Do male crickets strategically adjust the number and viability of their sperm under sperm competition?

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Sperm competition theory predicts that males should strategically adjust sperm ejaculate expenditure according to the number of competing ejaculates, because sperm production is costly. That is, males should provide females with larger or higher-quality ejaculates when one rival male is present, but decrease ejaculate size or quality as the number of rivals exceeds one. We tested this hypothesis in the laboratory by subjecting male domestic crickets (Acheta domesticus) to increased sperm competition risk (one rival male) or intensity (two rival males) and then measuring total sperm number and viability (proportion of living sperm) of the ejaculate. In addition, we tested whether male ejaculate expenditure covaried with their own or their mate’s phenotypic quality. Contrary to theoretical predictions, males did not prudently adjust the number of sperm they transferred to mates based on either sperm competition risk or intensity. We also found that smaller males had higher proportions of living sperm in their ejaculate relative to larger males, suggesting that male A. domesticus can adjust their ejaculate quality based on their perceived reproductive prospects.

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strong support for prudent allocation of sperm number under more viable sperm (Loher & Rence 1978; Sakai et al. 1991; Reinhardt
the current spermatophore to form a new one with younger and age, males might adjust viability passively by periodically discarding
might be strategically adjusted in relation to social conditions present during courtship and copulation. Similarly, sperm viability
number of sperm because sperm number is generally correlated (e.g. Simmons et al. 2007; Thomas & Simmons 2007).
thus sperm viability (i.e. the proportion of living sperm) should be important in determining fertilization success. Because high-quality ejaculates could require more resources to produce or maintain than low-quality ejaculates, sperm viability may trade off with other essential life-history traits, such as immunity (Simmons & Roberts 2005; Dowling & Simmons 2012). Therefore, in addition to sperm number, males should also strategically adjust their ejaculate quality in response to sperm competition (e.g. Hunter & Birkhead 2002; Garcia-Gonzalez & Simmons 2005; Snook 2005).
indeed, males apparently produce higher-quality ejaculates under high sperm competition risk (e.g. Simmons et al. 2007) and lower-quality ejaculates under increasing sperm competition intensity (e.g. Simmons et al. 2007; Thomas & Simmons 2007).
Studies on crickets (Orthoptera: Gryllidae) generally provide strong support for prudent allocation of sperm number under increased sperm competition risk (Gage & Barnard 1996; Schaus & Sakaluk 2001; Mallard & Barnard 2003; Lyons & Barnard 2006) but not under increased sperm competition intensity (Gage & Barnard 1996; Schaus & Sakaluk 2001). Crickets are ideal organisms for examining questions related to sperm competition because polyandry is widespread within the group (Bretman & Tregenza 2005) and females store sperm from multiple males for prolonged periods in their spermatheca (Simmons 1986). A male’s fertilization success is proportional to the relative number of his sperm stored by the female (Parker 1990). Cricket sperm are transferred to the female in a spermatophore, which is made by the male prior to encountering a female; thus, the number of sperm allocated to the female should reflect the social conditions (e.g. number of rival males) present during spermatophore formation rather than those present during courtship and copulation. Similarly, sperm viability might be strategically adjusted in relation to social conditions experienced by the male at the time of spermatophore production or, given that sperm viability likely decreases with spermatophore age, males might adjust viability passively by periodically discarding the current spermatophore to form a new one with younger and more viable sperm (Loher & Rence 1978; Sakai et al. 1991; Reinhardt & Siva-Jothy 2005). The allocation of resources to sperm viability via frequent spermatophore replacement might be a process separate from the allocation of resources to sperm number; thus, male crickets might strategically adjust sperm number, sperm viability, or both in response to sperm competition risk and intensity.
In the present study, we experimentally tested the hypothesis that male Acheta domestica (Linnaeus 1758) crickets strategically adjust ejaculate expenditure under increased immediate sperm competition risk and intensity (sensu Engqvist & Reinhold 2006). First, we predicted that male A. domestica respond to increased sperm competition risk by allocating larger numbers of sperm and investing more in sperm viability in the presence of one rival male. Second, we predicted that males respond to increased sperm competition intensity by allocating fewer sperm and investing less in sperm viability as the perceived number of rival males exceeds one. In addition, we tested whether male ejaculate expenditure covaries with their own or their mate’s phenotypic quality. Because female A. domestica prefer larger mates (Crankshaw 1979; Gray 1997; Savage et al. 2005; Stoffer & Walker 2012), mating opportunities for smaller males are often limited. Also, spermatophore production in crickets is costly (Kerr et al. 2010), so males may adjust their ejaculate quality based on their perceived reproductive prospects, where attractive males invest less per ejaculate to ensure the availability of resources for future reproductive events (e.g. Bussière 2002; Engqvist 2011), and disfavoured males invest more per ejaculate due to their limited future mating success (sensu Parker 1990; Tazzyman et al. 2009). However, because male A. domestica produce spermatophores prior to encountering potential mates, strategic ejaculation based on female quality would require a male to discard his current spermatophore and postpone mating for a minimum of 40 min until a new spermatophore is produced (A. M. Worthington, personal observation). We controlled for this behaviour and thus predicted that (1) smaller males would allocate more sperm and/or invest more in sperm viability than larger males and (2) sperm number and viability would not significantly correlate with female phenotype as predicted in other taxa (Wedell et al. 2002; Parker & Pizzari 2010; Kelly & Jennions 2011).

METHODS

Experimental Animals
Experimental crickets were obtained from a commercial dealer (Fluker’s Cricket Farms Port Allen, LA, U.S.A.) at 4–5 weeks of age. The sexes were separated prior to their imaginal moult to ensure virginity. Females were housed in large communal bins (44 x 33 x 40 cm) and males were reared individually in clear plastic 250 ml containers (10 cm diameter x 4.5 cm depth). All crickets were housed in an environmentally controlled room maintained at 28 °C on a 12:12 h light:dark cycle. All crickets were supplied with cotton-plugged water vials and dry cat food (Special Kitty Premium Cat Food) ad libitum. Males were checked daily for maturity.

Experimental Set-up
Sexually mature (7 days posteclosion), virgin males were randomly assigned to one of three experimental treatments in which they experienced zero, one or two rivals for 5 consecutive days. The experimental apparatus comprised two clear plastic cages (12 x 20 x 15 cm; Hagan Critter Cages, Mansfield, MA, U.S.A.) placed inside an anechoic chamber (38 x 28 x 18 cm polystyrene box with 3.8 cm thick walls). The anechoic chamber prevented males within a trial from hearing the calls of males in other trials. For each trial, the focal male was randomly assigned to one of the two internal cages and the designated number of rivals (0, 1 or 2) was placed in the other cage. Each cage received two pieces of cat food, two water vials and two small pieces of egg carton for shelter, and was fitted with a perforated lid to permit continual visual, acoustic and olfactory contact between focal and rival males while preventing physical interaction between them. A total of 93 focal and 102 stimulus rival males were used in this experiment. Cages were checked once daily for mortality. Rival males that died during a trial were replaced immediately (N = 10), and trials were terminated if the focal male died (N = 4). Focal males were exposed to rivals in the experimental apparatus for 5 days. On day 5 at 1400 hours, the focal male was removed from the experimental apparatus and immediately placed in a mating arena (250 ml clear plastic container with lid) with a randomly chosen virgin female (aged 7–35 days posteclosion) from the same-sex holding bin. Pairs were observed under a 25 W red light at 28 °C and given 90 min to copulate. We terminated trials when the female failed to respond to the courteship song within the allotted time, or spermatophore transfer to the mounted female was unsuccessful (N = 15). To

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ensure that we included only spermatophores formed in the presence of rivals in our analysis, we terminated trials when the male either failed to produce a courtship song, or attempted to discard his spermatophore to produce a new spermatophore \( (N = 16) \). For each mating, we recorded the latency to copulate \( (\text{time from the introduction of the female until spermatophore transfer}) \), copulation duration \( (\text{time that the female spent mounted on the male}) \), and the focal male age \( (\text{number of days posteclosion}) \).

**Sperm Quantity and Viability**

We used fine forceps to remove the spermatophore immediately after attachment to the female. The spermatophore was then placed into a 1.5 ml microcentrifuge tube containing 175 \( \mu l \) of Grace’s medium \( (\text{Sigma-Aldrich, St Louis, MO, U.S.A.}) \), crushed with fine forceps three times in quick succession, left undisturbed for 10 min to allow the sperm to discharge into the Grace’s solution, and then mixed by vortexing for three 1 s pulses \( (\text{see Gress & Kelly 2011}) \). Our procedure ensured that the integrity of the spermatophore was sufficiently compromised so as to permit the evacuation of its contents without causing significant sperm mortality. Our procedure generally followed that used by behavioural ecologists with some modification, as outlined in Gress & Kelly \( (2011) \). By using standardized methods and treating each spermatophore identically, any differences among males in sperm number or viability can be attributed to our experimental treatments. We pipetted 20 \( \mu l \) of the diluted ejaculate into a 1.5 ml microcentrifuge tube. Sperm viability was assayed using the LIVE/DEAD® assay \( (\text{Molecular Probes, L-7011}) \). We added 1.25 \( \mu l \) of 1 mM SYBR® \( 14 \) (diluted 1:50 with Grace’s medium) to the sperm sample and then incubated it in the dark for 10 min before adding 2.5 \( \mu l \) of propidium iodide and incubating for an additional 10 min. We then pipetted a 10 \( \mu l \) sample into a well of a disposable haemocytometer \( (\text{INCYTO C-Chip, Covington, GA, U.S.A.}) \). Sperm were visualized using fluorescent microscopy \( (\text{Leica DM 2500; Leica Microsystems GmbH, Wetzlar, Germany}) \) at 400× magnification and all sperm within five predetermined grid squares were counted as living \( (\text{stained green}) \) or dead \( (\text{stained red}) \). All sperm counts were made by B.E.G., who was blind to experimental treatment. The numbers of live and dead sperm in each spermatophore were calculated by multiplying the mean sperm count by its dilution factor.

**Male and Female Phenotypic Quality**

All male–female pairs were euthanized by freezing at \( -20^\circ C \). We used pronotum width \( (\text{body size}) \) and body fat content \( (\text{i.e. condition}) \) as measures of phenotypic quality for focal males, and pronotum width and fresh body mass as measures of phenotypic quality for their female mates. We used body mass to measure phenotypic quality of female mates because males may be able to assess body mass of a mounted female during copulation, whereas female body fat content would be difficult for males to assess directly. Pronotum width was measured for all crickets to the nearest 0.01 mm using callipers \( (\text{diameter} \text{MaxSp}i2000; \text{Swiss Precision}) \). Male crickets were dried at 60 °C for 24 h and weighed to the nearest 0.01 mg using an electronic balance \( (\text{Denver Instruments TP-64}) \). Body fat was then extracted using petroleum ether \( (\text{Fisher Scientific}) \) reflux in a Soxhlet apparatus for 12 h. Individuals were again dried at 60 °C for 24 h and then reweighed. Body fat content \( (\text{mg}) \) was obtained by subtracting the dry mass measures before and after fat extraction. Female fresh body mass was weighed to the nearest 0.01 mg using an electronic balance.

**Statistical Analyses**

We used ANCOVA to examine the effect of different numbers of rival males \( (0, 1 \text{ or } 2) \) on the size \( (\text{sperm number}) \) and quality \( (\text{sperm viability}) \) of ejaculates produced by focal males. Each ANCOVA included phenotypic measures of males (pronotum width, body fat content, age) and females (pronotum width, mass) as well as mating behaviours \( (\text{latency to mate, copulation duration}) \) as covariates. The ANCOVA examining the effect of rival presence on sperm viability also included the total number of sperm as a covariate, as ejaculates with more sperm can exhibit greater viability due to sampling artefacts \( (\text{Holman 2009; Gress & Kelly 2011}) \). In separate ANCOVAs, we also examined the effect of rival male presence and male and female phenotypic traits on the latency to mate and copulation duration. We checked whether each ANCOVA met model assumptions by examining residuals. Relationships between dependent variables and covariates from ANCOVA models.

![Figure 1. Box plots of (a) number and (b) viability of sperm produced by Acheta domesticus males exposed to zero, one or two males for 5 days prior to copulating with a female.](image-url)
were visualized using partial regression plots (Moya-Laraño & Corcobado 2008).

All statistical analyses were performed in R version 2.12 (R Development Core Team 2009) with α = 0.05. To compare the biological importance of our findings, we calculated standardized effect sizes by converting the t statistic for each covariate into Pearson’s r and by converting raw summary statistics (mean, standard deviation, sample size) for the experimental treatments into Hedge’s d using MetaWin 2.0 (Rosenberg et al. 2000).

RESULTS

Effects of Sperm Competition Risk and Intensity on Ejaculate Size and Quality

There was no significant effect of the number of perceived rival males (Fig. 1a), male phenotype (i.e. body size, age, body fat content), female phenotype (i.e. body size, body mass) or mating behaviour (i.e. latency to mate, copulation duration) on the number of sperm ejaculated by males (Table 1). Similarly, there was no significant effect of the number of perceived rival males (Fig. 1b), male age, female phenotype or mating behaviour on the viability of sperm ejaculated by males (Table 1). However, ejaculates produced by males with smaller pronota (Fig. 2) had significantly greater sperm viability (Table 1).

Effect of Perceived Sperm Competition on Mating Behaviours

The presence of rival males did not significantly affect latency to mate or mating duration (Table 2). However, females of smaller body mass showed longer latencies to mate (Table 2).

DISCUSSION

Contrary to prediction, we found no evidence that male A. domesticus strategically adjust the number and viability of their sperm under sperm competition? (delBarco-Trillo 2011; Kelly & Jennions 2011). In fact, the effect of a single rival male on total sperm allocation in our study ($d_0$ vs 1 rival = $-0.4267$) was in the direction opposite to that found in meta-analyses by Kelly & Jennions (2011; $d_1$ = $+0.521$) and delBarco-Trillo (2011; $d_2$ = $+0.315$). Furthermore, we found no significant decrease in sperm number as sperm competition intensity increased from one to two rivals. Our observed treatment effect of two rival males ($d_3$ vs 2 rivals = $+0.1658$) was again in the direction opposite to that calculated by Kelly & Jennions (2011; $d_4$ = $-0.032$) for a wide variety of animal taxa, but they too found no significant evidence supporting this prediction.

This is in contrast to the general pattern found across animal taxa (delBarco-Trillo 2011; Kelly & Jennions 2011). In fact, the effect of a single rival male on total sperm allocation in our study ($d_0$ vs 1 rival = $-0.4267$) was in the direction opposite to that found in meta-analyses by Kelly & Jennions (2011; $d_1$ = $+0.521$) and delBarco-Trillo (2011; $d_2$ = $+0.315$). Furthermore, we found no significant decrease in sperm number as sperm competition intensity increased from one to two rivals. Our observed treatment effect of two rival males ($d_3$ vs 2 rivals = $+0.1658$) was again in the direction opposite to that calculated by Kelly & Jennions (2011; $d_4$ = $-0.032$) for a wide variety of animal taxa, but they too found no significant evidence supporting this prediction.

<table>
<thead>
<tr>
<th>Table 1 Effect of perceived risk and intensity of sperm competition, male phenotype, female phenotype and mating behaviour on the number (df = 48) and viability (df = 47) of sperm produced by A. domesticus</th>
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</thead>
<tbody>
<tr>
<td>Sperm number</td>
</tr>
<tr>
<td>0 vs 1 rival</td>
</tr>
<tr>
<td>1 vs 2 rivals</td>
</tr>
<tr>
<td>Male body size (pronotum width, mm)</td>
</tr>
<tr>
<td>Male body fat content (mg)</td>
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<tr>
<td>Male age (days posteclosion)</td>
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<tr>
<td>Female body mass (mg)</td>
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<tr>
<td>Female body size (pronotum width, mm)</td>
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<tr>
<td>Latency to mate</td>
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<tr>
<td>Copulation duration</td>
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<tr>
<td>Sperm viability</td>
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<td>1 vs 2 rivals</td>
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<td>Latency to mate</td>
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<tr>
<td>Copulation duration</td>
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<td>Total sperm number</td>
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</table>

* Pearson’s r for covariates (calculated based on model t values) and Hedge’s d for differences between experimental treatments (calculated based on raw summary statistics).
There was also no significant effect of sperm competition risk or intensity on sperm viability; however, the effects of sperm competition risk ($d_0$ vs 1 rival = +0.4797) and intensity ($d_1$ vs 2 rivals = −0.2252) on sperm viability were in the direction predicted by theory for sperm number (Parker & Pizzari 2010). Similarly, in another study investigating strategic adjustment of sperm viability in crickets, Thomas & Simmons (2007) found that male Teleogryllus oceanicus allocated low-viability sperm when mating with virgin females (no risk), increased sperm viability when mating with singly mated females (high risk) and reduced sperm viability when mating with multiply mated females (high intensity). Despite seeming counterintuitive, transferring ejaculates having large quantities of dead sperm might maximize male fitness since producing viable sperm is costly and trades off with other fitness-related life-history traits, such as immunity (Simmons & Roberts 2005; Dowling & Simmons 2012).

By manipulating exposure to rivals 5 days prior to mating, we may have altered male perception of me

Our results contradict Gage & Barnard (1996), who found that male A. domesticus produced more sperm under increased sperm competition risk. One possible explanation for why we found no effect of sperm competition risk or intensity in our study is that the use of one rival male versus two rival males was an insufficient stimulus for the focal male. This is unlikely, however, because Gage & Barnard (1996) saw an increase in sperm number after exposure to just one rival male. Perhaps exposing focal males to potential rivals prior to mating did not increase the focal male’s perception of sperm competition risk. This protocol, however, has been effective in mating did not increase the focal male (1996) saw an increase in sperm number after exposure to just one male.

Female A. domesticus prefer larger males as mates (Crankshaw 1979; Gray 1997; Savage et al. 2005; Stoffe & Walker 2012), and smaller males are thus predicted to increase their reproductive success by allocating more resources into their ejaculates than are larger males (sensu Parker 1990; Tazzymann et al. 2009). Our finding that ejaculates of smaller male A. domesticus had greater sperm viability than those larger males supports this prediction and concurs with other empirical evidence that the ejaculates of smaller male A. domesticus have more living sperm than those of larger males (Klaus et al. 2010). Female A. domesticus apparently exert cryptic female mate choice through premature removal of the spermatophore (earlier removal means fewer sperm transferred; e.g. Fleischman & Sakaluk 2007), but there is no evidence that spermatophore removal in this species is dependent on male body size (Mault & Sakaluk 2008), as in other cricket species (Simmons 1986; Batenan et al. 2001). Females, however, appear to preferentially store younger, more viable sperm (Reinhardt & Siva-Jothy 2005); thus, by increasing their sperm viability, smaller males should accrue significant paternity benefits. That male A. domesticus can adjust ejaculate quality based on their perceived reproductive prospects should not be surprising given that male T. oceanicus crickets adjust their ejaculate quality in response to sperm competition risk and intensity (Simmons 2007), female mating status (Thomas & Simmons 2007), female receptivity (Dowling & Simmons 2012) and dominance status (Thomas & Simmons 2009). Evidence of disfavoured males increasing ejaculate quality has also been found in other taxa and includes increases in sperm motility (Cage et al. 1995; Froman et al. 2002), longevity (Gage et al. 1995) and velocity (Rudolfson et al. 2006). Although investing in higher ejaculate quality may require more resources and have significant costs associated with it, males that face limited future reproductive opportunities might strategically invest in high-quality ejaculate to increase fertilization success in current reproduction and thus maximize their fitness.

In conclusion, we found no support for sperm competition models predicting that males prudently adjust the number of sperm they transfer to mates based on either sperm competition risk or sperm competition intensity. We did, however, find that the ejaculates of smaller males had significantly more viable sperm than those of larger males, adding to the growing body of literature suggesting that males can adjust ejaculate quality based on their reproductive prospects.

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