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Disentangling effects of mating, nuptial gifts and accessory gland proteins on reproduction in female crickets

Ian G. Rines [a], Audrey E. Harrod [a], John Hunt [b], Ben M. Sadd [a], Scott K. Sakaluk [a, *, 1]

[a] School of Biological Sciences, Behavior, Ecology, Evolution and Conservation Section, Illinois State University, Normal, IL, U.S.A.
[b] School of Science, Western Sydney University, Hawkesbury Campus, Penrith, New South Wales, Australia

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Accessory gland proteins contained within male ejaculates influence female reproduction and survival in insects. Nuptial food gifts offered by male crickets and katydids, the consumption of which may also alter female behaviour and physiology after mating, also contain accessory gland proteins. However, because nuptial feeding promotes the transfer of sperm and ejaculatory substances, it is unclear whether it is accessory gland proteins in the ejaculate, nuptial gifts or both that mediate these effects. Here we evaluate the effects of mating, nuptial gifts and accessory gland proteins on female reproduction in a gift-giving cricket (*Gryllodes sigillatus*) using a crossed experimental design. We injected females of varying mating experience with male accessory gland extract, permitting some females to consume the nuptial food gift, while experimentally preventing others from doing so. Mating resulted in a significant decrease in female sexual receptivity, an effect likely mediated by accessory gland proteins contained in the male's ejaculate. Consumption of the nuptial food gift resulted in the premature cessation of nuptial feeding following the female's next mating, leading to a concomitant decrease in sperm transfer by a rival male. This is a novel finding, demonstrating that fitness benefits to males of nuptial gift provisioning can also accrue over later copulations by their mates. Neither injection of accessory gland extract nor nuptial feeding influenced female oviposition; the absence of any effect of the injection of accessory gland proteins on female reproduction suggests that their efficacy may depend on their direct introduction into the female reproductive tract. More research is required to identify the specific accessory gland proteins in ejaculates and nuptial gifts that modulate female behaviour and physiology, potentially illuminating the evolution of these mechanistic tactics underlying sexual conflict.

In insects, mating can have a multitude of effects on females beyond the simple receipt of sperm (Arnqvist & Nilsson, 2000). Mating can elicit a suite of physiological and behavioural changes during and after copulation in both males and females (Fowler et al., 2019). Such effects can be beneficial to females, as in bed bugs (*Cimex lectularius*), in which male ejaculates increase female reproductive rate but offset a cost of reproductive senescence (Reinhardt et al., 2009). However, these effects can also be detrimental to females, as when mating leads to physical injury (Crudgington & Siva-Jothy, 2000; Johnstone & Keller, 2000) or the transmission of sexually transmitted diseases (Knell & Webberley, 2004). There are numerous pathways by which these effects can be mediated: the physical act of mating itself (Crudgington & Siva-Jothy, 2000), the influence of sperm in the female reproductive tract (South & Lewis, 2011), compounds in the ejaculates of males such as accessory gland substances (Perry et al., 2013; Sirot, 2019; Worthington et al., 2015) and, in certain insect species in which males synthesize nuptial food gifts provisioned to females, compounds orally consumed by females that affect their postcopulatory behaviour and subsequent receptivity (Arnqvist & Nilsson, 2000; Sakaluk et al., 2019; Vahed, 2007).

The influence of male-derived ejaculatory substances on female insect longevity, reproduction and sexual receptivity has especially been a major focus of previous research (Gillot, 2003; Leopold, 1976; Perry et al., 2013), most notably in *Drosophila* (Wolfner, 1997, 2002). In particular, seminal fluid proteins produced by male accessory glands are known to influence the expression of genes mediating female reproduction, induce oogenesis and ovulation, promote sperm storage and influence female sexual receptivity, among other effects (Avila et al., 2011). While some of these effects are beneficial to females, such as

* Corresponding author.
E-mail address: sksakal@ilstu.edu (S. K. Sakaluk).
[1] Present address: Behavior, Ecology, Evolution & Conservation Section, 4120 Biological Sciences, Illinois State University, Normal, IL 61720-4120, U.S.A.

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when egg laying is synchronized with the availability of sperm (Murtaugh & Denlinger, 1987), some male-induced changes in female behaviour seem to be primarily in the male's fitness interest, as when they decrease or abolish female receptivity to future matings with rival males (Craig, 1967; Fuchs et al., 1969). Given that the changes induced by male seminal fluid proteins may not always be to the benefit of the recipient female's fitness, it is thought that these proteins play a major role in mediating sexual conflicts over future mating (Chapman, 2018; Hollis et al., 2019; Sirot et al., 2015).

Although accessory gland products are typically transferred to females in a male's ejaculate along with his sperm, this is not the only avenue through which such substances can be introduced to females. The nuptial food gifts offered by certain male crickets and katydids (Lehmann et al., 2018; Pauchet et al., 2015), which are orally ingested by females after mating, are also replete with accessory gland proteins. Comparative evidence suggests that the consumption of nuptial gifts may also alter female behaviour and physiology after mating (Arnqvist & Nilsson, 2000; Sakaluk et al., 2019; Vahed, 2007). However, because nuptial feeding typically promotes increased transfer of sperm and other ejaculatory substances (Sakaluk, 1984; Vahed, 1998; Wedell, 1993), whether it is accessory gland proteins in the ejaculate, in the nuptial gift or both that mediate these effects remains unclear.

The decorated cricket, *Gryllodes sigillatus*, is an ideal model system with which to disentangle the competing effects of mating, accessory gland proteins in the male's ejaculate and compounds ingested during nuptial feeding on female postcopulatory behaviour, receptivity and oviposition. In this species, males offer a nuptial food gift to females that comes in the form of a spermatophylax, a gelatinous mass forming part of the male's spermatophore and consumed by the female after mating (Sakaluk, 1984). Spermatophylax feeding deters the female from prematurely removing the sperm ampulla, the sperm-containing portion of the spermatophore, and thus serves to promote increased sperm transfer (Sakaluk, 1984, 1985, 1987) and male fertilization success (Calos & Sakaluk, 1998; Eggert et al., 2003; Sakaluk, 1986; Sakaluk & Eggert, 1996).

A recent proteomics analysis of the decorated cricket spermatophylax has revealed a suite of 30 different proteins, at least 18 of which arise from genes expressed in male accessory glands (Pauchet et al., 2015). Females are exposed to these spermatophylax proteins during nuptial gift feeding (Sakaluk et al., 2019), in addition to accessory gland proteins contained in the ejaculate transferred via the sperm ampulla (Simmons et al., 2013, 2014). However, the role that these unique and abundant spermatophylax proteins might play in influencing female physiology and behaviour remains unknown. Spermatophylax consumption is known to influence the oviposition schedule of females, increasing oviposition of female *G. sigillatus* early in their lives (Kasuya & Sato, 1998), and there is evidence that it may also lead to a decrease in female sexual receptivity, albeit in an unrelated species (Sakaluk, 2000; Sakaluk et al., 2006).

Here, we evaluate the effects of mating, nuptial feeding and male accessory gland proteins on female reproductive behaviour using a crossed experimental design in which we injected females of varying mating status with male accessory gland extract, an approach that has been employed to good effect in other taxa (Gillot, 2003; Sirot et al., 2021; Villarreal et al., 2018; Yamane et al., 2008). We hypothesized that male accessory gland proteins, in the spermatophylax, the ampulla or both alter female reproduction. To test this hypothesis, we conducted two experiments, one in which we assessed the effects of accessory gland proteins and mating, with or without the consumption of the spermatophylax, on female future receptivity and postcopulatory behaviour and another in which we quantified the effects of accessory gland protein injection and spermatophylax consumption on female oviposition behaviour. We predicted that females receiving injections of accessory gland proteins would exhibit reduced sexual receptivity compared with control females, but that this effect would be more evident in previously mated females than in unmated females, due to the receipt of proteins via both mating and injection. In line with previous findings showing that spermatophylax consumption can increase the rate of oviposition (Kasuya & Sato, 1998), we further predicted that injection of accessory gland proteins would accelerate egg laying, but that this effect might be contingent on whether females had recently consumed a spermatophylax.

METHODS

Experimental Animals

Experimental *G. sigillatus* were the descendants of approximately 500 adult crickets collected in Las Cruces, New Mexico, U.S.A. in 2001 and used to initiate a laboratory colony maintained at a population size of approximately 5000 and allowed to breed randomly (Ivy & Sakaluk, 2005). After hatching, nymphs were initially reared in 6-litre plastic bins filled with egg carton to increase rearing surface area and provisioned with finely ground cat food (Purina® Complete Cat Chow) ad libitum and water in glass vials plugged with moist cotton. Approximately 3 weeks later, nymphs were transferred to 19-litre plastic bins, provided with water as above, but fed whole Purina® Complete Cat Chow and Envigo® 2018 CM Teklad Certified Global 18% protein rodent diet pellets ad libitum. All crickets were reared at constant temperature (32 °C) and photoperiod (16:8 h light:dark cycle). Immature crickets were checked daily for the moult to the penultimate instar and then isolated to control for age of subjects and to ensure that they remained unmated. Isolated females were held individually in deli containers (450 ml), whereas males were housed together in 19-litre containers with ample food and water.

Preparation of Accessory Gland Extracts

We dissected accessory glands from sexually mature, unmated males at 7 days postadult moult. Males were kept on ice for up to 2 min and then dissected in a dish containing ice-cold Ringer's saline solution. Accessory glands were removed using sterilized forceps and a dissecting probe, homogenized in 100 µl of Ringer's saline solution in a sterile 1.5 ml microcentrifuge tube and centrifuged at 10 000 revolutions/min for 10 min at 4 °C. We removed 75 µl of the supernatant containing accessory gland proteins, but not tissue fragments, from extracts derived from five pooled accessory glands. Total protein concentration was measured using a Pierce™ BCA Protein Assay Kit. We added 200 µl of the assay working reagent to 25 µl samples in triplicate in an optically clear 96-well plate. Samples were incubated at 37 °C for 30 min in darkness before absorbance was measured at 562 nm using a ThermoScientific MultiSkan GO microplate spectrophotometer. Following blank subtraction, we calculated protein concentrations per pool based on bovine serum albumin standards. The same protocol was followed with dissected wing stridulatory muscle to create a sham control for protein injection per se. Protein concentrations of all pooled extracts were adjusted to 61 µg/ml, based on the lowest concentration measured. All extracts were then stored at −80 °C and thawed on ice when used for injections. Protein integrity was also confirmed by running extracts on a 4–12% SDS page gel, which showed intact proteins in both accessory gland and wing stridulatory muscle extracts.

Experiment 1: Effects on Female Receptivity and Postcopulatory Behaviour

We employed a fully factorial design in which females of varying mating status were injected with male accessory gland proteins or assigned as controls. Specifically, females were assigned to one of three injection treatments at 7 days postadult eclosion: (1) injection of Ringer's saline (a control for the vehicle), (2) injection of wing stridulatory muscle protein extract (a control for any effect of a protein injection per se) or (3) injection of accessory gland protein extract. Females were cold anaesthetized on ice in 1.5 ml tubes for a maximum of 2 min. Crickets were injected with 2 µl of the respective treatment solution between the sixth and seventh pleurite of the abdomen using a needle formed from a heat-pulled glass microcapillary tube (external diameter 1 mm, internal diameter 0.50 mm). During a daily block of injections, needles were cleaned with 70% ethanol and rinsed with Nanopure™ water between each injection, and a new needle was used for each injection treatment.

Injection treatments were replicated within three distinct mating regimes. Specifically, 6-day-old unmated adult females were assigned to one of three mating treatments prior to accessory gland injection: (1) unmated, (2) mated once and allowed to consume the spermatophylax (i.e. mated normally) or (3) mated once but prevented from consuming the spermatophylax (i.e. mated but prevented from nuptial feeding). This design enabled us to discern whether any effect of accessory gland proteins was contingent on whether a female had previously mated and, if so, whether injection of accessory gland proteins interacted with spermatophylax consumption to influence a female's subsequent receptivity. We placed females that were allowed to mate normally with a male in a small mating arena (described below) and observed them until mating was complete. The female was then permitted to consume the spermatophylax; only females that consumed the spermatophylax for at least 30 min were retained in the experiment. Females that were allowed to mate but prevented from consuming the spermatophylax after spermatophore transfer were confined to a 1.5 ml microcentrifuge tube for 30 min to prevent spermatophylax consumption (Ryan & Sakaluk, 2009). Subsequently, we removed the spermatophylax with fine forceps and allowed the female to remove and consume the ampulla of her own volition as was the case with the normally mated females. Thus, females in both mated groups retained their sperm ampulla for at least 30 min, which is sufficient to supply females with ample sperm and ejaculatory substances (Sakaluk, 1984). We assigned 178 females to the various treatments; sample sizes for specific treatment combinations are reported in Table 1.

We staged mating trials involving experimental females and randomly selected outbred males 3 h after females received their injections. Although this period allowed the female to recover from injection, it is also biologically relevant as the intercopulatory interval of males allowed constant access to receptive females is approximately 3 h, which necessarily constrains female mating frequency (Sakaluk, 1985), and females often mate more than once a night under natural conditions (Sakaluk et al., 2002). Moreover, females are not likely to be immediately influenced by compounds transferred at mating, and so providing a brief recovery period allowed time for any effect of accessory gland proteins to materialize. Mating trials took place during the dark phase of the daily light cycle in a room maintained at 30 °C, a time during which male sexual signalling and mating behaviour normally occurs (Burpee & Sakaluk, 1993; Sakaluk, 1987). Matings were staged under red light for observation in small, clear mating arenas (8 × 3 × 6 cm) lined with moistened paper towel to provide traction to experimental subjects. In each mating trial, males were introduced first into the mating arena and allowed a few minutes to acclimate, after which females were introduced. Females were uniquely labelled but observed blind to treatment. Males that did not initiate courtship within the first 10 min of being introduced to the female were removed and replaced with a different male.

Table 1
Sample sizes for specific treatment combinations in experiments 1 and 2

Injection treatment	Mating treatment	*N*
Experiment 1: Effects on female receptivity and postcopulatory behaviour		
Accessory gland	No spermatophylax	20
Accessory gland	Spermatophylax eaten	21
Accessory gland	Virgin	20
Saline	No spermatophylax	20
Saline	Spermatophylax eaten	19
Saline	Virgin	19
Wing muscle	No spermatophylax	19
Wing muscle	Spermatophylax eaten	20
Wing muscle	Virgin	20
Experiment 2: Effects on female oviposition		
Accessory gland	No spermatophylax	15
Accessory gland	Spermatophylax eaten	14
Saline	No spermatophylax	15
Saline	Spermatophylax eaten	16
Wing muscle	No spermatophylax	15
Wing muscle	Spermatophylax eaten	15

We recorded the time at which the female mounted the male in relation to the initiation of male courtship (a necessary prelude to copulation), the time at which successful spermatophore transfer occurred and the beginning and end of spermatophylax consumption. From these measures, we calculated two critical metrics: (1) latency to mount (the time from when a male initiated courtship until the female mounted him) and (2) the time the female spent feeding on the spermatophylax after mating. These measures served as proxies for female sexual receptivity and the length of time for sperm transfer, respectively, as the duration of spermatophylax consumption is directly linked to the duration of ampulla attachment, which in turn determines the number of sperm transferred (Sakaluk, 1984). Females were considered sexually unresponsive in any trial in which the male courted for longer than 30 min without the female mounting, at which point the trial was terminated, as receptive females typically mount within approximately 15 min of being courted (Sakaluk, 1987); these observations were included as right-censored values in subsequent analyses.

Experiment 2: Effects on Female Oviposition

As in experiment 1, we employed a fully factorial design in which females of different mating status were injected with male accessory gland proteins or assigned as controls. Females were randomly assigned to the same three injection treatments as described in the previous experiment. However, here, injection treatments were replicated within only two mating regimes: (1) females mated once and allowed to consume the spermatophylax (i.e. mated normally) and (2) females mated once but prevented from consuming the spermatophylax (i.e. mated but prevented from nuptial feeding). In addition, ampulla attachment time was standardized for all females at 25 min by removing the ampulla with forceps, controlling for differential receipt of sperm or ejaculatory substances. We assigned 90 females to the various treatments; sample sizes for specific treatment combinations are reported in Table 1.

Approximately 2.5 h after mating, females within the two mating treatments were given their prescribed injections, as outlined above. Females were then isolated in individual containers with a moistened cotton wool pad as an oviposition substrate,

water, food and egg carton substrate. The oviposition pad was replaced every 12 h for 7 consecutive days. Individual oviposition pads were frozen and later thawed to count eggs, which was done blind to treatment. After the 7-day oviposition period, females were frozen and their pronotum width measured as a proxy for structural body size using a stereomicroscope (Nikon SMZ800) equipped with a digital camera and imaging software (Nikon NIS-Elements Documentation v. 4.20).

Ethical Note

Our study adhered to the ASAB/ABS Guidelines for the use of animals in research, the legal requirements of the U.S.A. and all institutional guidelines at Illinois State University.

Statistical Analysis

We employed a Cox proportional hazards model to evaluate the effects of accessory gland protein injection on female latency to mating, with mating treatment, injection treatment and their interaction included as fixed effects. We assigned a value of 0 to the female if she mounted to designate that the observation was uncensored, and recorded her latency to mate following male courtship initiation. Females that had not mounted 30 min after males initiated courtship received a value of 1 to indicate right censoring of the values. We examined treatment effects on the duration of spermatophylax consumption duration using a generalized linear model with a lognormal (base e) response distribution. We analysed the effect of accessory gland protein injection on the temporal pattern of oviposition (eggs laid/h) using a repeated-measures general linear model with mating treatment, injection treatment, oviposition time period and their interactions included as fixed effects and female pronotum length included as a covariate. Female identity was included as a random effect to account for repeated measures of the same female across time. For the purposes of this analysis, oviposition period was apportioned into four time blocks, comprising the first 24 h (block 1), followed by three consecutive blocks of 48 h. An initial analysis utilizing seven blocks of consecutive 24 h periods proved resistant to identifying an appropriate response distribution, due to an overabundance of zero values. One female did not lay any eggs the entire week and was excluded from the analysis. All analyses were conducted using SAS software version 9.4 (SAS Institute, Cary, NC, U.S.A.).

RESULTS

Experiment 1: Effects on Female Receptivity and Postcopulatory Behaviour

There was no significant effect of accessory gland injection treatment on a female's latency to mount a male in a future staged mating (Wald $\chi^2_2 = 2.76$, $P = 0.25$), but there was a significant effect of female mating treatment (Wald $\chi^2_2 = 12.35$, $P = 0.0021$; Fig. 1). Specifically, unmated females mounted courting males more quickly than previously mated females, regardless of whether the latter had been permitted to consume the spermatophylax ($\chi^2 = 13.44$, $P = 0.0007$) or not ($\chi^2 = 27.94$, $P < 0.0001$). However, there was no significant difference in the latency to mount by mated females that were prevented from feeding on the spermatophylax and by those permitted to do so ($\chi^2 = 1.94$, $P = 0.41$). There was also no significant interaction between mating treatment and injection treatment on female latency to mount (Wald $\chi^2_4 = 0.86$, $P = 0.93$).

There was no significant effect of accessory gland injection treatment on the time females spent feeding on the spermatophylax

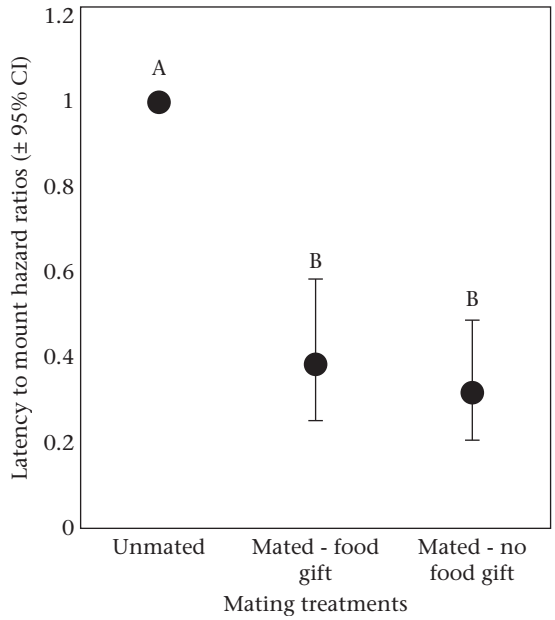

Figure 1. Hazard ratios of female latency to mount following initiation of male courtship by female prior mating treatment. Hazard ratios are presented relative to the unmated reference group, with a hazard ratio below 1 signifying an increased latency to mount. Different letters above treatments signify significant differences in pairwise comparisons ($P < 0.05$).

($F_{2,145} = 1.38$, $P = 0.25$). However, there was a significant effect of female mating treatment on the duration of spermatophylax consumption ($F_{2,145} = 5.98$, $P = 0.0032$; Fig. 2). Post hoc pairwise comparisons revealed that mated females that were prevented from consuming the spermatophylax after their initial mating fed on the spermatophylax of the subsequent mating for a significantly longer duration than previously unmated females ($t_{151} = 2.50$, $P = 0.036$) or mated females that were permitted to consume the spermatophylax during the earlier mating ($t_{151} = 3.34$, $P = 0.0030$). The

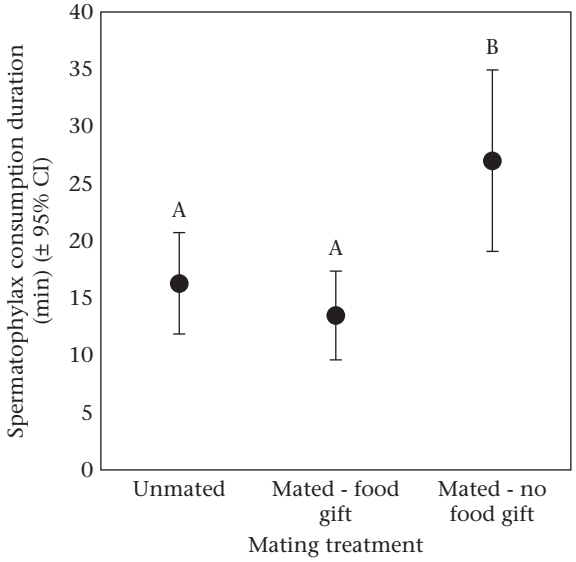

Figure 2. Spermatophylax consumption duration by females in different mating treatments. For mated females, this represents their second mating. Points represent predicted marginal means (least squares means ± 95% confidence intervals). Different letters above treatments signify significant differences in pairwise comparisons ($P < 0.05$).

spermatophylax consumption duration of unmated females did not significantly differ from previously mated females that were permitted to consume the spermatophylax after their initial mating ($t_{151} = -0.95$, $P = 0.61$). There was also no significant interaction between mating and injection treatments in their influence on spermatophylax consumption duration ($F_{4,145} = 1.26$, $P = 0.29$).

Experiment 2: Effects on Female Oviposition

There were no significant effects of accessory gland injection treatment ($F_{2,84} = 0.17$, $P = 0.85$), mating status ($F_{1,84} = 0.69$, $P = 0.41$) or their interaction ($F_{2,84} = 0.50$, $P = 0.61$) on the rate of egg laying. However, the rate of egg laying varied significantly over time ($F_{3,252} = 75.6$, $P < 0.0001$; Fig. 3). There were no significant interactions between time and either of the other fixed effects (injection * time: $F_{6,252} = 0.36$, $P = 0.91$; mating * time: $F_{3,252} = 0.81$, $P = 0.49$), although the three-way interaction between time, injection treatment and mating treatment was borderline nonsignificant in support of a more complex effect ($F_{6,252} = 2.00$, $P = 0.063$). Female pronotum length initially was included as a covariate but was omitted from the final analysis as it was not significant ($F_{1,83} = 0.70$, $P = 0.40$).

DISCUSSION

Our results reveal that the previous mating experience of a female can have a profound influence on her behaviour in a subsequent copulation and that receipt of sperm and consumption of the nuptial food gift independently influence how long a female spends feeding on the nuptial food gift after her next mating. In contrast, injection of accessory gland proteins had no significant effect on either female sexual receptivity or female propensity to consume the nuptial food gift. Neither the accessory gland injection treatment nor consumption of the spermatophylax after mating affected the temporal pattern of oviposition. We elaborate on the possible proximate mechanisms mediating these effects and their potential fitness consequences below.

The apparent decrease in female sexual receptivity after mating is consistent with what has been found in other insects (Avila et al., 2011), including other cricket species. In house crickets, *Acheta domesticus* (Koudele et al., 1987), and in the field crickets *Gryllus bimaculatus* (Loher et al., 1993), *Gryllus texensis* (Lickman et al., 1998) and *Teleogryllus oceanicus* (Moschilla et al., 2020; Tanner et al., 2019), mating leads to a diminished phonotactic response to male calling song, which is reflective of a decrease in female sexual receptivity. More directly, Judge et al. (2010) showed that mating leads to both a significant increase in the latency to a subsequent mating and a decreased probability of remating in *Gryllus pennsylvanicus*. That the decrease in sexual receptivity following mating might be mediated by seminal proteins or other ejaculatory substances transferred by the male was first hinted at by a study in which phototaxis of mated *G. bimaculatus* was reinstated upon removal of the female's spermatheca, the primary storage organ for sperm and, presumably, other ejaculatory products (Loher et al., 1993). In line with this possibility, Fleischman and Sakaluk (2004) observed that multiply mated female *A. domesticus* took significantly longer to remate than singly mated females, suggesting that the accumulation of ejaculatory products in the female spermatheca could be influencing female sexual receptivity. However, definitive evidence that these effects are mediated, at least in part, by accessory gland proteins transferred in the male's ejaculate comes from a recent study using RNA interference to knock-down expression of genes encoding two proteins contained in the ejaculate of male *T. oceanicus* (Moschilla et al., 2020): females mated to males in which expression had been knocked down subsequently showed greater phototactic responsiveness than females mated to control males.

Whether or not the female was permitted to consume the spermatophylax after mating had no influence on the sexual receptivity of the female beyond the effect of mating per se. This result aligns with that of a previous study in which females were permitted to consume the spermatophylax after mating or experimentally prevented from doing so (Sakaluk et al., 2006); here too, there was no difference between the two treatments in the latency to remating. However, spermatophylax consumption significantly influenced the time spent feeding by the female on the nuptial food gift at her next mating. Females permitted to consume the spermatophylax normally after mating spent significantly less time feeding on the spermatophylax after a subsequent mating than mated females that were experimentally prevented from nuptial feeding after an initial mating. This result reveals a two-fold fitness advantage to males arising from the consumption of the spermatophylax by their current mate. First, by delaying female removal of the sperm dispensing ampulla, it promotes an increase in the number of sperm transferred to the current mate, which is the primary determinant of a male's fertilization success, particularly when his sperm must compete with sperm of the female's other mating partners (Calos & Sakaluk, 1998; Eggert et al., 2003; Sakaluk, 1986; Sakaluk & Eggert, 1996). Females routinely mate with many different males (Sakaluk et al., 2002), and the sperm of

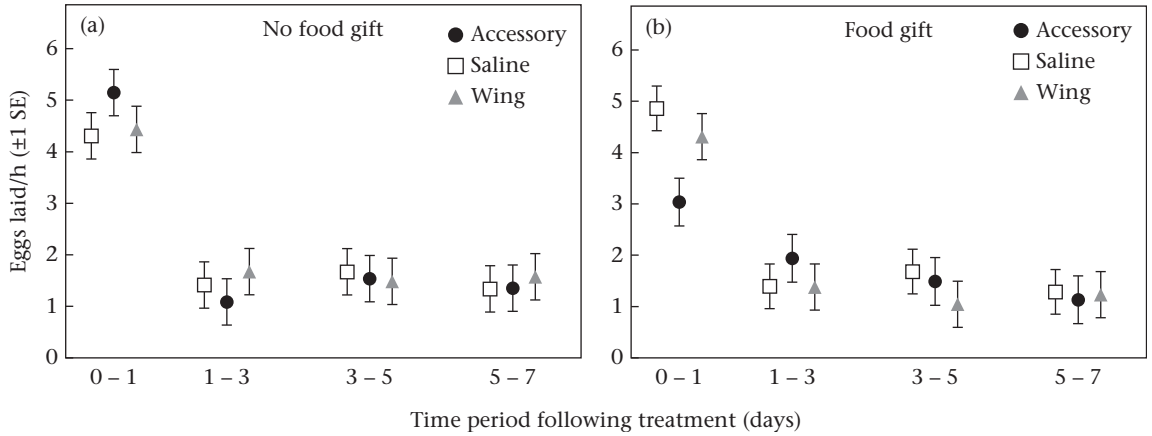

Figure 3. Mean number of eggs laid/h by females across different time periods following accessory gland infection treatment. (a) Mated females prevented from consuming the spermatophylax. (b) Mated females permitted to consume the spermatophylax. Data points represent predicted marginal means (least squares means ± 1 SE).

the different males are recruited for fertilization in direct proportion to their relative abundance in the female's spermatheca (Sakaluk, 1986; Sakaluk & Eggert, 1996). Second, the female spends less time feeding on the spermatophylax of a rival male at her next mating, and consequently terminates sperm transfer sooner (Sakaluk, 1984) to the benefit of her previous mate. This effect of nuptial feeding on the female's acceptance of a subsequent gift is, to the best of our knowledge, the first showing that the fitness benefits of nuptial feeding can also accrue over future matings by the female.

Neither injection of accessory gland extract nor consumption of the spermatophylax influenced the number of eggs laid by females or the temporal pattern of oviposition. The absence of an effect of the injection of accessory gland tissue aligns with the result of an earlier study in which accessory gland proteins extracted from spermatophores were injected into the abdomen of female *G. bimaculatus* (Green & Tregenza, 2009). Neither female phonotaxis (a proxy for female receptivity) nor the number of eggs laid was influenced by this treatment, leading the authors to suggest that any effects of accessory gland proteins on female reproduction may require their direct transmission into the female reproductive tract. That the injection of accessory gland extract had no effect on any aspect of female behaviour or reproduction in the current study supports this suggestion, especially given that later work has shown that knock-down expression of genes encoding seminal proteins alters female receptivity in *T. oceanicus* (Moschilla et al., 2020).

There was also no effect of spermatophylax consumption on the number of eggs laid, a result consistent with previous studies showing that experimental manipulation of the number of food gifts consumed daily had no influence on total female fecundity (Ivy & Sakaluk, 2005; Kasuya & Sato, 1998; Will & Sakaluk, 1994). However, Kasuya and Sato (1998) found that the number of spermatophylaxes consumed had a significant effect on the schedule of oviposition, with an increase in spermatophylax consumption associated with a higher rate of egg laying early in the oviposition period. Although a transitory increase in oviposition rate could, in theory, benefit a male via an increase in the number of eggs fertilized by him before the female remates with a rival, we observed no such increase in this study.

In conclusion, both previous mating experience and spermatophylax consumption influence important facets of female reproduction that reverberate on male fitness, including female receptivity, nuptial feeding behaviour and sperm transfer. One especially novel finding is the cascading effect of spermatophylax consumption on female acceptance and feeding on future gifts, as this influences male fitness through the penalty exacted in terms of reduced sperm transfer of future rival males. These effects are likely mediated, at least in part, by accessory gland proteins contained in male nuptial food gifts, ejaculates or both. Paradoxically, injection with accessory gland extracts had no effect on any aspect of female reproduction or behaviour, but this is likely because seminal proteins could not access potential receptors within the female reproductive tract (Green & Tregenza, 2009) or, alternatively, that compounds secreted by females within their reproductive tract are necessary for the proper functioning of seminal products (McDonough-Goldstein et al., 2022; Meslin et al., 2017). Regardless, our findings necessitate the identification of the underlying mechanisms mediating these effects, with a particular emphasis on identifying the accessory gland proteins involved as there is a limited number of these (Pauchet et al., 2015). Combining studies such as the one presented here with targeted molecular approaches will expand our understanding of how specific accessory gland proteins in ejaculates and nuptial gifts modulate female behaviour and physiology. This is an important endeavour in increasing our understanding of the evolution of sexual conflict and the mechanistic strategies underlying it.

Author Contributions

I. G. Rines: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing-original draft, Visualization, Funding acquisition; **A. E. Harrod**: Investigation, Writing-review and editing; **J. Hunt**: Conceptualization; Resources, Writing-review and editing, Funding acquisition; **B. M. Sadd**: Conceptualization, Methodology, Supervision, Project administration, Formal analysis, Writing-review and editing, Visualization, Funding acquisition; **S. K. Sakaluk**: Conceptualization, Methodology, Supervision, Project administration, Data curation, Formal analysis, Writing-review and editing, Funding acquisition.

Data Availability

Raw data are archived in the Mendeley Data Repository: https://doi.org/10.17632/dh2pdkby89.1 (Sakaluk, 2022).

Declaration of Interest

None.

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