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Males adjust their manipulation of female remating in response to sperm competition risk

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To reduce the potential for sperm competition, male insects are thought to inhibit the post-mating reproductive behaviour of females through receptivity-inhibiting compounds transferred in the ejaculate. Selection is expected to favour phenotypic plasticity in male post-copulatory expenditure, with males investing strategically in response to their perceived risk of sperm competition. However, the impact that socially cued strategic allocation might have on female post-mating behaviour has rarely been assessed. Here, we varied male perception of sperm competition risk, both prior to and during mating, to determine if a male's competitive environment impacts the extent to which he manipulates female remating behaviour. We found that female Australian field crickets (*Teleogryllus oceanicus*) mated to males that were reared under sperm competition risk emerged from a shelter in search of male song sooner than did females mated to males reared without risk, but only when mating occurred in a risk-free environment. We also found that females reared in a silent environment where potential mates were scarce emerged from the shelter sooner than females exposed to male calls during development. Collectively, our findings suggest complex interacting effects of male and female sociosexual environments on female post-mating sexual receptivity.

1. Introduction

Although females often derive substantial benefits from polyandry [1–3], female remating also leads to greater post-copulatory competition between males [4,5]. Following remating, the initial male's sperm must compete for fertilization with that of any subsequent males, potentially leading to a reduction in his paternity share. Sexual selection is predicted to favour adaptations in males that increase their relative fertilization success by preventing rival males from gaining access to fertilization opportunities [4,6]. One way in which this can be achieved is by modifying a female's post-copulatory behaviour by inhibiting female sexual receptivity in order to prevent or delay remating [5,7–9].

In the Australian field cricket, *Teleogryllus oceanicus*, males are thought to manipulate the remating behaviour of females. Recently mated females engage in significantly less mate searching behaviour [10] and are less responsive to male song [11]. Similar to other insects [12–15], this post-mating inhibition of female sexual receptivity probably occurs, at least in part, as a function of proteins contained within a male's seminal fluid (sfps) [10]. At least two sfps, ToSfp022 and ToSfp011, appear to play a role in inhibiting female mate searching [10]. Generally, male *T. oceanicus* suffer a loss in paternity that is proportionate to the number of mates accepted by a female [16,17]. Therefore, the female refractory period probably reflects a sperm competition avoidance tactic by males. Here, we assess whether the manipulation of female remating behaviour exhibits socially cued plasticity, varying in response to a male's risk of sperm competition.

Selection is expected to favour phenotypic plasticity in post-copulatory expenditure to maximize the fitness returns of individual males [4]. Males are predicted

to invest strategically in each mating according to the sociosexual environment, modulating their reproductive allocation in response to criteria such as the level of competition [18,19]. Sperm have typically been the major focus of studies of strategic male allocation. Generally, males are predicted to increase their allocation of sperm when faced with a risk of sperm competition but reduce their allocation when the intensity of sperm competition increases [18], predictions for which there is now much support [20,21]. However, non-sperm components of the ejaculate, such as sfps, are likely to play a crucial role in the outcome of competition [22–24], and should therefore also be strategically allocated in response to sperm competition [25,26]. Empirical studies have observed socially cued variability in sfp allocation [27–29]. In *Drosophila melanogaster*, for example, males transfer greater quantities of sfps when exposed to high levels of competition [30]. But sfp allocation is probably complex, with male *Drosophila* making protein-specific adjustments in response to female mating status: transferring less of a fecundity-stimulating protein (ovulin), but not altering the quantity of a receptivity-inhibiting sfp (sex peptide—SP), when mating with a previously mated female [31]. Further, when males are exposed to a competitor male during copulation, more of both ovulin and SP are transferred to females [32].

Male *T. oceanicus* are also thought to make strategic adjustments to the composition of their ejaculates in response to competition [33,34]. When exposed to a risk of sperm competition, males produce ejaculates with greater sperm viability [35]. This corresponds to an increased expression of seven sfp genes [33], including two proteins previously found to influence female remating (ToSfp022 and ToSfp011) [10]. And many of the same genes that are upregulated in response to sperm competition risk are instead downregulated when males experience an increase in sperm competition intensity [34], corresponding to a reduction in sperm viability [36]. However, in general, the impact of male adjustments in sfp allocation on the subsequent reproductive behaviour of females has rarely been assessed.

Females might also be capable of responding strategically to their social environment [37,38]. For instance, while we expect that males might differentially inhibit female sexual receptivity according to their perception of sperm competition risk, varying the social environment might also adjust a female's perception of potential mating opportunities. Females that develop in an environment in which potential mates are scarce may be more responsive to additional mating opportunities [39], and mate more frequently in order to limit the risk of becoming sperm depleted [37]. Therefore, potentially, females reared in a low-male-density environment might oppose the post-copulatory tactics of males [38], maintaining high rates of remating behaviours in the face of the attempted male manipulation. Although such predictions make intuitive sense, the influence of the female social environment on the outcome of male post-copulatory strategy is not well established.

Here, we use the Australian field cricket, *T. oceanicus*, to determine if the risk of sperm competition impacts the extent to which a male manipulates female remating behaviour. In this species, males are known to use acoustic cues to detect the risk of sperm competition [33,35] and gustatory cues to detect the intensity of sperm competition [36,40], and adjust their allocation to the ejaculate accordingly. Here, we focus on risk and assess female mate searching behaviour following mating with males that were either exposed to the calls of rivals

during their development or were reared in acoustic isolation. We hypothesized that males reared in a competitive environment would allocate more resources into inhibiting female remating, resulting in females that express significantly less mate searching behaviour than those mated to males reared in a non-competitive environment. We also assessed the post-mating behavioural response of females according to males' perception of competition at the time of mating itself, allowing us to observe the extent to which males make short-term adjustments to their manipulation of female remating to match the immediate competitive conditions. Finally, we conducted an additional experiment to determine whether a female's perception of male availability during her development impacts her remating behaviour, and thus whether male manipulation of female remating might be affected by the social environment of females themselves.

2. Methods

(a) Social manipulation

Crickets (male and female) were drawn from an outbred laboratory stock population that originated from Carnarvon, Western Australia. First-instar nymphs were taken after hatching and assigned to one of two treatments that manipulated their perception of sperm competition risk throughout their development. Acoustic cues to future sperm competition have been shown to be effective in generating plasticity in male allocation to sperm quality, and to seminal fluid gene expression in *T. oceanicus* [33,35]. Thus, our sperm competition 'risk' treatment raised males exposed to calling song, while in our 'no-risk' treatment, males were reared in silence. Two 5 min pre-recordings of male song were collected from two populations of 30 sexually mature males each housed together with an equal number of females. The recordings, which contained a mixture of calling and courtship song, were broadcast on a continuous loop to 'risk' treatment crickets throughout their development. From the penultimate nymphal instar, crickets were housed in individual plastic containers (7 × 7 × 5 cm), and monitored daily until adult eclosion. Upon adult eclosion, the sound-producing forewings of males from both treatments were removed to prevent them from producing their own calls. All individuals remained in their respective acoustic treatments until used in experimental trials. All crickets were kept in a constant temperature room at 26°C on a 12:12 h light:dark cycle and supplied with cat chow and water ad libitum.

Concurrently, at the penultimate nymphal stage, male and female crickets were removed from stock populations and housed individually. As with the treatment individuals, the sound-producing forewings of stock males were removed upon adult eclosion. Crickets from the stock culture would have perceived the calls of sexually active crickets throughout their development.

(b) Effects of male social environment on female remating behaviour

A total of 198 males were used to determine if a male's perceived risk of sperm competition impacts the extent to which he manipulates female post-mating behaviour. A full factorial design assessed the impact of sperm competition risk when perceived at two different stages: during development and during mating. At 7 days post-adult eclosion, males from both developmental treatments (risk and no-risk) were left to mate with a single stock female each. Pairs were left to mate for a period of 12 h. At the time of mating, females were 22 days of adult age. Mating was conducted in either one of two environments representing a male's

immediate risk of sperm competition: 'risk' or 'no-risk'. The competitive environments experienced during mating were identical to the developmental environments. 'Risk' matings occurred with the pair placed near a speaker broadcasting male song, whereas 'no-risk' pairs mated in silence. Previous work has found that the strongest inhibitory effect of mating on female sexual receptivity occurs after 1 day [10]. We therefore measured the mate searching behaviour of females 1 day after mating completion.

Ultimately, we assessed how post-mating female behaviour was affected when perceived risk of sperm competition varied both during development and at the time of mating. Between 48 and 52 males were assigned to each of the two mating treatments (risk, no-risk) within each of the two developmental environments (risk, no-risk).

(c) Effects of female social environment on female remating behaviour

In a separate experiment, we assessed whether the acoustic environment experienced by a female during her development impacts her post-mating behaviour. Thus, females were also reared in one of the two developmental environments, exposed to the calling song of males (song) or reared in 'silence'. Twenty-two days after adult eclosion, females from both treatments were mated with a single stock male each. Pairs were left to mate for a period of 12 h. At the time of mating, males were 7 days post-adult eclosion. All matings were conducted in the silent environment. After mating completion, females were placed back into their respective acoustic environments until their mate searching behaviour was assessed 1 day later. A total of 94 females were assigned to one of the two acoustic treatments (song = 46, silence = 48).

(d) Mate searching behavioural trials

We adopted an experimental design for the behavioural trials that has been used in previous studies [41–44] and is described in detail in the electronic supplementary material. The trials consisted of an open field test, where the mate searching behaviour of individual females was observed. The behavioural arena measured 32 × 46 cm and contained a single shelter made from the PVC pipe (height: 8.5 cm; diameter: 8 cm) located in one corner. Using tracking software (EthoVision v8.5; Noldus Information Technology Inc), the arena was segmented into three separate areas representing varying levels of exploration: 'near', 'middle' and 'far' (see electronic supplementary material, figure S1). Each behavioural trial began by placing an individual cricket into the shelter within the arena, with the door closed (electronic supplementary material, figure S1). Then, following a simulated predatory disturbance, the door of the shelter was opened and the trial began. During the course of each trial, the mating calls of male crickets were broadcast from outside the arena. Therefore, females were expected to emerge from the shelter to search for calling males despite exposing themselves to the apparent predator.

(e) Statistical analysis

We collected data on two metrics of a female's mate searching behaviour: latency to emerge from a shelter, and mobility. Latency to emerge refers to the amount of time taken for an individual to leave its shelter following the initiation of the trial. Those that did not emerge from the shelter after a 10 min period were assigned the maximum time allowed for emergence of 600 s, and were excluded from the analysis of mobility. For those females that did emerge from the shelter, the total distance moved throughout the arena, time spent in the open arena as a whole and time spent in each pre-determined exploratory section (near, middle, far) were entered into a principal components analysis (PCA) to obtain a single measure of mobility (table 1). The PCAs for

Table 1. Principal components analyses of the behavioural components for female mobility in the experiment observing the effects of the male social environment on female remating behaviour (experiment 1) ($n = 81$) and the experiment observing the effects of the female environment on female post-mating behaviour (experiment 2) ($n = 66$).

	effects of male social environment	effects of female social environment
eigenvalue	3.58	3.73
variance	71.53	74.69
explained (%)		
behaviour loadings		
distance moved	0.453	0.465
total time in arena	0.523	0.504
'near' duration	0.380	0.385
'middle' duration	0.464	0.481
'far' duration	0.402	0.388

both experiments returned single components with eigenvalues greater than 1, and that explained greater than 70% of the variance in mobility. All behaviours loaded equally on the PC (table 1).

The effects of sperm competition risk on the mate searching behaviour of mated females were assessed using two general linear models, one for latency to emerge from the shelter in search of calling song and the other for the mobility PC scores. Male sperm competition risk both during development and mating were entered as factors and female body mass as a covariate. Models included an interaction effect between male developmental and mating treatments. However, the Box–Cox transformations could not resolve non-normal residuals in the latency to emerge model and non-parametric permutation testing was used instead. Significant interaction effects in the initial models were followed by subsequent assessments of the differences in behaviour between the treatments in the mating environment (risk, no-risk) within each of the two developmental environments (risk, no-risk). Non-parametric *post hoc* analyses were conducted using pairwise Wilcoxon rank-sum tests.

The effects of the female's social environment on her mate searching behaviour were again assessed using two general linear models, one for latency to emerge and the other for the mobility PC scores. The female acoustic environment (song, silence) was entered as a factor and female body mass as a covariate. The Box–Cox transformations could not resolve non-normal residuals in the latency to emerge model and non-parametric permutation testing was used instead. Non-parametric *post hoc* analyses were conducted using pairwise Wilcoxon rank-sum tests.

3. Results

(a) Effects of male social environment on female remating behaviour

Permutation testing for the latency to emerge model revealed a significant interaction effect between a male's perceived risk of sperm competition during development and mating (table 2 and figure 1). There were also significant main effects of sperm competition risk when experienced at both stages separately (table 2). Data for the two developmental treatments were

Table 2. Results of the permutation testing for female latency to emerge from the shelter and the general linear model for mobility as a factor of a male's exposure to sperm competition risk both during development and during mating.

behaviour		d.f.	mean square	iter./F	p-value
latency to emerge	development	1	218 004	5000	0.018
	mating	1	24 8067	5000	0.006
	mass	1	7925	222	0.430
	development $\times$ mating	1	162 207	5000	0.025
	error	193	33 132		
mobility	development	1	4.26	1.18	0.280
	mating	1	6.04	1.68	0.199
	mass	1	0.10	0.03	0.871
	development $\times$ mating	1	0.39	0.11	0.742
	error	76	3.59

Figure 1. Latency to emerge from the shelter and mobility in the open arena of females recently mated to males that experienced variability in their perception of sperm competition during development. Closed circles indicate females mated to males that experienced 'no-risk' of sperm competition during mating itself; open circles indicate females mated to males that experienced 'risk' during mating. The median and interquartile range are shown with sample sizes in parentheses.

analysed separately to disentangle the interaction effect. Females mated to 'risk' reared males emerged from the shelter in search of calling song significantly sooner when mating occurred in a 'no-risk' compared to a 'risk' environment ($Z = 3.18$, $p = 0.001$) (figure 1). However, for females mated to 'no-risk' reared males, there was no significant difference in emergence times between those mated in 'risk' and 'no-risk' environments ($Z = 0.01$, $p = 0.990$) (figure 1).

An initial general linear model revealed no significant interaction effect between sperm competition risk in the

developmental and mating environments on the mobility PC scores of mated females (table 2 and figure 1). There were also no significant main effects on the mobility of females (table 2).

(b) Effects of female social environment on female remating behaviour

Permutation testing revealed that a female's environment during development significantly impacted her latency to emerge from a shelter 1 day after mating (table 3 and figure 2). Females reared in the 'silent' environment emerged from the shelter in search of calling song significantly sooner than females reared in the 'song' environment (figure 2). However, there was no significant effect of the rearing environment on female mobility (table 3 and figure 2).

4. Discussion

We sought to determine the extent to which the social environment of males and females mediates female post-mating behaviour. Our data indicate the potential for male Australian field crickets to plastically adjust their inhibition of female sexual receptivity according to their perceived risk of sperm competition. Males appear to make complex strategic decisions, taking into account both the long- and short-term competitive conditions experienced during development and courtship. Females mated to males reared under sperm competition risk were more sexually receptive, emerging from a shelter in search of calling song sooner, but only when mating occurred in a 'no-risk' environment. In other words, because mating in a non-competitive environment represents a lower likelihood of experiencing sperm competition than would be expected for their developmental conditions, males reared under 'risk' appeared to invest less in inhibiting female remating. Therefore, when making strategic adjustments in post-copulatory allocation, males assess the immediate competitive conditions relative to their developmental environment.

The socially cued manipulation of female mate searching probably occurs through the strategic allocation of male seminal fluid proteins (sfps). Sfps, which are produced primarily

Figure 2. Box plots showing the latency to emerge from the shelter and mobility in the open arena for recently mated females reared in either the ‘silent’ or ‘song’ social environments. Sample sizes are shown in parentheses.

Table 3. Results of the permutation testing for female latency to emerge from the shelter and the general linear model for mobility as a factor of female social environment during development and mass.

behaviour		d.f.	mean square	iter./F	p-value
latency to emerge	developmental environment	1	839 790	5000	<0.001
	mass	1	9422	62	0.635
	error	91	40 725		
mobility	developmental environment	1	7.58	2.10	0.152
	mass	1	7.72	2.14	0.149
	error	63	3.61		

in male accessory glands, are one of the major components of the ejaculate in insects [23]. They are involved in a vast array of functions including regulating sperm storage and forming mating plugs as well as influencing many female physiological, behavioural and life-history traits [22]. In a number of insect species, including *T. oceanicus*, male sfps have been demonstrated as the mechanism through which males inhibit female post-mating sexual receptivity [10,12–14,22]. Further, males are believed to strategically allocate resources into the production of sfps. Male *T. oceanicus* upregulate the expression of a collection of sfps when experiencing sperm competition risk [33], including those previously found to inhibit female mate searching behaviour (ToSfp022 and ToSfp011) [10]. Therefore, we suggest that it is this socially cued modulation in sfp allocation that probably induced the variability in female mate searching observed here.

Previous studies have discussed the possibility of males responding differently to sperm competition risk in the pre-mating and mating environments [45,46]. In *D. melanogaster*, an increase in sperm competition intensity in the pre-mating environment led to a decrease in the expression of three sfp genes that influence female remating latency and oviposition rate [45]. But when competition was applied during courtship itself, males did not differentially express sfp genes [45]. Therefore, males are perhaps limited in their ability to modify protein production once courtship begins. However, the expression of sfp encoding genes does not necessarily translate into the final composition of the seminal fluid transferred to females. Males may simply transfer differing

amounts of proteins that have already been expressed [25,38,45]. Here, males reared in song would have probably increased the expression of influential sfps during their development [33]. And yet, when mating occurred in a ‘no-risk’ environment, these competitively reared males inhibited female mate searching to a lesser degree. Therefore, males may be capable of adjusting the composition of their ejaculates during courtship, conserving the already produced sfps for use when the prevailing risk of sperm competition is high. When mating in a ‘risk’ environment, female receptivity appeared at a minimum regardless of the developmental environment of their mate, suggesting that it is the immediate perception of risk that is the most important cue impacting male allocation decisions.

We also demonstrate the potential for females to adjust their remating response according to their own sociosexual environment. Females reared in silence emerged from a shelter in search of an additional mating opportunity sooner than females exposed to male song during their development. These data suggest that, when accustomed to an environment in which potential mates are scarce, females are more responsive to remating (also see [39]). The socially cued increase in female post-mating receptivity would probably allow for the persistence of high rates of remating, maintaining the potential for sperm competition despite the attempted male manipulation. In other words, our results suggest that the male inhibition of female remating may be constrained by the strategic response of females [37,38]. The outcomes of male attempts to manipulate female remating are therefore

dynamic, influenced by both a male's strategic decision-making and the socially cued plasticity of female receptivity.

In conclusion, our findings highlight the complexity of strategic reproductive plasticity. Our data provide evidence that male crickets adjust the extent to which they manipulate female mate searching behaviour in response to their perception of sperm competition risk. We show that males are capable of making finely tuned changes to their post-copulatory tactics, taking into account the competitive conditions both during development and at the time of mating when making strategic decisions. We also demonstrate the ability of females to respond strategically to their environment, potentially maintaining high rates of remating in spite of the attempted male manipulation.

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Ethics. This study was conducted on insects, which do not require formal animal ethics approval.

Data accessibility. The full dataset has been uploaded to the Dryad Digital Repository: <https://doi.org/10.5061/dryad.4j0zpc885> [47].

Authors' contributions. L.W.S., J.L.T. and J.A.M. designed the study. J.A.M. conducted the study, analysed the data and wrote the first draft of the manuscript. L.W.S. and J.L.T. critically revised the manuscript. All authors contributed to and approved the final version of the paper and agree to be held accountable for the work.

Competing interests. We declare we have no competing interests.

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