phonotactic responsiveness and sexual receptivity of non-virgin female crickets is diminished relative to virgins (Cade, 1979; Loher, 1981; Koudele *et al*., 1987; Loher *et al*., 1993; Lickman *et al*., 1998). These results suggest that female sexual receptivity changes in response to the gradual accumulation of sperm or ejaculatory products in the female's spermatheca over multiple matings. This accumulation could influence the female's own endocrine system in either of two ways: (1) the physical expansion of the spermatheca could trigger stretch

receptors that effect a change in the level of hormones regulating receptivity (Sugawara, 1979), or (2) chemical 'messengers' in the male's ejaculate could signal the receipt of sperm, activating that component of the female's endocrine system controlling oviposition and sexual receptivity (review in Eberhard, 1996). It is known, for example, that juvenile hormone titres increase dramatically in mated females that have been provided with a suitable oviposition substrate (review in Loher & Zaretsky, 1989), and that juvenile hormone levels moderate both female phonotaxis and sexual receptivity (review in Strambi *et al*., 1997). The oviposition-promoting substances passed from males to females in the ejaculate promote short-term oviposition, but it has been suggested that long-term control of oviposition behaviour may be dependent on having both prostaglandin and sperm stored in the female's reproductive tract (Murtaugh & Denlinger, 1987). Also, when the female's spermatheca is denervated, the effect of mating on oviposition is abolished (Murtaugh & Denlinger, 1985). This suggests that there is neuronal feedback between the spermatheca and the region of the nervous system controlling at least some aspects of female reproductive behaviour.

If our interpretation concerning the difference in mating propensity of virgin and non-virgin females is correct, it raises an obvious conundrum: if the female refractory period is mediated by the accumulation of sperm and accessory-gland substances, then why did we not see a decrease in the remating propensity of those experimental females that received sperm relative to those that did not? The answer must be that the time over which changes in hormone titres effect a change in female behaviour must, at the very least, exceed the 24-h period over which rematings were staged. In support of this possibility, Koudele *et al*. (1987) found no difference in the phonotactic responsiveness of virgin females and mated female *A. domesticus* tested up to 24 h after a single mating.

The absence of any immediate or short-term effects of varying ejaculate transfer on female receptivity suggests either that males do not transfer receptivity-inhibiting substances in their ejaculates, or that females have evolved resistance to any such substances as might be expected if males and females were locked into a recurrent cycle of antagonistic coevolution (Holland & Rice, 1998). If the former is true, it may be that the costs of manufacturing an anti-aphrodisiac exceed the benefits of inducing a temporary reduction in female receptivity, particularly if males are assured of some fertilization success irrespective of a female's subsequent mating activity. In

crickets, a male's fertilization success is dependent largely on the number of sperm that he transfers because the sperm of a female's various mating partners are recruited for fertilizations in direct proportion to their relative abundance in the female's spermatheca (Sakaluk, 1986; Simmons 1987; Sakaluk & Eggert, 1996). This means that males can anticipate some fertilizations so long as they have some sperm represented in a female's spermatheca. In contrast to this pattern, most species of insects show a high degree of last-male sperm precedence (Simmons & Siva-Jothy, 1998), in which males lose all prospects of future paternity once a previous mating partner remates. Although we know too little about the distribution of receptivity-inhibiting substances across insects to make a definitive test, we predict that such substances are most likely to occur in species exhibiting last-male sperm precedence than in species, like crickets, in which fertilizations are determined by lottery.

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Zusammenfassung

Wir testeten die Hypothese, daß männliche Heimchen (*Acheta domesticus* L.) in ihrem Ejakulat Substanzen übertragen, die die Rezeptivität der Weibchen einschränken, indem wir experimentell die einem Weibchen übertragene Menge an Ejakulat manipulierten und ihre anschliessende Tendenz zur erneuten Paarung kontrollierten. Sowohl bei jungfräulichen als auch nicht-jungfräulichen Weibchen hatte die Länge der Zeitspanne, über die die Spermatophore nach der anfänglichen Paarung ans Weibchen angeheftet blieb, keinen Effekt auf die Latenzzeit bis zu einer erneuten Kopulation des Weibchens. Dieses Ergebnis war unabhängig davon, ob die Weibchen sofort nach der ersten Paarung oder erst 24 h später Gelegenheit zu einer erneuten Paarung erhielten. Wir schließen daraus, daß männliche *A. domesticus* in ihrem Ejakulat keine Substanzen übertragen, die die Rezeptivität der Weibchen einschränken, jedenfalls keine kurzfristig wirkenden. Es gab aber einen deutlichen Unterschied in der Wiederverpaarungstendenz von jungfräulichen und nicht-jungfräulichen Weibchen, wobei nicht jungfräuliche Weibchen sehr viel später erneut kopulierten. Diese Ergebnisse lassen vermuten, daß die Rezeptivität der Weibchen sich nur allmählich über mehrere Paarungen hinweg verändert, vielleicht durch die Akkumulation von Spermien oder ejakulatorischer Produkte im Fortpflanzungstrakt des Weibchens.