ethology international journal of behavioural biology

Song and Sperm in Crickets: A Trade-off between Pre- and Post-copulatory Traits or Phenotype-Linked Fertility?

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Received: August 18, 2010
Initial acceptance: September 8, 2010
Final acceptance: October 20, 2010
(L. Ebensperger)

doi: 10.1111/j.1439-0310.2010.01857.x

Abstract

When females mate multiply (polyandry) both pre- and post-copulatory sexual selection can occur. Sperm competition theory predicts there should be a trade-off between investment in attracting mates and investment in ejaculate quality. In contrast, the phenotype-linked fertility hypothesis predicts a positive relationship should exist between investment in attracting mates and investment in ejaculate quality. Given the need to understand how pre- and post-copulatory sexual selection interacts, we investigated the relationship between secondary sexual traits and ejaculate quality using the European house cricket, *Acheta domesticus*. Although we found no direct relationship between cricket secondary sexual signals and ejaculate quality, variation in ejaculate quality was dependent on male body weight and mating latency: the lightest males produced twice as many sperm as the heaviest males but took longer to mate with females. Our findings are consistent with current theoretical models of sperm competition. Given light males may have lower mating success than heavy males because females take longer to mate with them in no-choice tests, light males may be exhibiting an alternative reproductive tactic by providing females with more living sperm. Together, our findings suggest that the fitness of heavy males may depend on pre-copulatory sexual selection, while the fitness of light males may depend on post-copulatory fertilization success.

Introduction

Many choosy females mate multiply and, when multiple mating occurs, sperm competition can result in sexual selection continuing beyond copulation (Parker 1998; Simmons 2001). Paternity success can thus be influenced by secondary sexual characteristics (pre-copulatory sexual selection) and by traits that influence fertilization success (post-copulatory sexual selection). When variance in paternity success is influenced by both pre- and post-copulatory sexual selection, sperm competition theory predicts that these forms of sexual selection may work to counteract one another (Møller 1998; Danielsson 2001; Andersson & Simmons 2006). Specifically, sperm competition theory predicts there should be a negative relationship between the investment in ejaculate quality and the investment in all other reproductive traits, including those that influence mate attraction (Parker 1998). In support of this idea, several empirical studies have revealed negative relationships between exaggerated sexual traits and ejaculate quality (plumage in red-backed fairy-wrens *Malurus melanocephalus*: Rowe et al. 2010; dominance and pheromones in crickets *Teleogryllus oceanicus*: Thomas & Simmons 2009; secondary sexual characteristics in wrasse *Symphodus melops*: Uglem et al. 2001; salmon *Salmo salar*: Vladić & Järvi 2001; Vladić et al. 2002; Arctic charr *Salvelinus alpinus*: Liljedal et al. 1999; guppies *Poecilia reticulata*: Evans 2010; and coho salmon *Oncorhynchus kisutch*: Pitcher et al. 2009).

Conversely, several other empirical studies have revealed positive relationships between the

investment in ejaculate quality and the investment in secondary sexual characteristics (eyespan in stalk-eyed flies *Teleopsis dalmanni*: Rogers et al. 2008; colour spots and sigmoidal courtship displays in guppies *P. reticulata*: Matthews et al. 1997; Pitcher & Evans 2001; Pilastro et al. 2002; plumage brightness in greenfinch *Carduelis chloris*: Merila & Sheldon 1999; antler size in red deer *Cervus elaphus*: Malo et al. 2005). These positive relationships support the phenotype-linked fertility hypothesis, which predicts that signalling males are advertising two things: (1) their ability to provide enough sperm to fertilize an entire complement of ova and (2) their sperm quality as it relates to fertilization efficiency (Sheldon 1994). The phenotype-linked fertility hypothesis suggests that females have been selected to prefer exaggerated male secondary sexual characteristics to ensure their higher fertility.

Predicting the directionality of the relationships between pre- and post-copulatory sexually selected traits can thus be complex because of the alternative association patterns suggested by sperm competition theory (negative association) and the phenotype-linked fertility hypothesis (positive association) (Rowe et al. 2010). Given the relationship between secondary sexual characteristics and ejaculate quality can provide information about the evolution of mating signals, we investigated the relationship between cricket mate attraction signals and their ejaculate quality. We focused on crickets because (1) they are often used as model organisms to understand the mechanisms of sexual selection (Hedrick 1986, 1988; Wagner & Harper 2003; Wagner & Basolo 2007), (2) females usually choose high effort signalling males (Gray 1997; Rodríguez-Muñoz et al. 2010), (3) females mate multiply, generating sperm competition, and (4) much is known about how ejaculate quality (i.e., sperm number and quality) influences fertilization success (Sakaluk & Eggert 1996; Schaus & Sakaluk 2001, 2002) and sperm competition success (Schaus & Sakaluk 2001; Garcia-Gonzalez & Simmons 2005; Reinhardt & Siva-Jothy 2005; Thomas & Simmons 2007). Cricket acoustic mate attraction signals thus represent a suite of pre-copulatory sexually selected traits, while ejaculate quality represents a suite of post-copulatory sexually selected traits.

To distinguish between sperm competition theory and the phenotype-linked fertility hypothesis, we quantified the relationship between long-distance acoustic mate attraction signals and ejaculate quality. We continuously recorded all acoustic mate attraction signals produced by males when they were between 7 and 14 d post-final moult to measure the quality and quantity of the signals. On day 14, we mated these virgin males to virgin females using no-choice tests and quantified the time it took pairs to successfully mate (as an indirect measure of female choice). We used no-choice tests because Shackleton et al. (2005) revealed that latency to mating predicts actual mating success while simultaneously removing potential male–male competition effects. As soon as mating occurred, we removed the spermatophore from the female and quantified the number and viability of each male's sperm. Because sperm number and viability are two potentially non-independent variables of ejaculate quality (Holman 2009) that have been shown to influence fertilization efficiency in insects (Simmons 2001; Wagner & Harper 2003; Garcia-Gonzalez & Simmons 2005; Snook 2005), we also combined sperm number and viability into a measure of the number of living sperm. We were thus able to test whether exaggerated male mate attraction signals had a positive or negative relationship with sperm number, viability and/or number of living sperm.

Methods

We obtained late-instar nymph *Acheta domesticus* from a local breeder in Ottawa, Ontario, Canada. To ensure their virginity, we isolated male and female crickets from the stock culture and raised them in single sex groups in 55-l bins until they reached adulthood. Nymphs were checked daily for adult eclosion, and adults were held individually in 420-ml containers. Both nymph and adult crickets were given water and food (Harlan Teklad Laboratory (Madison, WI, USA) Diet food #8604: 24% crude protein, 4% crude fat, 4.5% crude fibre) *ad libitum* and were held in a temperature-controlled room (30.5 ± 2°C). In total, we used 34 male and 36 female crickets in our experimental trials, of which 30 of each sex successfully completed mating trials. Four of the males performed auto-spermatophore extrusion upon first entering mating trials. Auto-ejaculation may be used to discharge old and defective sperm to maintain the stores of fertile sperm and signal production of new sperm (Kumashiro et al. 2003; Wagner et al. 2004; Reinhardt & Siva-Jothy 2005; White et al. 2008). We excluded these four males from our study because their spermatophores may not have been intended for fertilization. We used two extra females because on two occasions the male did not court, so the female was removed and a new virgin female was placed in the container. In both cases, the male immediately courted the second female.

Acoustic Mate Attraction Displays

Upon reaching day 7 of adulthood, males were placed into individual containers in an Electronic Acoustic Recording system. The recording system consisted of 32 individually recording microphones, each with a single LED light that provided males with a 12-h:12-h L:D cycle. Each male was separated from its neighbours by an acoustically isolated enclosure (a 2″ thick Styrofoam box that was internally lined with 1″ thick acoustic foam) that contained the microphone and the LED light. This design minimized the ability of individuals to detect the signalling of their neighbours, which could have been perceived as competition (Bertram et al. 2007). We continuously recorded long-distance acoustic mate attraction signals from seven to fourteen days post-final moult using real-time electronic analysis. This analysis was paused for only 15 min each day as we replenished the food and water supplies. The recording system monitored all 30 males simultaneously by sampling the acoustic environment surrounding each individual cricket and discounting it from actual male signalling. The microphones were continuously monitored using CricketSong software (Cambridge Electronic Design Ltd., Cambridge, UK).

We processed the resulting audio files using Spike2 6.10 software (Cambridge Electronic Design Ltd.). For each male, we determined mean values for pulse duration, amplitude, interchirp duration, time spent signalling, interpulse duration, number of pulses per chirp, carrier frequency and chirp duration for each full day of recording (measuring all sound pulses produced) to determine whether daily acoustic signalling parameters were affected by male weight and age. These daily values were then averaged across the week by weighting the averages by the number of pulses produced each day to determine whether song production can predict sperm quality and quantity.

Mating Behaviour

Male crickets were weighed on day 14 of adulthood. We then placed them in an empty 420-ml container with a virgin female of known mass and of approximately the same age (±2 d). The cricket pair was then observed for up to an hour to determine whether mating occurred. We recorded the time it took for the pair to mate, from their initial meeting to the successful transfer of the spermatophore. We used latency to mate in no-choice tests because Shackleton et al. (2005) outlined the benefits of using no-choice tests and highlighted the difficulty in controlling for male–male competition when using two-choice tests. No-choice tests (Pilastro et al. 2002) measure the time it takes a female to mate when she is placed with a single male. This approach can be beneficial because the female's choice is based on the full complement of chemical, acoustic and physical mating cues that are provided by a male. Further, female choice is determined from actual mating, not quantified indirectly from time spent near a male or first male approached. Shackleton et al. (2005) provide an extensive review of the benefits of using no-choice tests and the extensive number of taxa where no-choice tests are utilized.

Sperm Viability and Number Assays

We immediately removed the spermatophore from the female and placed it into 20 μl of Beadle saline (128.3 mM NaCl, 4.7 mM KCl, 23 mM $CaCl_2$). We then immediately ruptured the spermatophores and gently forced them through a pipette five times to prevent sperm agglutination. To assess the number of sperm produced, we counted sperm from the spermatophore solution using a 'improved Neubauer chamber' haemocytometer under 40× magnification (Zeiss AxioImager.M2m; as per Pitcher et al. 2007). Sperm count was expressed as the total number of sperm per spermatophore for each male. To assess sperm viability, we used a live/dead sperm viability assay (Invitrogen Molecular Probes; as per Garcia-Gonzalez & Simmons 2005; Thomas & Simmons 2007). It has been suggested that this fluorescent staining assay might have a negative effect on sperm viability and therefore might produce artificially low estimates of living sperm (Holman 2009). However, because we assay for sperm viability within 30 min of spermatophore transfer, we assume that any deleterious effects of the staining procedure are consistent among spermatophores and should not influence the variation among males in living sperm observed. Five microlitres of the spermatophore solution was mixed with 5 μl of 1:50 diluted 1 mM SYBR-14 stain and left in the dark for 10 min before 2 μl of 2.4 mM propidium iodide was added.

The sample was then incubated in the dark for an additional 10 min before being observed under a fluorescence microscope with a blue excitation filter (λ = 470 nm at 60%). This assay stained live (i.e. viable) sperm green with SYBR-14, a membrane-permeant nucleic acid stain; dead sperm with damaged membranes were stained red with propidium iodide (Fig. 1). On very rare occasions, sperm were

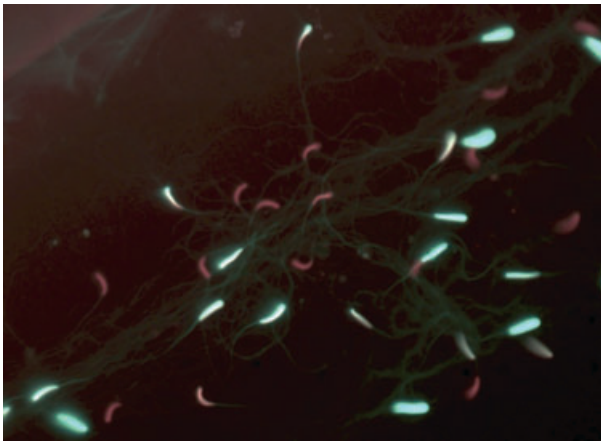

Fig. 1: House cricket sperm stained with the live/dead sperm viability assay under a blue excitation filter (λ = 470 nm at 60%). Green is considered living sperm (viable) with intact cellular membranes, while red is considered dead sperm with damaged membranes.

stained both colours and, as the meaning of these moribund cells is unclear, they were not counted (Garner et al. 1997; Pitcher et al. 2007). To minimize the effect of loss of viability that occurs with sperm age, images of the sperm were taken immediately after staining, and we determined the live/dead ratio by viewing the images after the assay was completed (Holman 2009). We counted 500 sperm per male, and sperm viability was calculated as the percentage of live sperm.

Thus, the measures we used to quantify sperm quality were total number of sperm, sperm viability, and the number of living sperm. To quantify the number of living sperm, we multiplied sperm viability by the total number of sperm. We included number of living sperm as one of our measures because sperm viability and sperm number are suspected to be non-independent of one another as they could share dependence on environmental and genetic factors (Holman 2009). As such, combining the two measurements may offer a more accurate measure of ejaculate quality than either assay alone.

Statistical Analyses

We conducted statistical analyses in JMP 8.0.2.2 (SAS Institute Inc., Cary, NC, USA). Data on weekly averaged acoustic signalling parameters and number of living sperm were Box-Cox transformed to meet the assumptions of normality necessary for parametric statistical analysis. We obtained the mean, standard deviation, minimum, maximum and coefficient of variation for all sperm characteristics, all eight acoustic signalling parameters as well as male weight, female weight and mating latency. We used multiple regressions to quantify the factors influencing sperm viability, number of living sperm and total number of sperm. To test the prediction that males with attractive mating displays have higher sperm viability, more living sperm and/or higher total sperm count, we included all eight signalling parameters in our three models. We included male weight, female weight and the interaction between the two in our model of mating latency. We also ran another series of multiple regression models to ascertain how male weight, female weight and the interaction between male and female weight influenced the number of living sperm, total number of sperm and sperm viability. To examine the effects of male weight and age on acoustic signalling behaviour, we used multivariate analyses of variance with repeated measures (MANOVA) on the mean daily acoustic signalling parameters. We included male weight, male age and the interaction between the two in the acoustic signalling parameter models. As we performed eight different MANOVAs, we used a False Discovery Rate B-Y adjustment (FDR_{BY}) of $p < 0.0184$ to correct for multiple statistical comparisons (Waite & Campbell 2006). We opted to use the FDR_{BY} adjustment instead of the Bonferroni adjustment because the Bonferroni adjustment has been shown repeatedly to be overly conservative (e.g., Benjamini et al. 2001; Nakagawa 2004; Narum 2006).

Results

Males exhibited extensive variation in their post-copulatory sperm traits, pre-copulatory mating signals, body sizes and mating latencies (Table 1). Most males produced fairly viable sperm (average viability = 58%), but viability ranged from extremely low viability (7%) to completely viable (100%). The average number of sperm in each spermatophore was 12 550, but ranged from 1000 to 56 500. Acoustic mate signalling behaviour was also highly variable across males. All males included in the study signalled acoustically, but some signalled for only a few minutes per day while others signalled for an average of 13 h per day (Table 1). Most males produced chirps that were two or three pulses in duration, at a carrier frequency near 4.9 kHz. Females were 40% heavier than males on average, but both males and females exhibited substantial variation in their body weights. Overall, there was a twofold difference among males and among females in their weights. Of all variables measured, sperm number

Table 1: Variation in acoustic signalling parameters, sperm quality measures, cricket weight and mating latency

Variable	$\bar{x}$	SD[a]	Minimum	Maximum	CV[a]
Sperm viability (proportion)	0.580	0.230	0.060	1.000	39.868
Number of living sperm	1.233×10^4	9.857×10^4	3.300×10^3	3.650×10^4	79.970
Total number of sperm	1.992×10^4	1.255×10^4	1.000×10^3	5.650×10^4	62.992
Pulse duration (ms)	13.690	4.640	4.690	20.850	33.896
Amplitude (dB)	53.050	12.160	24.520	76.190	22.916
Interchirp duration (ms)	1135.330	631.410	444.840	2885.780	55.615
Time spent signalling (min)	349.370	206.820	1.180	778.780	59.198
Interpulse duration (ms)	48.460	5.470	25.630	55.920	11.293
Pulses per chirp	2.380	0.300	2.020	3.100	12.444
Carrier frequency (Hz)	4930.750	293.250	4431.500	5791.890	5.947
Chirp duration (ms)	75.210	18.130	32.920	112.330	24.109
Male weight (mg)	345.760	59.870	224.500	476.600	17.316
Female weight (mg)	486.490	72.170	293.700	602.000	14.835
Mating latency (min)	20.370	10.490	5.000	47.000	51.504

[a]SD is the standard deviation and CV is the coefficient of variation.

and total number of living sperm exhibited the highest coefficients of variation. Mating latency and average number of minutes signalling over a male's lifetime also exhibited extremely high coefficients of variation.

There was no relationship between cricket acoustic long-distance signalling behaviour and ejaculate quality, as variation in signalling behaviour did not explain any of the variation in total number of sperm ($F_{1,29} = 0.189$, p = 0.990, $R^2_{adj} = -0.288$), sperm viability ($F_{1,29} = 0.781$, p = 0.624, $R^2_{adj} = -0.064$), or number of living sperm ($F_{1,29} = 0.247$, p = 0.976, $R^2_{adj} = -0.262$). Variation in ejaculate quality was, however, dependent on male weight and mating latency. There was a negative relationship between ejaculate quality and male weight, as lighter males produced significantly more sperm and more living sperm than heavier males (Table 2; Figs 2 and 3). Further, males that took a long time to mate females produced significantly more sperm and significantly more living sperm than males that took a short time to mate (Table 2). Female weight and the interaction between male and female weight did not explain significant variation in ejaculate quality (Table 2).

The relationship between mating latency and ejaculate quality could have been partially driven by the fact that male weight negatively affected mating latency (whole model ANOVA: $F_{1,29} = 2.983$, p = 0.049, $R^2_{adj} = 0.170$). Smaller males took significantly longer to successfully mate than heavier males ($F = 5.069$, p = 0.033). Female weight and the interaction between male and female weight did not explain any of the variation in mating latency (female weight: $F = 2.632$, p = 0.117; interaction: $F = 0.195$, p = 0.663).

Variation in one acoustic mating signal parameter, pulse duration, was dependent on variation in male weight (Table 3). Heavy males produced acoustic signals with longer pulse durations than light males.

Table 2: The influence of male weight, female weight and mating latency on measures of ejaculate quality using multiple regression

Response variable	$F_{1,29}$	R^2_{adj}	p	Effects	F	p
Number of living sperm	3.410	0.249	0.023	Male weight (mg)	11.750	0.002
				Mating latency (min)	4.645	0.041
				Male weight × female weight	0.084	0.774
				Female weight (mg)	0.011	0.919
Total number of sperm	3.200	0.338	0.030	Male weight (mg)	10.010	0.004
				Mating latency (min)	6.301	0.019
				Male weight × female weight	0.006	0.937
				Female weight (mg)	<0.001	0.989
Sperm viability	0.739	−0.037	0.574	Male weight (mg)	2.572	0.121
				Mating latency (min)	0.526	0.475
				Male weight × female weight	0.137	0.714
				Female weight (mg)	0.097	0.758

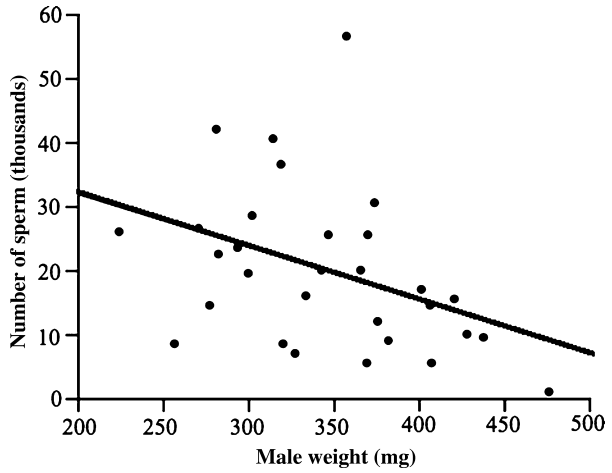

Fig. 2: The relationship between body weight and total number of sperm produced. Light males produced more total sperm than heavy males (raw data shown).

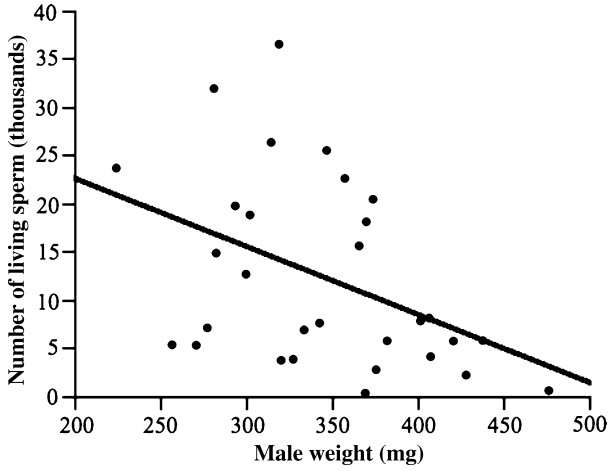

Fig: 3. The relationship between body weight and number of living sperm produced. Light males produced more living sperm than heavy males (raw data shown).

None of the other acoustic signalling parameters were influenced by male weight, age or the interaction between weight and age (Table 3).

Table 3: The influence of male weight and age on acoustic mate attraction signal parameters using multivariate analyses of variance with repeated measures. The relationship between pulse duration and male weight was significant at the False Discovery Rate B-Y adjustment of $p < 0.0184$ required to account for these eight tests; none of the other seven acoustic parameters were influenced by male weight or age

Acoustic parameter	Effects	*F*	df	p
Pulse duration (ms)	Weight (mg)	6.714	1, 26	0.016
	Age (day)	0.845	7, 20	0.564
	Weight × age	1.002	7, 20	0.458
Amplitude (dB)	Weight (mg)	2.273	1, 26	0.144
	Age (day)	0.425	7, 20	0.875
	Weight × age	0.430	7, 20	0.872
Interchirp duration (ms)	Weight (mg)	1.274	1, 26	0.269
	Age (day)	0.387	7, 20	0.899
	Weight × age	0.500	7, 20	0.823
Time spent signalling (min)	Weight (mg)	0.683	1, 28	0.416
	Age (day)	1.562	7, 22	0.199
	Weight × age	0.885	7, 22	0.534
Interpulse duration (ms)	Weight (mg)	0.492	1, 26	0.489
	Age (day)	1.257	7, 20	0.320
	Weight × age	1.191	7, 20	0.352
Pulses per chirp	Weight (mg)	0.272	1, 26	0.606
	Age (day)	0.915	7, 20	0.515
	Weight × age	0.808	7, 20	0.591
Carrier frequency (Hz)	Weight (mg)	0.150	1, 26	0.702
	Age (day)	1.684	7, 20	0.170
	Weight × age	1.338	7, 20	0.284
Chirp duration (ms)	Weight (mg)	0.006	1, 26	0.939
	Age (day)	0.589	7, 20	0.757
	Weight × age	0.553	7, 20	0.784

Discussion

We examined ejaculate quality in relation to the quality and quantity of acoustic mate attraction signals of the house cricket to distinguish between sperm competition theory and the phenotype-linked fertility hypothesis. Sperm competition theory predicts there should be a trade-off between investment in ejaculate quality and investment in mate attraction (Parker 1998), while the phenotype-linked fertility hypothesis predicts males with exaggerated secondary sexual traits should produce higher-quality ejaculates (Sheldon 1994). We did not find any direct support for either of these hypotheses, as there were no significant relationships between male acoustic mate attraction signalling parameters and sperm viability, number of living sperm or total number of sperm. However, male weight had a negative effect on ejaculate quality. This negative relationship provides indirect support for sperm competition theory, as female house crickets prefer heavy males over light males in two-choice tests (Gray 1997) and in no-choice tests (present study).

Lighter male crickets are more likely to lose aggressive competitions (Brown et al. 2006), produce less attractive acoustic signals (present study; Gray 1997), are less likely to attract females (Gray 1997) and take longer to successfully mate a female (present study) than heavier males. Consequently, lighter males may lose (or never gain) quality territories from which they can project their mate attraction signals. Light males are likely therefore to experience low lifetime reproductive success. One way in which

light males may be exhibiting an alternative mating tactic is by packing their ejaculates full of living sperm when they do mate to maximize their reproductive success. Our findings support this idea as light males produced significantly more living sperm than heavy males.

Mating latency was calculated as the time from when the virgin female was placed with the virgin male to the time when mating occurred. Given virgin male crickets are thought to invest equally in courtship effort during their first mating (Bateman & Fleming 2005), our mating latency measure should be fairly indicative of female choice. However, our mating latency measure did not incorporate a measure of when the male started to court the female. Given mating latency is influenced by the male's decision to court (Simmons 1988), our mating latency measure may include the aspects of both male and female choice. However, the number of sperm a male produces should not be affected by pre-copulatory signalling as sperm enters a spermatophore during spermatophore formation, which occurs prior to courtship and mounting (Hall et al. 2000; Kumashiro et al. 2003). Future work should ensure courtship timing is quantified, so mating latency can be calculated as the time from courtship to mating.

Theoretical models and empirical data associated with sperm competition support the idea that males who are least likely to be successful in pre-copulatory mate choice should be selected to increase their investment in their ejaculates to maximize their paternity success (Parker 1998; Cornwallis & Birkhead 2006). For example, in hooknose Pacific coho salmon (*O. kisutch*), males with the most intense red colouration are often more dominant. These dominant males have better access to females' nests and thus experience enhanced fertilization success. Pitcher et al. (2009) revealed that hooknose males with duller colouration produce higher-quality ejaculates (greater sperm motility and longevity) than males with intense red colouration. Pitcher et al.'s (2009) findings suggest a trade-off between investment in pre-spawning displays and investment in ejaculate quality. Similarly, ejaculate quality is negatively related to social dominance in male domestic fowl (*Gallus gallus domesticus*). Dominant males have greater access to females but subordinate males have higher sperm mobility (Pizzari et al. 2007). Further, bluegill sunfish (*Lepomis macrochirus*) sneaker males are smaller and less desirable to females than parental males and so obtain most of their fertilization success by covertly ejaculating in a parental male's nest. Sneaker males invest more into sperm number than the larger, more attractive parental males (Neff et al. 2002). Collectively, these findings suggest that the production of high-quality ejaculates may be an evolutionary adaptation to increase paternity success when mating opportunities are limited as a result of poor ability to compete via pre-copulatory sexual selection (Neff et al. 2002).

These empirical findings bring to light a problematic question. If drab or small males have been selected to maximize their paternity success by producing higher-quality sperm, why would not bright or large males do this as well? Life-history theory and sperm competition theory suggest that increased pre-copulatory investment should result in a trade-off with post-copulatory ejaculate investment (Parker 1998). Males that invest heavily into growing large and/or producing very attractive pre-copulatory sexually selected signals may therefore be incapable of increasing their investment in their ejaculates.

Conclusions

Male house crickets' acoustic mate attraction signals did not explain any of the variation in ejaculate quality. Instead, variation in ejaculate quality was negatively influenced by male weight. Light males produced more living sperm than heavy males. Conversely, heavy males produced long-distance acoustic mate attraction signals with longer pulse durations. Given heavy males mated more quickly than light males, our findings suggest that the fitness of heavy males may depend on pre-copulatory mate choice, while the fitness of light males may depend on post-copulatory fertilization success.

Acknowledgements

We are grateful to Jeff Dawson for helping to prepare materials, Shelley Hepworth for training and use of her microscope, Emily Whattam and Vanessa Rook for assistance with acoustic recording analyses, Byron Cavanaugh, Jay Fitzsimmons and two anonymous reviewers for useful comments on the manuscript and Louis Gagnon for animal care. Funding was provided by a Natural Science and Engineering Research Council of Canada (NSERC) Canada Graduate Scholarship to LPF, a NSERC Discovery Grant to SMB & TEP, the Canadian Foundation for Innovation to SMB & TEP, the Ontario Research Fund to SMB & TEP, Carleton University and the University of Windsor.

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