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

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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.

22 Keywords: accessory gland proteins, crickets, mating behaviour, nuptial food gifts, oviposition,
23 sexual conflict, sexual receptivity

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, Bradley, Moxon, & Chapman, 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, Naylor, & Siva-Jothy, 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, Sirot, & Wigby, 2013; Worthington, Jurenka, & Kelly, 2015; Sirot, 2019), and, in certain insect species in which males synthesize nuptial food gifts provisioned to females, compounds orally consumed by females that affect their post-copulatory behaviour and subsequent receptivity (Arnqvist & Nilsson, 2000; Vahed, 2007; Sakaluk, Duffield, Rapkin, Sadd, & Hunt, 2019).

The influence of male-derived ejaculatory substances on female insect longevity, reproduction, and sexual receptivity has especially been a major focus of previous research (Leopold, 1976; Gillot, 2003; 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, Sirot, LaFlamme, Rubinstein, & Wolfner, 2011). While some of these effects are beneficial to females, such as 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, Craig, & Despommier, 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 (Sirot, Wong, Chapman, & Wolfner, 2015; Chapman, 2018; Hollis et al., 2019).

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 (Pauchet et al., 2015; Lehmann, Lehmann, Neumann, Lehmann, Scheler, & Jungblut, 2018), 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; Vahed, 2007; Sakaluk et al., 2019). However, because nuptial feeding typically promotes increased transfer of sperm and other ejaculatory substances (Sakaluk, 1984; Wedell, 1993; Vahed, 1998), 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 post-copulatory 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 (Sakaluk, 1986; Sakaluk & Eggert, 1996; Calos & Sakaluk, 1998; Eggert, Reinhardt, & Sakaluk, 2003).

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, Avery, & Weddle, 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; Yamane, Miyatake, & Kimura, 2008; Villarreal, Pitchera, Helinski, Johnson, Wolfner, & Harrington, 2018; Sirot et al., 2021). We hypothesized that male accessory gland proteins, in the spermatophylax, the ampulla, or both, alter female reproduction. To test this hypothesis, we conducted

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 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 L 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 three weeks later, nymphs were transferred to 19 L 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 (16h:8h L:D). 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 L containers with ample food and water.

Preparation of Accessory Gland Extracts

Accessory glands were dissected from sexually mature, unmated males at 7 days post-adult moult. Males were kept on ice for up to two minutes 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 RPM for 10 minutes at 4°C. 75 µL of the supernatant containing accessory gland proteins, but not tissue fragments, were removed from extracts derived from five pooled accessory glands. Total protein concentration was measured using a Pierce™ BCA Protein Assay Kit. 200 µL of the assay working reagent were added to 25 µL samples in triplicate in an optically clear 96 well plate. Samples were incubated at 37°C for 30 minutes in darkness before absorbance was measured at 562 nm using a ThermoScientific MultiSkan GO microplate spectrophotometer. Following blank subtraction, protein concentrations per pool were calculated 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 Post-copulatory Behavior

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 seven days post adult eclosion: i) injection of Ringer's saline (a control for the vehicle), ii) injection of wing stridulatory muscle protein extract (a control for any effect of a protein injection per se), or iii) injection of accessory gland protein extract. Females were cold anesthetized on ice in 1.5 mL tubes for a maximum of two minutes. Crickets were injected with 2 µL of the respective treatment solution between the 6th and 7th 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: i) unmated, ii) mated once and allowed to consume the spermatophylax (i.e., mated normally), and iii) 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 interacts with spermatophylax consumption in their influence on a female's subsequent receptivity. Females that were allowed to mate normally were placed with a male in a small mating arena (described below) and observed until mating was completed and permitted to consume the spermatophylax thereafter; only females that consumed the spermatophylax for at least 30 minutes were retained in the experiment. Females that were mated but prevented from consuming the spermatophylax after spermatophore transfer were confined to a 1.5 mL microcentrifuge tube for 30 minutes to prevent spermatophylax consumption (Ryan & Sakaluk, 2009). Subsequently, the spermatophylax was removed with fine forceps and the female was allowed 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). A total of 178 females was assigned to the various treatments; sample sizes for specific treatment combinations are reported in Table 1.

Mating trials involving experimental females and randomly selected outbred males were staged three hours after females received their injections. This period allowed the female to recover from injection but is also biologically relevant as the intercopulatory interval of males allowed constant access to receptive females is approximately three hours, which necessarily constrains female mating frequency (Sakaluk, 1985), and females often mate more than once a night under natural conditions (Sakaluk, Schaus, Eggert, Snedden, & Brady, 2002). Moreover, females are not likely to be immediately influenced by compounds transferred at mating, and

178 so providing a brief recovery period allowed time for any effect of accessory gland proteins to
179 materialize. Mating trials took place during the dark phase of the daily light cycle in a room
180 maintained at 30 °C, a time during which male sexual signalling and mating behaviour normally
181 occurs (Sakaluk, 1987; Burpee & Sakaluk, 1993). Matings were staged under red light for
182 observation in small, clear, mating arenas (8 x 3 x 6 cm) lined with moistened paper towel to
183 provide traction to experimental subjects. In each mating trial, males were introduced first into
184 the mating arena and allowed a few minutes to acclimate, after which females were
185 introduced. Females were uniquely labelled but observed blind to treatment. Males that did
186 not initiate courtship within the first 10 minutes of being introduced to the female were
187 removed and replaced with a different male.

188 We recorded the time at which the female mounted the male in relation to the initiation
189 of male courtship (a necessary prelude to copulation), the time at which successful
190 spermatophore transfer occurred, and the beginning and end of spermatophylax consumption.
191 From these measures, we calculated two critical metrics: 1) latency to mount (the time from
192 when a male initiated courtship until the female mounted him) and 2) the time the female
193 spent feeding on the spermatophylax after mating. These measures served as proxies for
194 female sexual receptivity and the length of time for sperm transfer, respectively, as the
195 duration of spermatophylax consumption is directly linked to the duration of ampulla
196 attachment, which in turn determines the number of sperm transferred (Sakaluk, 1984).
197 Females were considered sexually unresponsive in any trial in which the male courted for
198 longer than 25 minutes 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: i) females mated once and allowed to consume the spermatophylax (i.e., mated normally), and ii) 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 minutes by removing the ampulla with forceps, controlling for differential receipt of sperm or ejaculatory substances. A total of 90 females were assigned to the various treatments; sample sizes for specific treatment combinations are reported in Table 1.

Approximately 2.5 hours 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 hours 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).

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. For each female, mounting was designated by a 1 together with the specific time post male courtship initiation. Females that had not mounted 25 minutes after males initiated courtship received a 0 at this specific time 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. The effect of accessory gland protein injection on the temporal pattern of oviposition (eggs laid per hour) was analysed 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 hours (block one), followed by three consecutive blocks of 48 hours. An initial analysis utilizing seven blocks of consecutive 24-h periods proved resistant to identifying an appropriate response distribution, due to an over-abundance 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).

243

244 **RESULTS**

245 *Experiment 1: Effects on Female Receptivity and Post-copulatory Behavior*

246 There was no significant effect of accessory gland injection treatment on the latency of females
247 to mount a male in a future staged mating (Wald χ^2 = 2.75, *df* = 2, *P* = 0.25), but there was a
248 significant effect of female mating treatment (Wald χ^2 = 12.33, *df* = 2, *P* = 0.0021, Fig.1).
249 Specifically, unmated females mounted courting males more quickly than previously mated
250 females, regardless of whether the latter had been permitted to consume the spermatophylax
251 (χ^2 = 12.77, *P* = 0.0011) or not (χ^2 = 28.03, *P* < 0.0001). However, there was no significant
252 difference between the time to mounting of mated females that were prevented from feeding
253 on the spermatophylax and those permitted to do so (χ^2 = 2.11, *P* = 0.38). There was also no
254 significant interaction between mating treatment and injection treatment on female latency to
255 mount (Wald χ^2 = 1.37, *df* = 4, *P* = 0.85).

256 There was no significant effect of accessory gland injection treatment on the time
257 females spent feeding on the spermatophylax ($F_{2,145}$ = 1.38, *P* = 0.25). However, there was a
258 significant effect of female mating treatment on the duration of spermatophylax consumption
259 ($F_{2,145}$ = 5.98, *P* = 0.0032, Fig. 2). Post hoc pairwise comparisons revealed that mated females
260 that were prevented from consuming the spermatophylax after their initial mating fed on the
261 spermatophylax of the subsequent mating for a significantly longer duration than previously
262 unmated females (t_{151} = 2.50, *P* = 0.036) and mated females that were permitted to consume
263 the spermatophylax during the earlier mating (t_{151} = 3.34, *P* = 0.0030). The spermatophylax
264 consumption duration of unmated females did not significantly differ from previously mated

265 females that were permitted to consume the spermatophylax after their initial mating (t_{151} = -
266 0.95, P = 0.61). There was also no significant interaction between mating and injection
267 treatments in their influence on spermatophylax consumption duration ($F_{4,145}$ = 1.26, P = 0.29).
268
269 *Experiment 2: Effects on Female Oviposition*
270 There were no significant effects of accessory gland injection treatment ($F_{2,84}$ = 0.17, P =
271 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
272 of egg laying. However, the rate of egg laying varied significantly over time ($F_{3,252}$ = 75.6, P <
273 0.0001, Fig. 3). There were no significant interactions between time and either of the other
274 fixed effects (Injection*Time: $F_{6,252}$ = 0.36, P = 0.91; Mating*Time: $F_{3,252}$ = 0.81, P = 0.49),
275 although the three-way interaction between time, injection treatment, and mating treatment
276 was borderline non-significant in support of a more complex effect ($F_{6,252}$ = 2.00, P = 0.063).
277 Female pronotum length initially was included as a covariate but was omitted from the final
278 analysis as it was not significant ($F_{1,83}$ = 0.70, P = 0.40).
279
280 **DISCUSSION**
281 Our results reveal that the previous mating experience of a female can have a profound
282 influence on her behaviour in a subsequent copulation, and that receipt of sperm and
283 consumption of the nuptial food gift independently influence how long a female spends feeding
284 on the nuptial food gift after her next mating. In contrast, injection of accessory-gland proteins
285 had no significant effect on either female sexual receptivity or female propensity to consume
286 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, Stout, & Reichert, 1987), and the field crickets *Gryllus bimaculatus* (Loher, Weber, & Huber, 1993), *G. texensis* (Lickman, Murray, & Cade, 1998), and *Teleogryllus oceanicus* (Tanner, Garbe, & Zuk, 2019; Moschilla, Tomkins, & Simmons, 2020), mating leads to a diminished phonotactic response to male calling song, which is reflective of a decrease in female sexual receptivity. More directly, Judge, Tran, & Gwynne (2010) showed that mating leads to both a significant increase in the latency to a subsequent mating and a decreased probability of remating in *G. 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 & 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

309 the ejaculate of male *T. oceanicus* (Moschilla et al., 2020): females mated to males in which
310 expression had been knocked down subsequently showed greater phototactic responsiveness
311 than females mated to control males.

312 Whether or not the female was permitted to consume the spermatophylax after mating
313 had no influence on the sexual receptivity of the female beyond the effect of mating per se.
314 This result aligns with that of a previous study in which females were permitted to consume the
315 spermatophylax after mating or experimentally prevented from doing so (Sakaluk et al., 2006);
316 here too, there was no difference between the two treatments in the latency to remating.
317 However, spermatophylax consumption significantly influenced the time spent feeding by the
318 female on the nuptial food gift at her next mating. Females permitted to consume the
319 spermatophylax normally after mating spent significantly less time feeding on the
320 spermatophylax after a subsequent mating than mated females that were experimentally
321 prevented from nuptial feeding after an initial mating. This result reveals a two-fold fitness
322 advantage to males arising from the consumption of the spermatophylax by their current mate.
323 First, by delaying female removal of the sperm dispensing ampulla, it promotes an increase in
324 the number of sperm transferred to the current mate which is the primary determinant of a
325 male's fertilization success, particularly when his sperm must compete with sperm of the
326 female's other mating partners (Sakaluk, 1986; Sakaluk & Eggert, 1996; Calos & Sakaluk, 1998;
327 Eggert et al., 2003). Females routinely mate with many different males (Sakaluk et al., 2002)
328 and the sperm of the different males are recruited for fertilization in direct proportion to their
329 relative abundance in the female's spermatheca (Sakaluk, 1986; Sakaluk & Eggert, 1996).
330 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 the injection of accessory gland extract, nor consumption of the spermatophylax influenced the number of eggs laid by females, nor 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 of 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 (Will & Sakaluk, 1994; Kasuya & Sato, 1998; Ivy & Sakaluk, 2005). However, Kasuya & 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 (Meslin et al., 2017; McDonough-Goldstein, Pitnick, & Dorus, 2022). 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.

Data Archiving

Upon acceptance of the manuscript, the raw data will be archived in the Mendeley Data Repository.

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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 post-copulatory 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

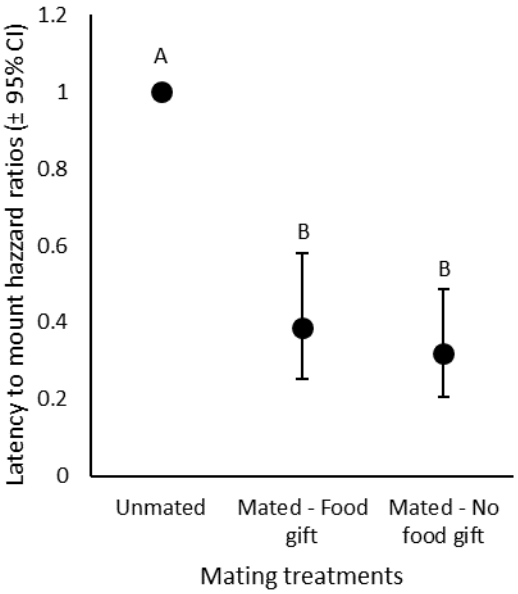

Figure 1. Hazard ratios of female latency to mount following initiation of male courtship by female prior mating treatment. Hazard ratios are presented relative to the unmated reference group, with a hazard ratio below 1 signifying an increased latency to mount. Different letters above treatments signify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

Figure 2. Spermatophylax consumption duration of females of different mating treatments. For mated females this represented their second mating. Points represent predicted marginal means (least squares means). Different letters above treatments signify significant differences in pairwise comparisons ($p < 0.05$).

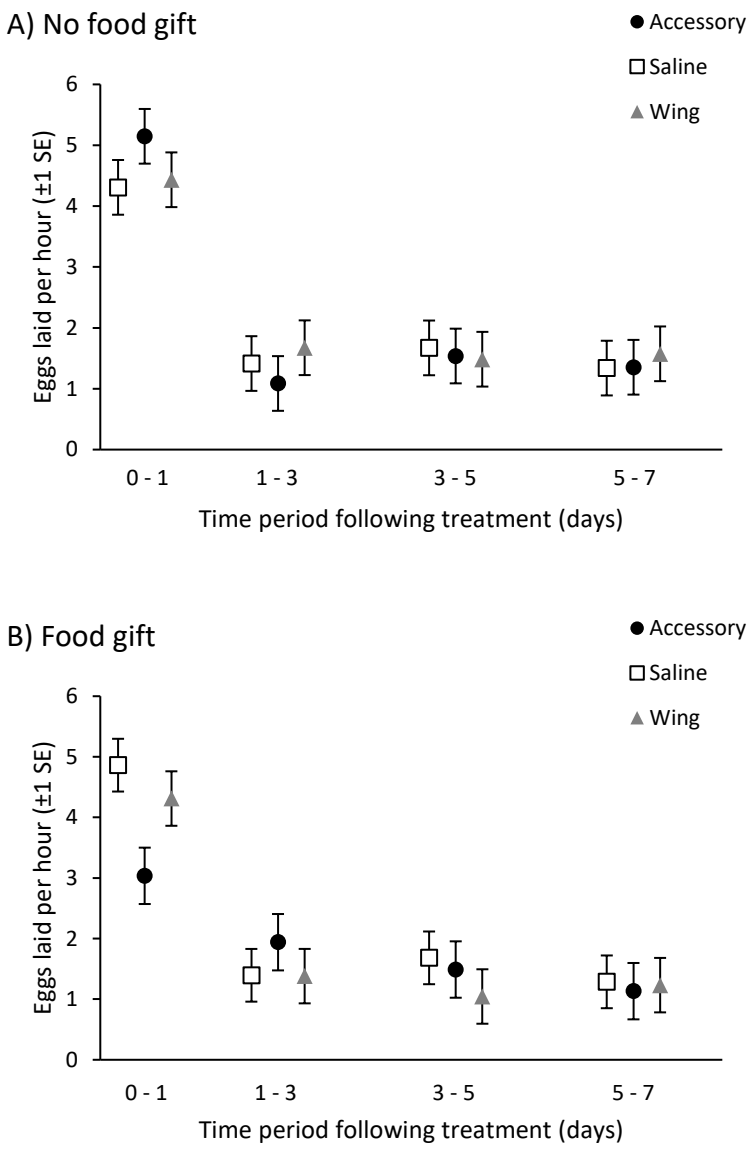

Figure 3. Mean number of eggs laid per hour by females across different time periods following accessory gland infection treatments. A) Mated females prevented from consuming the spermatophylax. B) Mated females permitted to consume the spermatophylax. Data points represent predicted marginal means (least squares means).