Animal Behaviour 77 (2009) 887–892

Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/yanbe

The influence of male ejaculates on female mate search behaviour, oviposition and longevity in crickets

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ARTICLE INFO

Article history:
Received 1 July 2008
Initial acceptance 3 October 2008
Final acceptance 20 December 2008
Published online 3 February 2009
MS. number: 08-00415

Keywords:
accessory gland
acoustic communication
acp
cricket
Gryllus bimaculatus
oviposition
phonotaxis
sexual selection
sexual conflict
spermatophore

In animals with internal fertilization, sperm are transferred in ejaculates, which include water and proteins produced by male accessory glands. These proteins help to protect and facilitate sperm passage, and in some species have been identified as having an influence on female behaviour and life history traits. They may increase oviposition rate, reduce sexual receptivity and decrease female life span. Virgin female field crickets *Gryllus bimaculatus* orient and move towards calling song produced by males. However, phonotaxis is greatly reduced after mating. We tested the hypothesis that female phonotaxis, oviposition and longevity are influenced by compounds in male ejaculates. We divided females into two groups: one injected with seminal proteins extracted from spermatophores from which sperm had been removed, and one injected with Ringer's solution. We measured female egg laying and phonotaxis before and after treatment, and recorded female longevity. We did not detect an effect of treatment on either egg laying or phonotaxis. However, females treated with seminal proteins moved less overall and died sooner than females in the control group. We therefore failed to find any evidence that postmating reductions in phonotaxis are due to effects of male seminal proteins. However, the reduction in female movement after treatment with seminal proteins could reduce their likelihood of subsequent matings.

The evolution of differences in gamete size between the sexes (anisogamy) is the basis of a fundamental dichotomy in reproductive investment between males and females. Females are typically the limiting sex, producing relatively few 'expensive' gametes, whereas males often produce very large numbers of sperm. The resulting competition for fertilizations underpins sexual selection and can also drive antagonistic coevolution between the sexes when male adaptations to intrasexual competition have detrimental effects on the reproductive success of their mates (Parker 2006). One source of such conflicts is situations where males attempt to manipulate the behaviour of their mate away from the optimum for her reproductive success. Such manipulation may include direct intervention by males to affect female behaviour. For instance, males of *Gryllus bimaculatus* prevent immediate female remating by mate guarding (Wynn & Vahed 2004), while male scorpions *Vaejovis punctatus* prevent female remating by using mating plugs (Contreras-Garduno et al. 2006). Males may also engage in less obvious manipulations by introducing compounds in seminal fluids, produced in the accessory glands, that influence female behaviour (Gillott 2003).

In *Drosophila melanogaster*, substances transferred in male ejaculate, accessory gland proteins (Acps), have been shown to reduce female sexual receptivity and longevity, and enhance egg production, ovulation and sperm storage (Wolfner 2002). Many of these Acps have targets within the reproductive tract; however, some enter the haemolymph and target other receptors (Ottiger et al. 2000). To date, most work concerning seminal products has been focused on detailed identification and functional analysis of those of *D. melanogaster*. However, if the evolution of manipulative compounds is driven by postmating selection, it is possible that they could be present in any species in which the female mates multiply. Females of the field cricket *G. bimaculatus* mate with multiple males over their reproductive life (Bretman & Tregenza 2005). Hence there is ample opportunity for postmating sexual selection. Additionally, Andres et al. (2006) found that, like seminal proteins transferred by male *D. melanogaster*, the seminal proteins of some gryllid species are also rapidly evolving and positively selected. Existing studies of field crickets suggest that in at least some species, male ejaculates may influence female oviposition; Destephano & Brady (1977) found a role for prostaglandins in egg production in the house cricket, *Acheta domesticus*. Furthermore,

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0003-3472/$38.00
doi:10.1016/j.anbehav.2008.12.017

Loher et al. (1981) found that males of *Teleogryllus commodus* transfer a prostaglandin synthesizing complex to females in their ejaculate. This complex synthesizes the production of PGE_2 from the precursor arachidonic acid, which subsequently stimulates ovipositional behaviour. There have been fewer studies concerning the effects of seminal proteins on sexual receptivity. Fleischman & Sakaluk (2004) found no evidence of an effect of spermatophore contents on sexual receptivity in *A. domesticus*. However, spermatophylaces given as nuptial gifts by male decorated crickets, *Gryllodes sigillatus*, and consumed by their mates, appear to contain substances that inhibit their sexual receptivity. Sakaluk et al. (2006) found that female *A. domesticus*, a non-nuptial gift-giving species, had reduced sexual receptivity after consuming the nuptial gift of *G. sigillatus*, whereas females of *G. sigillatus* had no such reduction. They concluded that conspecific females had evolved resistance to the manipulative compound, but that to maintain positive selection for its production, there must be variation in this resistance within populations of females.

In many species of orthopteran, male ejaculates provide nutritional benefits to females generally through nuptial gifts (Vahed 1998). However, ejaculates may also be harmful to females. If ejaculate components have a positive effect on male reproductive success through increasing the proportion of a female's investment that goes into their offspring, they will be favoured by selection even if they reduce the female's overall reproduction (interlocus sexual conflict, Tregenza et al. 2006; Wedell et al. 2006). There is strong evidence for such a conflict leading to a reduction in life span in female *D. melanogaster* (Chapman et al. 1995), but in field crickets, where the spermatophore is not accompanied by a nutritional gift, potential direct costs or benefits of ejaculates have received limited attention. Wagner et al. (2001) found that in *Gryllus lineaticeps,* females mating repeatedly lived longer than females mating only once. In contrast, in *G. bimaculatus*, Bateman et al. (2006) found that contact with males reduced female longevity but that there was no difference in life span between females where the spermatophore was removed immediately after mating and those where it was retained, suggesting a lack of any direct effects of ejaculates on longevity in this species.

Field cricket mate search behaviour is characterized by calling males and silent, phonotactic females. Females typically become sexually receptive and phonotactic a few days after adult eclosion. However, after mating, female attraction to male song is reduced; this effect was found in both *T. commodus* (Loher et al. 1981) and *Gryllus integer* (Lickman et al. 1998). In *G. bimaculatus*, phonotaxis is reduced after mating, but there is no reduction when spermatophores are removed before transferral of spermatophore contents, and furthermore, phonotaxis is restored when the ventral nerve cord is severed (Loher et al. 1993). It therefore appears that reduction in female phonotaxis is either triggered by some sort of mechanical filling of the spermatheca or perhaps under chemical control from substances present in male ejaculate. This reduction in mate search behaviour could therefore be controlled by the female, or alternatively, it could be induced by male manipulation. In this study we aimed to determine whether substances transferred by males in the spermatophore, hereafter referred to as seminal proteins (SPs), influence female phonotaxis, longevity and egg production in *G. bimaculatus*.

METHODS

Study Species

We used sixth-generation descendants from 30 nonvirgin female *G. bimaculatus* collected near Valencia, Spain. The crickets were fed standard rodent diet and raised in plastic boxes (25×16 cm and 14 cm high) at 28 ± 1 °C. To ensure that the females were virgin, we separated male and female nymphs at the penultimate instar. From hatching to the experimental period, we reared females in complete physical isolation from adult males. Upon adult eclosion, we placed females in individual containers (6×6 cm and 5 cm high) and supplied them with standard rodent diet and fresh water.

SP Experiment

We selected 100 females from the population and allocated them equally to one of two treatment groups: SP group: treated with SPs; Ringer's group: control treated with phosphate buffer solution (PBS, Ringer's solution).

SP Preparation

We collected 200 fully formed and hardened (sperm-provisioned) spermatophores from a group of approximately 50 males over a period of 7 days. We harvested spermatophores by gently squeezing the male's abdomen and removing the emerging spermatophore with a pair of forceps. We suspended the spermatophores in 100 µl of PBS and homogenized them using a pestle. We then spun the SPs in a centrifuge for 5 min at 3300 rpm and removed the supernatant. Spermatophores contain approximately 0.7 µl of water (mean of five spermatophores, fresh weight − weight after drying overnight in a drying oven at 35 °C), so 2 µl of suspended compound contained the spermatophore secretions of approximately 1.7 males. This ensured that females were receiving a dose of SPs expected to be adequate to elicit a response, allowing for a proportion of the SPs being bound to and therefore discarded with the sperm. We confirmed the presence of proteins in the spermatophore secretion by running a sample on a 12% SDS-PAGE. The sample was prepared using spermatophores from eight individuals and to the same concentration as the SPs used in the experiment.

Manipulations

We carried out manipulations when females were 10 days postadult eclosion. We performed the injections using a Narishige IM-6 microinjector (Narishige International, London, UK.) fitted with a µTIP 0.5 µm capillary. In *D. melanogaster*, some Acps targeting female receptivity and longevity enter the haemolymph and have targets outside of the reproductive tract (Ottiger et al. 2000). Many studies concerning the target and effect of Acps on female behaviour in *D. melanogaster* have involved injecting the compounds directly into the female abdominal cavity (reviewed in Kaufman & Lomas 1996). The targets of male ejaculates we wished to examine are the neurological regions responsible for phonotaxis, all of which are located outside the reproductive tract (Atkins & Stout 1994). We therefore injected females directly into the haemocoel, between the seventh and eighth abdominal sclerites. Prior to treatment, we anaesthetized females by placing them on ice for 5 min. We then carried out the following actions: SP group: each female was injected with 2 µl of suspended SPs; Ringer's group: each female was injected with 2 µl of PBS (Ringer's solution).

Female Response

Oviposition and longevity

All females used in the experiment were virgin; however, virgin females still regularly oviposit. Prostaglandins in *T. commodus* (Loher et al. 1981) typically elicit an ovipositional response within 2–24 h of mating; hence we measured female oviposition for 25

females from each treatment group 24 h before and 24 h after treatment. We allowed females to lay eggs in damp sand and then washed and counted the eggs. Females were then replaced in their individual containers (6 × 6 cm and 5 cm high) maintained at 28 ± 1 °C and a 16:8 h light: dark photoperiod with fresh food and water. We monitored crickets daily, replacing food and water as necessary, and finally recording their date of death.

Phonotactic response

We constructed a playback track to test female response to calling song. This track consisted of songs from three males. We used CoolEdit96 (Syntrillium Software Corporation, Scottsdale, AZ, U.S.A.) to normalize the amplitude to a peak of 70 dB (re 20 μPa) at a distance of 25 cm from the speaker. We broadcast each song consecutively from the left and right channel twice. Each calling bout lasted 30 s and bouts were separated by 15 s of silence. In total, the playback track lasted 9 min, and was played once in its entirety to each female. We looped the playback track and started it at a different point for each female to control for an interaction between song order and habituation to the apparatus.

We tested female phonotactic response immediately before treatment and 24 h afterwards using a trackball system (D-Sphere), developed by H. Dahmen of the University of Tuebingen. The device comprises a hollow polystyrene ball with a mass of 3.4 g, mounted on an air current rising from the bottom of an aluminium hemispherical cup, an arrangement that leads to very low frictional and inertial resistance. We held females in place on top of the sphere by attaching them by the pronotum to the end of a piece of wire using modelling wax. The wire holding the cricket was connected to a leaf spring made of thin card and held in place using a clamp and stand. The spring allowed the cricket to lift its body up and down on its legs with minimal impediment, but it could not turn left or right and hence its normal walking movements resulted in rotations of sphere. Trackball movement was detected through optical sensors (as used in an optical mouse). The response was recorded as the total rotation measured at 0.1 s intervals in two planes of movement, *X* and *Y*; the *X* component recorded the direction of preference whereas the *Y* component recorded the degree of forward effort exerted by the female; hence *X* and *Y* are hereafter referred to as the turning and forward components of movement, respectively. Each component had the potential to vary in magnitude independent of the other. We played songs from two speakers (cone diameter 2.5 cm) placed perpendicular to each other and 45° from the cricket's midline. We played crickets songs from one channel at a time.

RESULTS

Oviposition

We collected oviposition data from 22 females in the Ringer's group, and 23 females from the SP group. This is due to some deaths prior to the second data collection. The data were non-normal so we analysed the change in the number of eggs laid before and after the two treatments using a Mann–Whitney *U* test. The number of eggs laid in the 24 h before treatment minus the number laid after treatment did not differ significantly between the two groups ($Z = 1.29$, $N_1 = 22$, $N_2 = 23$, $P = 0.20$; Fig. 1).

Phonotactic Response

We analysed female response averaged across all songs. Females made a significant positive turning response to calling songs both before treatment (Wilcoxon signed–ranks test: $Z = 6.305$, $N = 89$, $P < 0.001$) and after treatment (Ringer's: $Z = 4.18$, $N = 41$, $P < 0.001$; SP: $Z = 4.64$, $N = 48$, $P < 0.001$). There was no significant difference between the amount of turning towards the song after treatment with Ringer's and that after treatment with SP (Mann–Whitney *U* test: $Z = 0.44$, $N = 89$, $P = 0.66$). There was also no significant difference in turning towards song before and after treatment group for either treatment group (paired *t* tests: Ringer's: $t_{10} = 2.35$, $P = 0.815$; SP: $t_{47} = 1.64$, $P = 0.11$; Fig. 2).

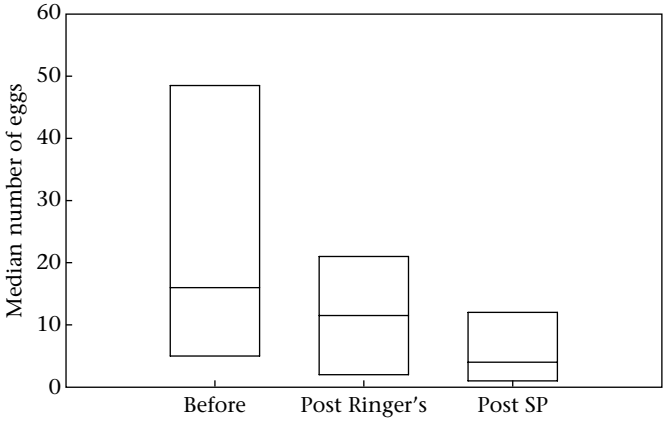

Figure 1. The numbers of eggs laid by females in the Ringer's treatment group and in the seminal protein (SP) group. Box plots represent the median number of eggs laid, and the upper and lower quartiles.

Before treatment, females moved forward significantly more during calling bouts than between calling bouts (when no calling stimulus was present; paired *t* test: square-root-transformed data: $t_{87} = 3.87$, $P < 0.001$). After Ringer's treatment, there was no significant difference between forward movement when song was played and forward movement when no song was played (paired *t* test: $t_{40} = 0.05$, $P = 0.964$), and there was a similar lack of forward movement in response to calling song after SP treatment ($t_{47} = 1.51$, $P = 0.14$). Females had a significantly reduced forward movement when a calling song was played after SP treatment compared with the response after Ringer's treatment (*t* test: $t_{87} = 2.66$, $P = 0.01$), but they also moved significantly less when there was no stimulus after treatment with Ringer's compared with treatment with SP ($t_{87} = 3.00$, $P = 0.004$). However, paired *t* tests did not reveal a significant difference in forward movement before and after treatment for either treatment group (Ringer's: $t_{40} = 1.14$, $P = 0.26$; SP: $t_{47} = 1.23$, $P = 0.22$; Fig. 3).

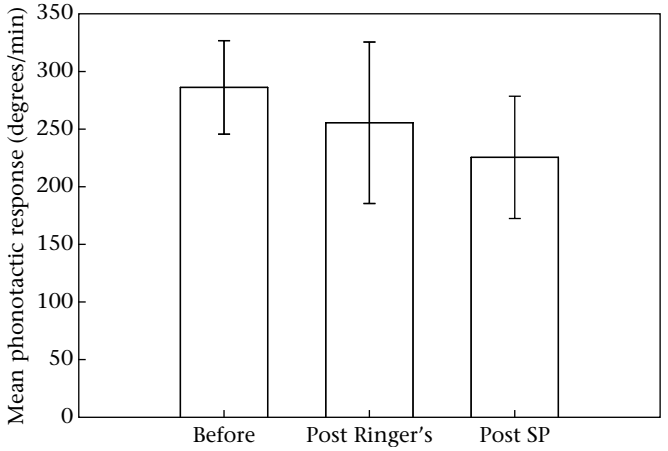

Figure 2. Orientation of females towards the calling song before and after treatment with Ringer's or seminal proteins (SP). Error bars represent 1 SE.

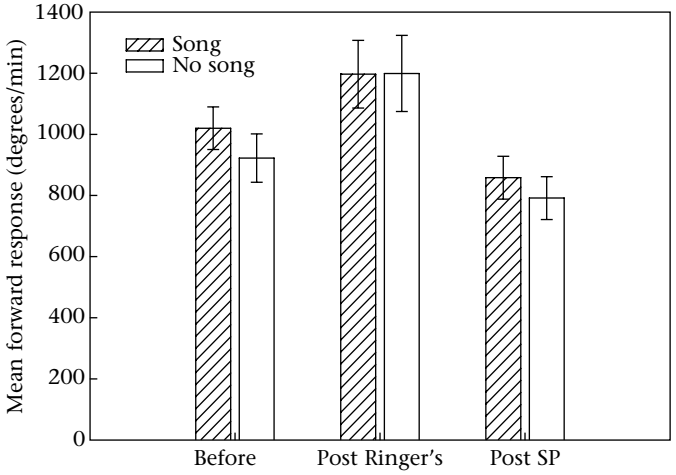

Figure 3. Forward movement of females in response to either a calling song stimulus or no song before and after treatment with Ringer's or seminal proteins (SP). Error bars represent 1 SE.

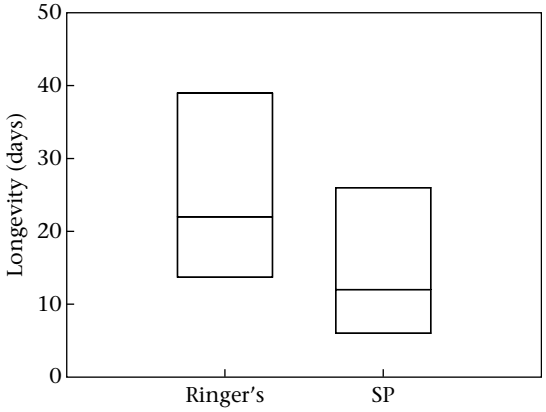

Figure 4. Longevity (days after adult eclosion) of females treated with Ringer's or seminal proteins (SP). Box plots represent the median, upper and lower quartile values.

Longevity

Owing to logistics with data collection, we recorded data on the adult longevity of 27 females in the Ringer's group and 34 females in the SP group. We were unable to normalize the data, so again we used a Mann–Whitney *U* test. Females in the SP group died sooner than females in the Ringer's group ($Z = 3.927$, $N = 61$, $P < 0.001$; Fig. 4.)

DISCUSSION

There was no significant effect of treatment group on number of eggs laid, and SPs did not influence the magnitude of phonotactic response measured as the amount of turning towards a male call. However, the magnitude of forward effort varied in response to both treatment and whether there was a sound stimulus. Before treatment, females showed an increased forward response when played a calling song compared to between calling bouts when there was no stimulus. After treatment, females in the SP group reduced their forward effort compared to the control group, both within and between calling bouts. Females of the SP group also died sooner than those in the control group.

There was no significant effect of treatment on oviposition. This could be explained in one of two ways: either SPs may not influence oviposition in this species, or they may need to be delivered directly into the female reproductive tract. In the grasshopper *Gomphocerus rufus*, mating inhibits female sexual receptivity but does not increase oviposition (Hartmann & Loher 1999). However, in *G. bimaculatus*, mating has been shown to increase oviposition (Bentur et al. 1977). Also, Bretman et al. (2006) found that female *G. bimaculatus* mated to more dominant males lay more eggs than those mated to submissive males. This could be caused by females allocating more resources to oviposition following a mating with a dominant male, but it is perhaps more likely that dominance status is associated with ability to manipulate oviposition through SPs.

We chose to deliver proteins directly into the haemocoel because this method has been used in studies of the effect of Acps on female behaviour in *Drosophila* species on the basis that targets have been identified outside of the reproductive tract. Ram et al. (2005) found that in *D. melanogaster*, Acps transferred during copulation have multiple targets, including the female's circulation and haemolymph. Ottiger et al. (2000) also found that in *D. melanogaster* Acps that influenced female receptivity targeted areas outside of the reproductive tract. In this experiment, we were investigating the possibility of male control over female phonotaxis. Phonotaxis is under neuronal control via the brain and, hence, if phonotaxis is influenced by SPs, we would expect the target of these proteins to lie outside the reproductive tract in the nervous system. However, in *T. commodus*, Loher et al. (1981) showed that SPs (which they refer to as prostaglandins) injected into the common oviduct region increased oviposition, but that rather than being influenced directly by the male compound, oviposition was increased by a secondary compound synthesized in the female reproductive tract. Hence, we cannot rule out the possibility that SPs in our experiment did not reach the appropriate targets on which to act to increase oviposition and future studies might include attempts to deliver direct to the spermatheca.

We found no detectable effect of the SPs on phonotactic orientation towards the song. This contrasts with the effect of mating on phonotactic response (Loher et al. 1993; Lickman et al. 1998; Bateman 2001; Lynch et al. 2005), but is in accordance with the previous study of *A. domesticus*, which concluded that seminal products do not reduce female receptivity in this species (Fleischman & Sakaluk 2004). Of course, it is possible that our negative result is because either the concentration of SPs was too low to have full effect or the site of delivery was inappropriate. During the protein extraction, some SPs may have been inadvertently discarded because of binding to the sperm. It is also possible that phonotactic reduction occurs via receptors in the reproductive tract or through compounds produced there in response to introduced SPs (since, as discussed above, neural control of phonotaxis occurs outside the reproductive tract). Alternatively, female phonotaxis may be reduced through multiple cues following mating, such as the presence of specific SPs in the haemolymph in conjunction with the stimulation of mechanoreceptors that detect that the spermatheca is full, a possibility that remains to be tested.

Females did move forward significantly less when injected with SPs than when injected with Ringer's. This effect was not detected by paired tests of movement before and after treatment; however, this may be an artefact of the second phonotactic test being a day later than the first, as Loher et al. (1993) found that female receptivity increases between 5 and 7 days after adult eclosion. Hence, a more appropriate test is to compare the two treatment groups after injection. Unpaired tests of the difference in forward movement between treatment groups revealed an effect of treatment. The reduction in forward effort after SP treatment was also significant even between calling bouts when no song was being played, suggesting that SPs reduce movement in general.

Alternatively, because only short bouts of silence separated calling songs, it could be that females remained faithful to the last calling song that they heard. Females of the bushcricket *Requena verticalis* have been shown to remain faithful to calling males even after they have stopped singing (Bailey et al. 2003). Female movement seems to be reduced overall after treatment with SPs. This could imply that females are generally less mobile after mating and therefore this may still act to reduce the number of subsequent mates found by the female. In *Drosophila*, Acps have been shown to influence the female postmating refractory period (Wigby & Chapman 2005). Once mated, Acps act on the female to reduce her receptivity to subsequent potential mates. This increases the overall paternity of the first male to mate with the female. This reduction in forward effort after injection with SPs could have similar effects in female crickets. If females move forward less after mating, they are less likely to discover further males. As previously mentioned, in *D. melanogaster,* Acps enter the haemolymph after being introduced into the reproductive tract, and have various targets (Ram et al. 2005). Similarly, Stanley-Samuelson & Loher (1985) found that radioactive prostaglandins injected into the body cavity of female *T. commodus* were quickly eliminated from the haemocoel and taken up by various other tissues such as the hindgut and ovaries. They also found that some compounds were taken up by the ventral nerve cord and flight muscles. Loher et al. (1993) found that in *G. bimaculatus*, the reduction in phonotaxis after mating could be reversed by severing the ventral nerve cord, suggesting that compounds found in male ejaculates do target these tissues, and specifically have the potential to affect female locomotion.

An alternative explanation for this reduction in motility after the SP treatment is that the effect could be caused by an immune response. Further evidence for this is provided by the observation that mortality was significantly increased in females in the SP group. However, in *D. melanogaster*, male sex peptide also increases female mortality (Wigby & Chapman 2005). Lung et al. (2002) identified a seminal protein in *D. melanogaster* (Acp62F) that exerts positive protective effects in the female reproductive tract, but is toxic and reduces female longevity upon entering the haemolymph. In the same species, Wigby & Chapman (2005) found that females that are continuously exposed to males have lower survival than those exposed to males for 48 h only, indicating a cost to mating that is not associated with oviposition and which is expressed as decreased longevity. It is therefore possible that the reduction in longevity of females of the SP group in our study was also due to the action of these proteins. Also, this reduction in forward effort was not mirrored in a significant reduction in turning towards song after treatment with SPs, which we would predict would also be affected if the immune system was challenged. Previous studies of the effect of mating on longevity in crickets found a positive effect in *G. lineaticeps* (2001), while in *G. bimaculatus* there was no effect of matings themselves (including ejaculates; Bateman et al. 2006). It is unclear why life span was affected in our study but not in Bateman et al.'s (2006) study, in which matings occurred naturally, since one might expect matings and ejaculates to be at least as harmful as ejaculates alone. One possibility is that the injection of extracted proteins directly into the haemocoel in our experiment allowed the expression of greater toxicity, unlike that experienced when ejaculates are transferred by the male into the reproductive tract which is adapted to receive them.

Although the influence of SPs on behaviour may sometimes be beneficial for the female (e.g. Boggs 1981), they are widely regarded as compounds produced by the male that are aimed at promoting male fitness, sometimes at the expense of female fitness (Chapman et al. 1995). If female crickets move less or orient less towards a calling song once mated as a result of male compounds, they are less likely to find more males, and hence the first male has an increased likelihood of paternity. This has obvious benefits for male fitness but the effects on female fitness are less clear. Female multiple mating has been shown to affect female fitness positively in some species (Tregenza & Wedell 2000). Specifically, egg-hatching success in *G. bimaculatus* is positively related to the number of brood sires (Tregenza & Wedell 1998). If males seek to reduce polyandry, it could be detrimental to females, so why have females not evolved resistance to these substances? Sakaluk et al. (2006) suggested that there is variation in female receptivity to 'antiaphrodisiacs' in the nuptial gifts of *G. sigillatus* both within and between populations. If males sometimes encounter receptive females either within their own population or through migration to a neighbouring population, this could maintain selection for these proteins. Alternatively, if females are exposed to greater threats through multiple mating, such as predation or sexually transmitted infections, a reduction in mate searching could promote female fitness. Several studies suggest that mate search behaviour in orthopteran species can be dangerous (Bateman 2001). For example, the survival probabilities for calling males and silent phonotactic females of the bushcricket *Poecilimon veluchianus* are equal (Heller 1992). Similarly, female mate choice in *A. domesticus* is affected by risk of predation (Hedrick & Dill 1993; Csada & Neudorf 1995). If mate search behaviour in *G. bimaculatus* is equally costly, then this receptivity to male-produced compounds could be maintained through positive selection. This in turn could produce density-dependent variation in success of male manipulation. In dense populations, male manipulation would be relatively unsuccessful as females would frequently encounter further mates by chance; however, in less dense populations, male manipulation could be more effective.

A final explanation for the observed changes in the SP treatment group when compared to the Ringer's group is that, rather than male manipulation, they may be female responses to a perceived mating. For example, a reduction in motility after SP treatment could represent an adaptation intended to reduce predation risk postmating. Searching for mates is likely to be energetically costly, and so females may benefit from reducing their search behaviour once they have copulated once. Also, there is evidence that predation risk can affect female mate choice in crickets (Hedrick & Dill 1993; Csada & Neudorf 1995) and it may also affect the costs of locomotion. Increased longevity could be the result of a trade-off between fecundity and the maintenance of somatic tissue. Although we did not find evidence for this in our oviposition data, females of many species invest more heavily in offspring production when mated, at the cost of other functions.

Our results suggest that in *G. bimaculatus*, male-produced compounds transferred to females in spermatophore contents may influence locomotion after mating, and may also reduce female longevity. We propose that in low-density populations, this may reduce female remating rates. Although polyandry increases female fitness in *G. bimaculatus*, it is possible that, in the wild, this reduction in mate search behaviour could be beneficial to both males and females. Further studies are needed to clarify the effect of SPs introduced directly into the reproductive tract and to assess whether any effects are fully under male control or whether they are female mediated in response to male proteins.

Acknowledgments

K.G. was funded by a BBSRC studentship award, T.T. was funded by a Royal Society Fellowship. Thanks to Michelle Hares for valuable laboratory assistance and David Hosken, Nina Wedell and four anonymous referees for comments on the manuscript.

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