Animal Behaviour 83 (2012) 369–375

Contents lists available at SciVerse ScienceDirect

Animal Behaviour

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

Glycine in nuptial food gifts of decorated crickets decreases female sexual receptivity when ingested, but not when injected

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ARTICLE INFO

Article history:
Received 7 August 2011
Initial acceptance 26 September 2011
Final acceptance 14 October 2011
Available online 12 December 2011
MS. number: A11-00624

Keywords:
amino acid
cricket
glycine
Gryllodes sigillatus
nuptial food gift
sexual conflict
sexual receptivity
spermatophylax

Sexual conflict over female remating behaviour has been implicated in the evolution and maintenance of nuptial food gifts in insects. Recent evidence suggests that the spermatophylax provided by male decorated crickets, *Gryllodes sigillatus*, a gelatinous adjunct to a male's spermatophore that is consumed by the female after mating, contains substances that inhibit female sexual receptivity, but that females have evolved resistance to these compounds. The identity of these substances remains unknown, but a significant component of the solid fraction of the gifts is composed of free amino acids, primarily glycine and proline. We tested the hypothesis that glycine is the refractory-inducing substance in two experiments in which we (1) injected female house crickets, *Acheta domesticus*, a species known to be susceptible to the refractory-inducing effects of spermatophylax consumption, with solutions containing glycine, proline, or a saline control and (2) fed female *A. domesticus* experimental gels containing glycine, proline, a mixture of both, or a saline control. The results of these experiments suggest that ingestion, but not injection, of glycine leads to a delay in remounting and remating by female *A. domesticus*. The absence of an effect of glycine injected into the haemocoel of the female suggests a mechanism by which glycine inhibits female sexual receptivity: glycine may be stimulating taste neurons that have downstream neurological effects on female behaviour.

An inevitable conflict between the sexes arises whenever females seek to mate with additional males. Although it may be in a female's reproductive interests to mate with multiple males (Arnqvist & Nilsson 2000), such behaviour opposes the reproductive interests of her various mating partners. By mating polyandrously, females may benefit by securing direct or indirect benefits from additional matings (Zeh & Zeh 2003). However, polyandry leads to increased sperm competition, which decreases male fitness (Parker 1970).

The sexual conflict over female remating has been implicated in the evolution and maintenance of nuptial food gifts in insects (Arnqvist & Nilsson 2000; Sakaluk et al. 2006; Vahed 2007). In a meta-analysis of 122 studies addressing the benefits of multiple mating in insects, Arnqvist & Nilsson (2000) found that although female reproductive success increased significantly with mating rate in gift-giving taxa, female mating rate in a number of gift-giving taxa appeared to be far lower than optimal. Arnqvist & Nilsson (2000) argued that this pattern was consistent with the incorporation of refractory-inducing substances in males' gifts. By inducing a period of nonreceptivity in females via a nuptial gift, males decrease the amount of sperm competition they face and thus sire a larger proportion of offspring. This conflict over female remating is believed to give rise to sexually antagonistic coevolution of male traits that induce nonreceptivity in females and of female traits that confer resistance to such substances (Holland & Rice 1998).

Nuptial food gifts constitute a form of male material donation to the female in the context of mating and can take the form of insect prey, glandular secretions and even parts of the male's soma (Thornhill 1976; Eggert & Sakaluk 1994; Vahed 1998). In decorated crickets, *Gryllodes sigillatus* (Orthoptera: Gryllidae), the nuptial food gift is a spermatophylax, a large, gelatinous, sperm-free mass that surrounds a smaller, sperm-containing ampulla. Together, the spermatophylax and the ampulla constitute the male's spermatophore, which is transferred to the female during copulation and remains attached outside her body at the base of her ovipositor (Alexander & Otte 1967; Sakaluk 1984, 1987). After mating, the female detaches the spermatophylax from the ampulla and feeds on it while the ampulla remains attached and is emptied of sperm (Sakaluk 1984). The spermatophylax functions as a device to protect the ampulla from being removed prematurely by keeping the female otherwise preoccupied (Sakaluk 1984).

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0003-3472/$38.00
doi:10.1016/j.anbehav.2011.11.005

In support of Arnqvist & Nilsson (2000)'s hypothesis, recent evidence suggests that the spermatophylax provided by male *G. sigillatus* contains receptivity-inhibiting substances, but this effect was apparent only when the spermatophylax was offered to females of a non-gift-giving cricket species, *Acheta domesticus* (Orthoptera: Gryllidae). When female *A. domesticus* were experimentally fed a *G. sigillatus* spermatophylax, they took longer to remate compared with females given no such opportunity to feed (Sakaluk et al. 2006). Female *A. domesticus* remain susceptible to the receptivity-inhibiting substances contained in the spermatophylax because they have been under no selection to respond to them, whereas female *G. sigillatus*, in contrast, do not show a delay in remating after spermatophylax consumption presumably because they have evolved resistance to these substances through a process of sexually antagonistic coevolution (Sakaluk et al. 2006).

It is unknown which substance(s) in the spermatophylax inhibits female sexual receptivity. Recent work investigating the chemical composition of the *G. sigillatus* spermatophylax has revealed that a significant fraction of its dry weight is composed of free amino acids (Warwick et al. 2009). Free amino acids are phagostimulants in many insects (Calatayud et al. 2002), which may account for the universal gustatory appeal of the spermatophylax to females of other cricket species (Sakaluk 2000). By including free amino acids in their spermatophylaxes, males may be able to 'trick' females into consuming a food gift that is otherwise of low nutritional value (Will & Sakaluk 1994; Ivy & Sakaluk 2005) and thereby transfer more sperm (Warwick 1999; Sakaluk 2000; Warwick et al. 2009).

Of the free amino acids identified in the spermatophylax, the major fraction was composed of the nonessential amino acids, glycine and proline (Warwick et al. 2009). The high glycine content of the spermatophylax hints at a possible explanation as to why female *A. domesticus* show decreased sexual receptivity after consuming a *G. sigillatus* spermatophylax: glycine may delay mating by signalling a recent feeding, or it may directly inhibit neuronal activity (Warwick et al. 2009). Glycine is a phagostimulant in mealybugs, and may elicit a similar response in crickets (Calatayud et al. 2002). If glycine acts as a phagostimulant, it may also signal that a female has recently fed and cause reduced receptivity due to the digestive process (Gwynne 2008). In addition, glycine has been implicated as an inhibitory neurotransmitter in gomphocerine grasshoppers (Heinrich et al. 1998). When grasshoppers were given microinjections of glycine directly into their central protocerebrum, their stridulatory behaviour was terminated prematurely, suggesting that there are inhibitory receptors that are activated by glycine (Heinrich et al. 1998).

We tested the hypothesis that glycine in the spermatophylax leads to a decrease in the sexual receptivity of female *A. domesticus* in two experiments in which we (1) injected glycine directly into the haemocoel of females and (2) permitted females to ingest experimental gels containing glycine. We predicted that females injected with glycine would show a greater latency to mating than females injected with proline or the saline vehicle, and that females permitted to ingest gels containing glycine after mating would be slower to remate than females permitted to consume control gels or gels containing proline.

METHODS

General Rearing Methods

Experimental individuals were the descendants of approximately 1500 house crickets purchased from a commercial supplier (Premium Crickets, Thomson, GA, U.S.A.). They were housed as nymphs in 62.5-litre plastic storage containers, maintained in an environmental chamber at 30 °C on a 14:10 h light:dark cycle. Crickets were provided with Fluker's cricket chow ad libitum and water supplied in 40 ml plastic tissue culture vials plugged with cotton dental rolls. Portions of egg carton were added as shelter and to increase the rearing surface area in each container. A small plastic container filled with moistened peat moss was added to each container to provide an additional source of water and to serve as an oviposition site for adult females. Every 1–2 days, newly eclosed adults were collected and housed in mixed-sex 18.9-litre plastic storage containers, provisioned as above, where they were held for 7–9 days prior to experimental trials to gain sexual experience. Twenty-four hours before experimental trials, males and females were housed separately by sex in 18.9-litre containers to ensure their sexual motivation.

Experiment 1: Effect of Glycine Injection on Female Sexual Receptivity

To determine the effects of glycine on female receptivity, we injected glycine directly into the haemocoel of females after an initial mating, and measured their latency to remating. Male and female crickets were randomly selected and paired for initial mating trials after the 24 h period of sexual isolation. Each pair was introduced into a Plexiglas mating chamber ($10 \times 2 \times 2.5$ cm) in a room illuminated by red light and maintained at 28 °C. Pairs were observed for 60 min during which time we recorded the following mating parameters: (1) latency to female mounting, measured as the time from when the male first courted the female to the time at which the female first mounted the male; (2) latency to mating, measured as the time from when males initiated courtship to the time at which the female dismounted the male with the spermatophore attached to her genital opening. We also recorded the time after mating at which the female removed the spermatophore as a proxy for the amount of sperm and other ejaculatory substances transferred (Sakaluk 2000). Pairs in which the female did not mount the male within 1 h of the initiation of courtship were discarded.

Twenty-three hours after a successful initial mating, the female was randomly assigned to one of four experimental injections: low glycine treatment ($N = 31$), high glycine treatment ($N = 31$), proline treatment ($N = 31$), or a saline control ($N = 31$). Each female was injected ventrally under the seventh abdominal sclerite with 5 μl of the assigned solution using a 10 μl Hamilton syringe and given 1 h to recover. Insect saline was used as a vehicle and prepared by adding the following compounds to 200 ml of autoclaved Millipore water: 0.393 g of NaCl, 0.019 g of KCl, 0.029 g of $MgCl_2$, 0.019 g of $CaCl_2$, 0.038 g of NaH_2PO_4, 0.007 g of $NaHCO_3$ and 0.12 g of glucose (Warwick 1999). Each day injections were scheduled, fresh 1 ml amino acid solutions were prepared using the following amounts of amino acids: 0.0124 g of glycine (low glycine treatment), 0.024 g of glycine (high glycine treatment) and 0.0207 g of proline (proline treatment), corresponding to roughly 10 times the actual amount of glycine, 20 times the actual amount of glycine and 10 times the actual amount of proline in the spermatophylax, respectively (see Warwick 1999). These amounts were chosen because not knowing the location of target receptors, we could not be certain that injected free amino acids would not be metabolized or excreted before reaching their intended targets; the excess amounts were intended to compensate for this possibility.

One hour after experimental injections, females were paired with another randomly selected male of the same age cohort and under the same conditions as established in initial matings. Pairs were observed for 60 min during which time we recorded (1) the latency to female remounting and (2) the latency to remating relative to the time at which the male initiated courtship. Pairs in which the female did not mount the male within 1 h of the initiation of courtship were

recorded as censored observations. We predicted that if glycine results in a decrease in female receptivity (as opposed to a generalized effect of amino acids), females injected with glycine should show a greater latency to remounting (and remating) than females injected with proline or the saline vehicle only.

Experiment 2: Effect of Ingestion of Glycine-containing Artificial Gels on Female Sexual Receptivity

A lack of an effect of glycine in the preceding experiment might mean that females need to ingest and, perhaps, metabolically process, glycine to elicit a response, as this is the normal route through which male-derived glycine is introduced into the female. To test for this possibility, synthetic food gifts were manufactured to approximate the size, consistency and select amino acid content of *G. sigillatus* spermatophylaxes to determine whether the ingestion of glycine, proline, or a combination of the two, affects female latency to remounting or remating. Synthetic food gifts were manufactured to contain only insect saline (saline control treatment, $N = 21$), the actual amount of glycine in the spermatophylax (low glycine treatment, $N = 21$), 10 times the actual amount of glycine in the spermatophylax (high glycine treatment, $N = 21$), the actual amount of proline in a spermatophylax (proline treatment, $N = 21$) and the actual amount of both glycine and proline in a spermatophylax (combination treatment, $N = 21$). The actual amounts of glycine and proline in a *G. sigillatus* spermatophylax were determined using the millimolar concentrations of the amino acids in a spermatophylax and the wet weight of spermatophylaxes reported in Warwick (1999).

Insect saline was prepared in 400 ml batches using the same proportions of solutes as described in the preceding experiment. From the autoclaved saline, concentrated amino acid solutions were made to reduce the error associated with delivering very small amounts of amino acids. A 10× glycine solution and 2× proline solution were made by adding 0.1295 g of glycine and 0.0443 g of proline to 50 ml insect saline, respectively. To arrive at the correct amounts of amino acid per synthetic food gift, these concentrated solutions required further dilution. For the low glycine treatment, the 10× glycine solution was diluted using insect saline to a concentration of 0.259 g/litre. Similarly, the 2× proline solution was diluted to a concentration of 0.443 g/litre. The combination treatment solution contained both glycine and proline in the aforementioned concentrations. For the high glycine treatment, the 10× solution was not diluted and insect saline was used unaltered for the saline control treatment.

In 50 ml flasks, 25 ml of each of the prepared treatment solutions were brought to a gentle boil. The flasks were then removed from heat and 2.60 g of pectin (Ball® No Sugar Needed, Ball Corp., Broomfield, CO, U.S.A.) was carefully added before returning the solution to a boil for 1 min. The final volume of the liquid gel was measured before pouring it into a glass petri dish and allowing it to cool. One by one, the gels were then microwaved for about 20 s to liquefy before using sterile 1 ml disposable syringes to dispense the gels in 0.03 ml quantities onto parchment paper. After cooling completely, the gels were transferred to labelled 1.5 ml centrifuge tubes and stored in a refrigerator until later use.

Initial mating trials were staged as described in the preceding experiment. Immediately after a successful mating, a gel from one of the treatments was offered on the tip of a dissecting needle to each female. An additional control was established consisting of randomly selected females that were offered no gel following spermatophore transfer (control treatment, $N = 23$). The sample sizes reported above only include those females that consumed at least 75% of the synthetic food gift; females ingesting less than this amount were eliminated from the study.

Twenty-four hours after ingestion of experimental gels, females were paired with another randomly selected male of the same age cohort and under the same conditions as established in initial matings. A 24 h period between initial and experimental matings was employed in this experiment because this is the interval over which ingestion of actual spermatophylaxes has been observed to influence a female's subsequent receptivity (Sakaluk et al. 2006). Pairs were observed for 60 min during which time we recorded (1) the latency to female remounting and (2) the latency to remating relative to the time at which the male initiated courtship. Pairs in which the female did not mount the male within 1 h of the initiation of courtship were recorded as censored observations. We predicted that if the ingestion of glycine alone results in a decrease in female receptivity (as opposed to a generalized effect of amino acids), females ingesting glycine should show a greater latency to remounting (and remating) than control females or those ingesting proline. If, instead, proline and glycine act synergistically to influence female receptivity, we predicted that experimental gels containing both amino acids would show the greatest receptivity-inhibiting efficacy compared with the other treatments.

Statistical Analysis

We used failure-time analysis to assess the effect of treatment on latency to female remounting and remating to accommodate the inclusion of right-censored data (i.e. observations in which females had not mounted or mated by the end of the observation period; Fox 1993). A Cox proportional hazards regression as implemented by PROC PHREG in SAS/STAT 9.2 software (SAS Institute Inc., 2010, Cary, NC, U.S.A.) was used for both experiments. Female latency to the initial mating as well as initial spermatophore attachment time were included as covariates. Latency to the first mating was used as a covariate because females may vary intrinsically in their propensity to respond to male courtship. Spermatophore attachment time was used as a covariate because male ejaculates may also contain receptivity-inhibiting substances (but see Fleischman & Sakaluk 2004).

Preplanned contrasts between treatments in both experiments were made using the CONTRAST statement in PROC PHREG. For the injection experiment, the following contrasts were constructed: (1) high glycine versus control, (2) both glycine treatments versus control, (3) both glycine treatments versus proline and (4) proline versus control. For the gel ingestion experiment the following contrasts were constructed: (1) saline versus control, (2) proline versus control, (3) both glycine treatments versus control, (4) both glycine treatments versus proline, (5) glycine/proline combination versus control, (6) glycine/proline combination versus proline and (7) glycine/proline combination versus low glycine. For each set of contrasts, we controlled the false discovery rate (the proportion of significant results that were type I errors) at $\alpha = 0.05$ (Benjamini & Hochberg 1995), an approach that conserves statistical power while maintaining some control over the number of type I errors (Nakagawa 2004; Verhoeven et al. 2005; Narum 2006; Waite & Campbell 2006).

RESULTS

Experiment 1: Effect of Glycine Injection on Female Sexual Receptivity

There was no significant effect of treatment on female latency to remount (Wald $\chi^2_3 = 0.75$, $P = 0.86$; Fig. 1a) or remate (Wald $\chi^2_3 = 1.60$, $P = 0.66$; Fig. 1b) following experimental injection. In addition, there was no significant effect of the female's initial mating latency on her latency to remount (Wald $\chi^2_1 = 1.01$, $P = 0.31$) or remate (Wald $\chi^2_1 = 1.76$, $P = 0.18$). The time at which

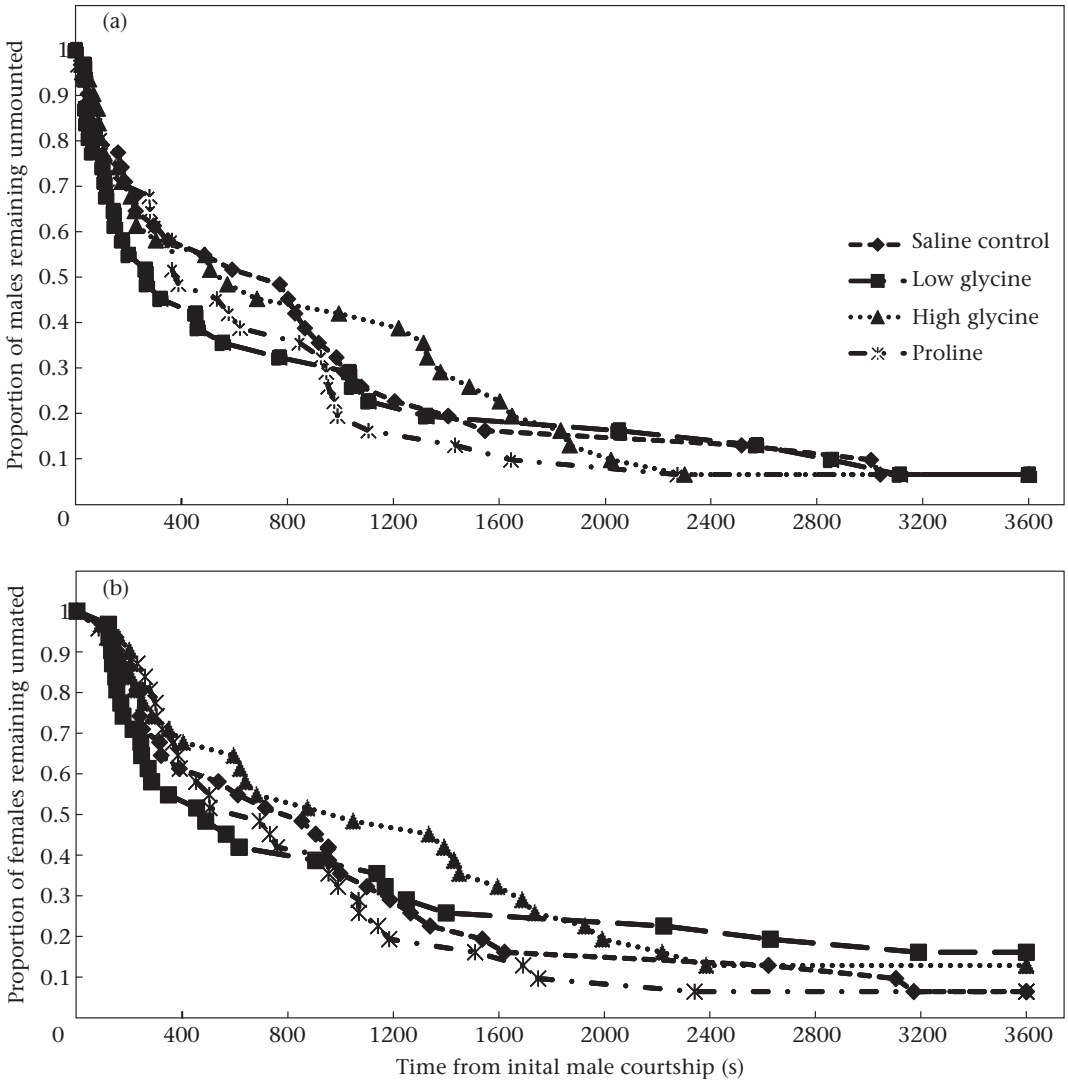

Figure 1. Survival curves for the injection experiment showing the proportion of females failing to (a) remount and (b) remate as a function of male courtship time (in seconds).

the female removed the spermatophore after the initial mating did not significantly affect the female's latency to remount (Wald $\chi^2_1 = 0.82$, $P = 0.37$), but it did significantly affect her latency to remate (Wald $\chi^2_1 = 4.16$, $P = 0.0415$). The positive parameter estimate ($\beta = 2.13 \times 10^{-4} \pm 1.04 \times 10^{-4}$) indicates that females that had a longer spermatophore attachment time after their initial mating remated sooner than females with a shorter spermatophore attachment time. None of the preplanned contrasts between treatment levels differed significantly for either the time to remounting or remating (Table 1).

Experiment 2: Effect of Ingestion of Glycine-containing Artificial Gels on Female Sexual Receptivity

There was a significant effect of treatment on female latency to remount after ingestion of experimental gels (Wald $\chi^2_5 = 19.84$, $P = 0.0013$; Fig. 2a), but the effect of treatment on female latency to remate was not significant (Wald $\chi^2_5 = 9.35$, $P = 0.096$; Fig. 2b). The female's latency to her initial mating significantly influenced both the female's latency to remount ($\beta = -2.87 \times 10^{-4} \pm 0.99 \times 10^{-4}$; Wald $\chi^2_1 = 8.43$, $P = 0.0037$) and remate ($\beta = -2.32 \times 10^{-4} \pm 1.02 \times 10^{-4}$; Wald $\chi^2_1 = 5.16$, $P = 0.0231$) following ingestion of experimental gels; essentially, females that took longer to mate in their initial trial also took longer to mount/mate in their experimental pairing. The time at which the female removed the spermatophore after the initial mating significantly affected her latency to remount ($\beta = -2.06 \times 10^{-4} \pm 0.98 \times 10^{-4}$; Wald $\chi^2_1 = 4.43$, $P = 0.0353$) but not her latency to remate (Wald $\chi^2_1 = 1.67$, $P = 0.20$). Longer spermatophore attachment durations following initial matings were associated with an increased latency to remount in experimental trials.

Table 1
Preplanned contrasts of effects of treatment on female latency to remounting and remating following experimental injection in house crickets, *Acheta domesticus*

Contrasts	*df*	Wald χ^2	*P*
Latency to remounting			
High glycine vs control	1	0.0063	0.9368
Both glycine levels vs control	1	0.0195	0.8891
Both glycine levels vs proline	1	0.4212	0.5163
Proline vs control	1	0.4788	0.4889
Latency to remating			
High glycine vs control	1	0.4433	0.5055
Both glycine levels vs control	1	0.4516	0.5016
Both glycine levels vs proline	1	1.4989	0.2208
Proline vs control	1	0.2526	0.6152

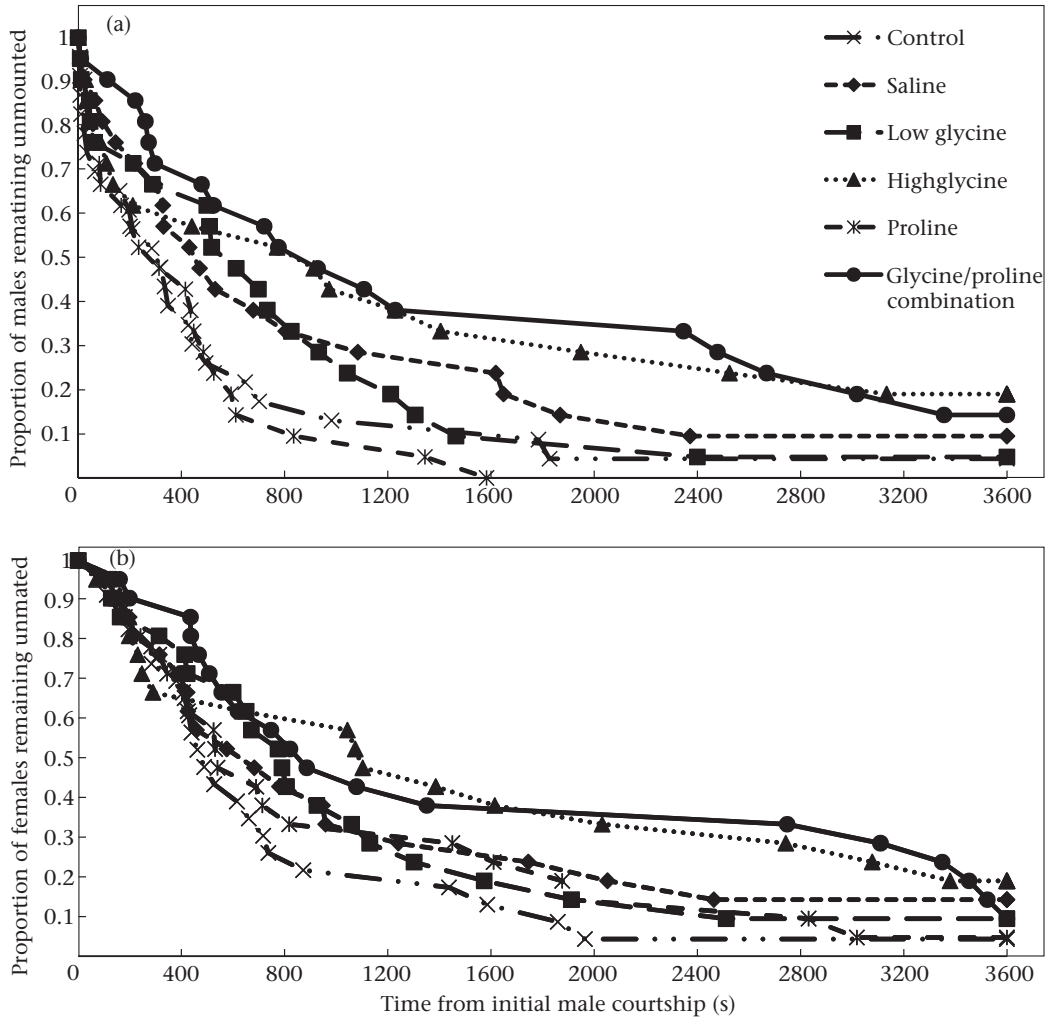

Figure 2. Survival curves from the gel experiment showing the proportion of females failing to (a) remount and (b) remate as a function of male courtship time (in seconds).

Preplanned contrasts revealed that females ingesting glycine-containing gels with or without proline took significantly longer to remount and remate than control females (Table 2). However, females ingesting gels containing only proline did not take significantly longer to remount or remate than control females. Females ingesting gels containing glycine with or without proline took significantly longer to remount than those fed proline only, but they did not take longer to remate. There was no difference in remounting or remating times of females fed gels containing a combination of proline and glycine and those fed gels containing the lower concentration of glycine.

DISCUSSION

The results of these experiments suggest that ingestion, but not injection, of glycine leads to a delay in remounting and remating behaviour of female *A. domesticus*. The ingestion of glycine gels (with or without proline) led to a delay in remounting and remating of females compared with control females. This delay in remounting was also apparent in females that ingested gels containing glycine: these females took longer to remount than females that ingested gels containing only proline. The ingestion of proline by itself had no effect on female latency to remounting or remating compared with controls. We conclude that the ingestion of glycine, but not proline, leads to a transient decrease in female sexual receptivity, and hence, that glycine in the spermatophylax is at least partly responsible for the decrease in female receptivity following spermatophylax consumption documented in previous studies (Sakaluk 2000; Sakaluk et al. 2006).

Table 2
Preplanned contrasts of effects of treatment on female latency to remounting and remating following ingestion of experimental gels in house crickets, *Acheta domesticus*

Contrasts	*df*	Wald χ^2	*P*
Latency to remounting			
Saline vs control	1	3.1720	0.0749
Proline vs control	1	0.0045	0.9466
Both glycine levels vs control	1	8.1243	**0.0044**
Both glycine levels vs proline	1	7.4252	**0.0064**
Glycine/proline vs control	1	12.1738	**0.0005**
Glycine/proline vs proline	1	12.3009	**0.0005**
Glycine/proline vs low glycine	1	2.6767	0.1018
Latency to remating			
Saline vs control	1	2.3506	0.1252
Proline vs control	1	1.7999	0.1797
Both glycine levels vs control	1	6.4848	**0.0109**
Both glycine levels vs proline	1	0.8669	0.3518
Glycine/proline vs control	1	7.0117	**0.0081**
Glycine/proline vs proline	1	1.9947	0.1579
Glycine/proline vs low glycine	1	0.7043	0.4014

Bold values represent significant effects after controlling for the false discovery rate using the Benjamini–Hochberg method.

Why was there a more pronounced effect of glycine on the latency to female remounting than on the latency to female remating? Mounting is a more sensitive proxy of a female's sexual receptivity because it is entirely under the female's control; mating cannot ensue unless a female mounts a courting male of her own volition. In contrast, mating (i.e. successful transfer of the spermatophore) is a function of both the female's sexual receptivity and the male's sexual competency. For example, mountings often end without successful spermatophore transfer because of the male's inability to thread the spermatophore tube into the genital opening of the female (Sakaluk 1987; for a detailed description of the mechanics of spermatophore transfer in crickets, see Kumashiro & Sakai 2001).

Our results also show that the mode of glycine introduction to females is biologically important. The injection of glycine directly into the haemocoel had no influence on remounting or remating by females, but ingestion of glycine at biologically relevant doses was sufficient to effect a delay in both. It may be that injected glycine was unable to reach its intended target, rendered too dilute in circulating haemolymph to elicit a response, or excreted by females shortly after its introduction into the haemocoel. It is also possible that the difference in female response to injected versus ingested glycine resulted from a difference in exposure times across the two experiments: females were injected 1 h before their second matings in experiment 1, whereas females were fed glycine-containing gels 24 h prior to their second matings in experiment 2. This difference in protocol was, however, deliberate and meant to account for the time taken for ingested gels to pass through the female gut and the concomitant liberation of glycine. In contrast, we reasoned that if females were injected 24 h in advance of their second matings, glycine might be lost from the haemocoel over this time period owing to processes described above, and that the likelihood of detecting an effect of injected glycine would be greatest shortly after its introduction into the haemocoel.

What, then, is the mechanism responsible for the inhibition of female receptivity following glycine ingestion? One possibility is that a metabolic product of glycine activates a target receptor in the gut or elsewhere that modulates female sexual receptivity. However, previous work in another cricket, *Gryllus rubens*, has demonstrated that 83% of radioactive glycine introduced into the alimentary canal is absorbed unaltered by the mid- and hindgut (Thomas & Nation 1984). Glycine is subsequently metabolized in the haemolymph, where it is incorporated into various compounds, including proteins (Zera & Zhao 2006). However, if one of these metabolic products was acting as an antiaphrodisiac, we would expect to see an effect of injecting as well as of ingesting glycine, because both would lead to the eventual metabolism of glycine.

Instead, there seems to be a process associated with the gustatory response that leads to a delay in remounting and remating by females because this delay was elicited only after ingestion and not after injection. However, the delay in remounting and remating behaviour cannot be explained by general digestive processes because there was no effect observed in females ingesting control or proline-containing gels. In insects, taste sensilla connect directly to the central nervous system and elicit action potentials after taste ligands (e.g. glycine) bind to receptors housed on gustatory neurons (Mullin et al. 1994). In western corn root worms, *Diabrotica virgifera virgifera* LeConte, γ-aminobutyric acid (GABA)/glycine sensitive gustatory neurons within sensilla are present on the maxillary galea (Mullin et al. 1994). Assuming similar glycine-activated neurons are present in cricket mouthparts, there could be a downstream effect of sensing glycine that inhibits female receptivity neurologically. Although most connections from gustatory neurons are made in the subesophageal ganglion and local motor circuits that control feeding, some do ascend to higher brain regions (Rogers & Newland 2003). Indeed, afferent neurons from the maxillary palps in the ensiferan Orthoptera have been shown to innervate the tritocerebrum, lobus glomerulatus, lateral protocerebrum and calyxes of the mushroom bodies (Ignell et al. 2000). Afferents in these regions could indirectly affect female sexual receptivity by modulating overall locomotor activity or by directly inhibiting sexual receptivity through some other mechanism.

If glycine alone is modulating female receptivity, what then is the function of the relatively large amounts of proline in the spermatophylax of *G. sigillatus*? As noted earlier, free amino acids act as phagostimulants (Calatayud et al. 2002), and chemical blends may enhance phagostimulatory power more than individual chemicals acting alone (Simpson & Raubenheimer 1996). It may be that the combination of proline and glycine acts to elicit a gustatory response while simultaneously influencing female receptivity. To test this hypothesis, one could utilize the gel technique employed here and compare feeding rates of individuals offered gels of varying free amino acid composition.

In addition to the effect of ingested glycine, our results suggest also that variation in a female's intrinsic motivation to mate and the duration of spermatophore attachment after an initial mating can influence the latency to remating. With respect to the former, a female's latency to mate was significantly repeatable, with females that took longer to mate in their initial trial also taking longer to mate in their experimental pairing. Although this might suggest the possibility of genetic variation in female responsiveness, it is equally likely that environmental factors such as female age or previous sexual experience play a role (Fleischman & Sakaluk 2004; Mautz & Sakaluk 2008). The duration of spermatophore attachment had inconsistent effects on the latency to remounting/remating across the two experiments: in experiment 1 (glycine injection), females that had longer spermatophore attachment durations after their initial mating remated sooner than females with shorter spermatophore attachment durations, whereas in experiment 2 (glycine ingestion), females that had longer spermatophore attachment durations after their initial mating took longer to remount than females with shorter spermatophore attachment durations. We are unable to reconcile these conflicting results, but if receptivity-inhibiting substances were transferred in males' ejaculates, we would expect a longer spermatophore attachment duration to result in a longer latency to remating. However, in a study designed specifically to test this possibility in which spermatophore attachment durations of females were experimentally manipulated, Fleischman & Sakaluk (2004) found no evidence that male *A. domesticus* transfer substances in their ejaculates that inhibit female sexual receptivity. Similarly, injection of seminal proteins derived from male spermatophores had no effect on phonotaxis of female field crickets, *Gryllus bimaculatus* (Green & Tregenza 2009).

Warwick and colleagues (Warwick 1999; Warwick et al. 2009) were the first to propose that free amino acids in the spermatophylax influence its gustatory appeal to females. They also implicated free amino acids as the proximate basis for the receptivity-inhibiting effect of spermatophylax consumption in *A. domesticus*, noting that elevated levels of free amino acids in the haemolymph inhibit locomotion (Simpson & Raubenheimer 2000). Specifically, they identified glycine, one of the two most abundant free amino acids in the spermatophylax, as a possible candidate given that glycine functions as a well-documented inhibitory neurotransmitter in vertebrates (review in Zafra & Giménez 2008). Although our results are consistent with a role for glycine in diminishing female sexual receptivity, we caution that glycine is probably not the only substance, or even the most predominant one, mediating this effect: in addition to free amino acids, the spermatophylax contains peptides and a number of unidentified proteins, any of which could function as refractory-inducing substances. Indeed, this role has been ascribed to accessory gland proteins transferred in the

ejaculates of males of other species, particularly *Drosophila* (Wolfner 1997; Miyatake et al. 1999; Ram & Wolfner 2007; Fricke et al. 2009). However, our results are inconsistent with a recent suggestion that the effect of spermatophylax consumption on female receptivity arises from a toxic effect of substances in the spermatophylax that reduces a female's motivation to mate (Gwynne 2008); given the ubiquity of glycine in the diet of insects and its role as a phagostimulant (Calatayud et al. 2002), it seems unlikely that its ingestion would have a toxic effect on females.

If glycine consumed in the spermatophylax helps mediate the decrease in sexual receptivity observed in female *A. domesticus*, why does it not have similar effects on female *G. sigillatus*, the intended target of any refractory-inducing substances? Previously we hypothesized that *G. sigillatus* females have evolved resistance to refractory-inducing substances contained in the spermatophylax to retain control of their mating rate through a process of sexually antagonistic coevolution (Sakaluk et al. 2006). In contrast, because female *A. domesticus* have not been subject to the selection imposed by male manipulation, they remain susceptible to receptivity-altering compounds contained in the spermatophylax. If, in fact, glycine is playing an important role in this regard, this would suggest that female *G. sigillatus* have evolved a mechanism that desensitizes them to glycine-mediated physiological effects. An obvious candidate in this respect might be an alteration in the conformation of the glycine receptor associated with the mouthparts. The continued inclusion of glycine in the spermatophylax is presumably maintained by variation in female *G. sigillatus* susceptibility, with males occasionally encountering females that succumb to the effects of glycine or other substances (Sakaluk et al. 2006).

In conclusion, our results suggest that glycine contained in the spermatophylax and ingested by females after mating promotes at least a transient sexual refractory period in female *A. domesticus*. Moreover, the absence of an effect of glycine injected into the haemocoel of the female suggests a mechanism as to how glycine inhibits female sexual receptivity: glycine may be stimulating taste neurons that have downstream neurological effects on female behaviour. In future studies, we hope to capitalize on these findings to identify the neural pathways activated by glycine taste receptors.

Acknowledgments

This research was funded by grants from the National Science Foundation to S.K.S., and two R.D. Weigel Research Grants from the Beta Lambda Chapter of the Phi Sigma Biological Honors Society to D.G.G. We thank Steve Juliano for statistical advice, Sandrine Clairardin for assisting in the serial dilutions, and Steve Juliano, Rachel Bowden and two anonymous referees for helpful comments on the manuscript.

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