Male-derived cuticular hydrocarbons signal sperm competition intensity and affect ejaculate expenditure in crickets

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Female sexual promiscuity can have significant effects on male mating decisions because it increases the intensity of competition between ejaculates for fertilization. Because sperm production is costly, males that can detect multiple matings by females and allocate sperm strategically will have an obvious fitness advantage. The presence of rival males is widely recognized as a cue used by males to assess sperm competition. However, for species in which males neither congregate around nor guard females, other more cryptic cues might be involved. Here, we demonstrate unprecedented levels of sperm competition assessment by males, which is mediated via the use of chemical cues. Using the cricket *Teleogryllus oceanicus*, we manipulated male perception of sperm competition by experimentally coating live unmated females with cuticular compounds extracted from males. We found that males adjusted their ejaculate allocation in response to these compounds: the viability of sperm contained within a male's ejaculate decreased as the number of male extracts applied to his virgin female partner was increased. We further show that males do not respond to the relative concentration of male compounds present on females, but rather to the number of distinct signature odours of individual males. Our results conform to sperm competition theory, and show for the first time, to our knowledge, that males can detect different intensities of sperm competition by using distinct chemical cues of individual males present on females.

**Keywords**: cuticular hydrocarbons; sperm competition; *Teleogryllus oceanicus*; sperm viability

1. INTRODUCTION

Sperm competition theory predicts that males should adjust their reproductive expenditure according to the risk (the probability of competing with another male's ejaculate; Parker et al. 1997) and the intensity (the actual number of competing ejaculates; Parker et al. 1996; Engqvist & Reinhold 2006) of sperm competition. Empirical support for sperm competition risk models has been documented in many taxa (Svärd & Wiklund 1989; Gage 1991; Gage & Baker 1991; Möller 1991; Stockley et al. 1997; Schaus & Sakaluk 2001). However, there is considerably less evidence indicating that males can vary ejaculate expenditure in response to incremental increases in the intensity of sperm competition, and this evidence is currently confined to externally fertilizing species (Pilastro et al. 2002). The lack of empirical evidence for intensity models in internally fertilizing species probably reflects the difficulty of identifying the mechanisms used by males to discriminate sperm competition intensity in these species (Engqvist & Reinhold 2005). For example, in externally fertilizing fish, males can gain information on both the risk and intensity of sperm competition from the number of rival males present during a particular spawning (Candolin & Reynolds 2002; Pilastro et al. 2002; Smith et al. 2003).

However, for species with internal fertilization in which males do not congregate around nor guard females, males must rely on other sensory cues.

One such cue may be related to a female's mating status. Empirical evidence from a wide range of insect taxa has shown that cuticular hydrocarbons (CHCs), contact pheromones found on the exoskeleton of most terrestrial arthropods, allow males to determine whether a female is mated or not (Simmons et al. 2003a; Carazo et al. 2004; Friberg 2006). In some species, differences in CHCs between unmated and mated females have been attributed to the presence of male pheromones on mated females (Scott 1986; Andersson et al. 2003). Sexual dimorphism of CHCs is common in terrestial arthropods (reviewed in Thomas & Simmons 2008b), and the presence of these male chemicals on mated females could be due to either direct production of these compounds by females following mating, or the transfer of compounds from males to females during copulation. Despite the strong empirical evidence that CHCs can play an important role in providing cues to female mating status (Friberg 2006), potential differences in the CHC profiles of females mated to one or more males have been almost completely neglected as a possible mechanism used by males to assess sperm competition intensity.

Previous work using the Australian field cricket *Teleogryllus oceanicus* has shown that males adjust their ejaculate expenditure in response to a female's mating status, although males do not adjust the total number of sperm ejaculated. Male *T. oceanicus* produce an ejaculate
containing sperm of lower viability (proportion of live and dead sperm) when mating with unmated females, increase the viability of sperm within their ejaculate when mating with singly mated females, but reduce the viability of sperm within their ejaculate when mating with multiply mated females (Simmons et al. 2007; Thomas & Simmons 2007). As yet, the cues used by males in assessing sperm competition intensity remain unknown. In this study, we perform two separate experiments to determine how male T. oceanicus assess sperm competition intensity.

Male *T. oceanicus* are acutely sensitive to sex-specific CHCs (Rence & Loher 1977). In our first experiment, we investigate whether the presence of male CHCs on females provides a cue to the intensity of sperm competition. For this experiment, we developed a bioassay whereby live unmated females were coated with CHCs extracted from males. We were able to mimic the different intensities of sperm competition by coating females with extracts consisting of either 0, 1, 5, 10 or 15 different males. By coating only unmated females we ensured that the chemical but presumably not the physiological state of females was altered, thereby controlling for any potentially confounding changes in female receptivity or behaviour following mating (Loher 1981). We found that males adjusted their ejaculate allocation in response to these chemical manipulations.

In the second experiment, we investigated more precisely how males assess the intensity of sperm competition using these odor cues. For example, it could be that males respond to variation in the concentration of male CHCs; the more a female 'smells' like a male, the more partners she is likely to have copulated. Alternatively, males may actually be able to assess the distinct signature odour of each individual male a female has mated. Crickets have the ability to modulate sperm expenditure in relation to learned odour cues (Lyons & Barnard 2006), and have been shown to have the capacity to recognize up to seven odour pairs at the same time (Matsumoto & Mizanumo 2006). Moreover, in *T. oceanicus*, CHCs can vary among individuals within a population, with more closely related individuals sharing more similar CHC profiles (Thomas & Simmons 2008a). We found that males responded to blends of different male odour signatures, rather than to the concentration of male-derived odours.

### 2. MATERIAL AND METHODS

#### (a) Experimental animals

Experimental animals were the offspring derived from female crickets collected from a banana plantation in Carnarvon, Western Australia. Crickets were maintained in a constant temperature room (25°C) and maintained on a 12 L:12 D cycle. They were supplied with water and fed cat food ad libitum. Sexes were separated before the penultimate instar. Following the imaginal moult, experimental crickets were housed individually in boxes (7 × 7 × 5 cm). To ensure sexual receptivity, crickets were left to mature for 14 ± 3 days before being used in experiments or to obtain extracts. In this species, females are highly promiscuous, and sperm competition conforms to a fair raffle in which the relative numbers of viable sperm from a given male determines his share of paternity (Garcia-Gonzalez & Simmons 2005).

#### (b) Cricket extracts

Cricket extracts were obtained by immersing virgin males in 5 ml of hexane (AR grade) for 5 min. Following immersion, the hexane was evaporated off. Extracts were administered to unmated females by using a glass pipette to detach the remaining residue from the edge of the glass vial and vortexing the female in the vial on low speed for 1 min. The uptake of CHCs via physical contact was first used in the studies of *Drosophila* (Coyne et al. 1994). In our experiments, the CHC residue was invariably lost from the glass vial, and thus assumed to have adhered to the vortexed female. Nonetheless, it is possible that variation in the amount of CHC adhering to the unmated females could be a source of error in our bioassay. For this reason, we explored the possible range in effect sizes that our manipulations might have been, using the 95% CIs on the observed effect size (Nakagawa & Cuthill 2007).

(i) **Experiment 1**

To test whether male CHCs present on females can convey information on sperm competition intensity, extracts were composed of either 0, 1, 5, 10 or 15 males immersed sequentially in the same 5 ml of hexane. Thus, the concentration of male CHCs applied to unmated females increased across our treatments from 0 to 15 male equivalents female

(ii) **Experiment 2**

To determine whether males respond to differences in the relative concentration of male CHCs, or to the distinct signature odour of each individual male present on females, we used three extract treatments that were administered to females as above. Previously, we have found significant between-family variation in male hydrocarbon profiles (Thomas & Simmons 2008a). Therefore, to maximize variation within the blends of hydrocarbons in this experiment, male crickets used to obtain extracts were drawn from the first generation offspring of 35 field collected females, which had been raised in full sibling family groups. Treatments A and B consisted of extracts derived from a single male, or from 10 non-sibling males, respectively. These two treatments differ in both the number (1 or 10) and concentration (1 or 10 male equivalents female

(c) **Experimental matings**

Experimental males were mated once to a random female immediately before being placed with a treated female. This initial non-experimental mating ensured that males were sexually mature and produced a fresh spermatophore in the presence of a
treated female. Spermatophores are discreet vessels containing sperm that remain attached outside the female following mating. Immediately following copulation, the spermatophore was removed from the female and ejaculate investment was measured. Each male was used in only one experimental mating. In experiment 1, a total of 98 males were used \( (n=17, 23, 16, 25 \) and 17 for females coated with extracts composed of 0, 1, 5, 10 and 15 males, respectively). In experiment 2, 83 males were used across the three treatment groups \( (n=26, 28 \) and 29 for treatments A, B and C, respectively).

**d** Ejaculate quality

As the response variable, we measured the quality of ejaculates \( \text{(proportion of live and dead sperm)} \) that males transferred to treated females. We did not measure absolute sperm numbers because this trait has no influence on the fertilization success of male *T. oceanicus* \( \text{(Simmons et al. 2003b)} \). By contrast, paternity success of *T. oceanicus* is determined by the proportion of live sperm in a male’s ejaculate \( \text{(García-González & Simmons 2005)} \). More importantly, however, male *T. oceanicus* have been shown to display phenotypic plasticity in the viability of sperm contained within their ejaculates in response to sperm competition risk and intensity \( \text{(Simmons et al. 2007; Thomas & Simmons 2007)} \), but not in the absolute numbers of sperm transferred at copulation \( \text{(Thomas & Simmons 2007)} \).

We measured sperm viability using the live/dead sperm viability assay. This assay stains live sperm green with SYBR-14, a permeant nucleic acid stain, and dead sperm red with propidium iodide. Using published methods \( \text{(García-González & Simmons 2005)} \) we ruptured the spermatophore in \( 20 \mu l \) of Beadle saline \( \text{(128.3 mM NaCl, 4.7 mM KCl and 23 mM CaCl} _2) \). We then mixed \( 5 \mu l \) of this sperm solution with an equal volume of \( 1 : 50 \) diluted \( 1 \) mM SYBR-14 and left the sample in the dark for 10 min before adding \( 2 \mu l \) of \( 2.4 \) mM of propidium iodide. Following a further 10 min incubation period, 500 sperm were scored under a fluorescence microscope at \( 20 \times \) magnification. Sperm counts were made blind to the experimental treatment. Sperm viability was calculated as a proportion: the number of sperm alive \( \text{(stained green)} \) divided by the total number of sperm counted.

**e** Data analysis

Data were analysed using generalized linear models \( \text{(GLM)} \) with treatment entered as the main effect, the number of live sperm as the dependent variable and the total number of sperm counted as the binomial denominator, and a logit link function. Owing to overdispersion, we used \( F \)-tests, rather than \( \chi^2 \), to test statistical significance \( \text{(Crawley 1993)} \). Sperm viability data are presented as mean proportions of live sperm \( \pm 1 \) s.e. Effect sizes and their 95\% CIs were calculated using Pearson’s \( r \) correlation \( \text{(Nakagawa & Cuthill 2007)} \).

**3. RESULTS**

**a** Experiment 1

Males adjusted their ejaculate according to the composition of extracts with which females were coated \( (F_{1,98}=6.06, \ p=0.016; \ \text{effect size, } r=0.526, \ 95\% \ CI=0.364–0.656) \); the viability of sperm contained in a male’s ejaculate decreased as the number of males contributing to the CHC mix applied to females was increased \( \text{(figure 1)} \). Using a quadratic model did not explain a significantly greater proportion of the variation in sperm viability \( (F_{1,95}=0.17, \ p=0.681) \).

![Figure 1](http://rspb.royalsocietypublishing.org/Downloaded from on April 28, 2017)

**b** Experiment 2

Consistent with experiment 1, we found that males adjusted their ejaculates according to the composition of the extract with which females were coated \( (F_{2,80}=6.047, \ p=0.004; \ \text{effect size, } r=0.560, \ 95\% \ CI=0.392–0.692; \text{figure 2)} \). In order to determine if treatment C differed significantly from treatment A or B, we conducted planned contrasts \( \text{(Ruxton & Beauchamp 2008)} \) using the effect estimate procedures provided in GLMSTAT v. 6.0 \( \text{(Beadh 2004)} \). Thus, treatment C was entered as level 1 in the GLM and thereby intrinsically aliased into the constant. Effect tests for treatments A and B were then calculated relative to treatment C. We found that the viability of sperm contained within ejaculates transferred by males in treatment B \( \text{(where 10 males contributed to the hydrocarbon mix that was administered at a concentration of 10 male equivalents female} ^{-1}) \) did not differ significantly from that of males in treatment C \( \text{(a mix of hydrocarbons extracted from 10 males, but at a concentration of} \)
Rival male odour affects ejaculate allocation

4. DISCUSSION

Male Drosophila (Scott 1986), butterflies (Andersson et al. 2003), bees (Kukuk 1985) and cockroaches (Sreng 2006) have all been demonstrated to transfer chemicals to females during or subsequent to mating. However, detection of these chemicals by conspecific males has rarely been investigated as a cue to sperm competition intensity. Assessment of sperm competition intensity in response to male odour cues has recently been demonstrated in meadow voles (delBarco-Trillo & Ferkin 2006) and beetles (Carazo et al. 2007); however, in both of these studies the nesting substrates were chemically laden with male odours. While such substrate-based cues may be relevant for species in which individual females have a fixed mating site, for species in which females are nomadic or mate away from the nest, males must rely on cues present on the female alone. Our study provides, to our knowledge, the first direct evidence that males can detect different intensities of sperm competition using chemical cues from rival males present on females.

Our study also provides striking evidence that males can detect and respond to individual odour cues from different numbers of male rivals. Rather than responding to simple differences in the concentration of male odours present on females, males responded to the actual number of individual males that the extracts were composed of. Previously, crickets have been shown to be capable of remembering up to seven different odour pairs (Matsumoto & Mizanumo 2006), and our study provides a biological context in which an ability to assess the number of different odour types may prove adaptive. The ejaculate response we have observed is unlikely to be open ended. Most likely, males will be limited in the number of distinctive signature odours they can accurately discriminate. In this regard, we note the increased variance in ejaculate response when females were coated with 15 distinct CHC odours (figure 1). Moreover, theory suggests that when female remating rates are very high, males may completely reject the mating attempts of multiply mated females (Engqvist & Reinhold 2006).

Given that males can discriminate between individual male odours, it may also be possible that they can discriminate phenotypic variation among them. For example, CHCs are known to differ between individuals relative to a number of traits, such as their social status (Kortet & Hedrick 2005), age (Lorenzi et al. 2004) and relatedness (Thomas & Simmons 2008a). Some traits, such as age, are known to influence a male's ejaculate quality: in T. oceanicus, younger males tend to have less viable sperm than older males (Garcia-González & Simmons 2005). Since paternity success in this species is determined by the proportion of live sperm in a male's ejaculate (Garcia-González & Simmons 2005), males that can assess the relative age of their competitor could potentially gain additional information on the level of sperm competition that he will face. It remains to be tested if such complex information is conveyed via these chemical cues, and acted upon by male T. oceanicus.

The finding that males display a general decline in ejaculate expenditure with increasing numbers of male extracts, is consistent with sperm competition intensity models (Parker et al. 1996; Engqvist & Reinhold 2006), and represents rare evidence that males of an internally fertilizing species respond to different intensities of sperm competition by altering their ejaculate allocation. The original sperm competition model was developed for externally fertilizing group-spawning species, and predicted that when more than two ejaculates compete for the fertilization of a given set of eggs, sperm expenditure should decrease as the number of competing males increases (Parker et al. 1996). Not surprisingly, the best evidence supporting this model comes from externally fertilizing fish, in which males release fewer sperm with an increasing number of male rivals (Pilastro et al. 2002). Most other studies, including all studies of internally fertilizing species, have either yielded conflicting results, or have not examined continuous variation in sperm competition intensity (Schaus & Sakaluk 2001; Pizzari et al. 2003; Byrne 2004; delBarco-Trillo & Ferkin 2006). While our results are in accordance with the predictions of the original model (Parker et al. 1996), they correspond more closely to the predictions of a more recent model that was developed specifically for systems with internal fertilization (Engqvist & Reinhold 2006). The predictions of this model are similar to the original sperm competition model for a species such as T. oceanicus, where females show high rates of remating (Simmons 2003), and sperm competition conforms to a fair raffle (Garcia-González & Simmons 2005). However, this newer model also predicts that the discrepancy in sperm expenditure between unmated and multiply mated females should be larger than the difference in sperm expenditure between unmated and once-mated females, a prediction for which our data provide strong support (figure 1).

Table 1. Treatment contrasts from the generalized linear model. (Treatment C (an extract composed of 10 different males at a concentration of only 1 male equivalent female $^{-1}$) is intrinsically aliased into the constant, and estimates represent differences between the constant and treatment A (1 male extract) or B (10 male extracts) on the logit scale (Crawley 2002). The Bonferroni-adjusted critical value for the two planned contrasts is 0.025. Effect sizes and their 95% CIs were calculated using Pearson’s $r$ correlation.)

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Consistent with previous research on *T. oceanicus*, our results show that males can alter the quality of their ejaculates. Exactly how males adjust the viability of sperm within their ejaculate remains to be determined; however, one possible mechanism could be that they differentially invest in seminal fluids. Seminal fluids may function to activate and/or nourish sperm during transportation and thereby influence the viability of sperm contained in the ejaculate. Seminal fluids are known to have important impacts on sperm quality (Poiani 2006); there is good evidence to suggest that production of seminal fluids is costly (Simmons 2001), and recent theoretical work (Cameron et al. 2007) predicted that males should invest strategically in seminal fluid compounds where these contribute to a male’s fertilization success.

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**REFERENCES**


