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The transfer of male cuticular hydrocarbons provides a reliable cue of the risk and intensity of sperm competition in decorated crickets

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Abstract

Theoretically, males should increase their ejaculate expenditure when the probability of sperm competition occurring (or risk) is high but decrease ejaculate expenditure as the number of competing ejaculates (or intensity) increases. Here we examine whether male decorated crickets (*Gryllodes sigillatus*) use cuticular hydrocarbons (CHCs) transferred to females by rival males at mating to assess the risk and intensity of sperm competition and adjust their ejaculate accordingly. Unmated females and those perfumed with CHCs extracted from one, three, or five males could be distinguished chemically, providing a reliable cue of the risk and intensity of sperm competition. In agreement with theory, males mating with these females increased sperm number with the risk of sperm competition and decreased sperm number with the intensity of sperm competition. Similarly, as the risk of sperm competition increased, males produced a larger and more attractive spermatophylax (an important non-sperm component of the ejaculate) but these traits did not vary with the intensity of sperm competition. Our results therefore demonstrate that both sperm and non-sperm components of the male ejaculate respond to the risk and intensity of sperm competition in different ways and that CHCs provide males with an important cue to strategically tailor their ejaculate.

Keywords: sperm competition, risk, intensity, sperm number, spermatophylax, cuticular hydrocarbons

Introduction

Whenever a female mates with two or more males and can store sperm from these matings, the sperm are forced to compete to fertilize the available eggs, a process known as sperm competition (Parker, 1970). Sperm competition is now widely recognized as a potent selective force that has driven the evolution of male behavior, morphology, and physiology, as well as a range of life-history traits (Birkhead & Møller, 1998; Parker, 1970; Simmons, 2001). Most empirical tests of sperm competition theory have focused by far on the numbers of sperm ejaculated. If sperm compete numerically, males that transfer the most sperm will have a competitive advantage over other males. However, because sperm are costly to produce (e.g., Danielsson, 2001; Nakatsuru & Kramer, 1982; Olsson et al., 1997; Warner et al., 1995), they are often in limited supply (e.g., Dewsbury, 1982; Levitan & Peterson, 1995; Pitnick & Markow, 1994). Consequently, the fertilization gains of investing in sperm are likely to differ across matings and will select for males that are able to invest their sperm strategically (e.g., Bretman et al., 2011; Engqvist & Reinhold, 2006; Parker, 1990, 1998; Wedell et al., 2002).

One of the most important factors known to influence the optimal allocation of sperm by a given male is the number of rival male(s) with which he must compete. This factor has received considerable theoretical attention, most frequently being modeled as a game between male competitors to find the evolutionary stable strategy (ESS) for sperm allocation when males have a finite resource budget to spend on reproduction (Parker & Pizzarri, 2010). The multitude of different sperm competition game models can be broadly divided into those considering sperm competition "risk" (whether or not sperm competition occurs) or "intensity" (the number of ejaculates competing for a given set of eggs) (Parker & Pizzarri, 2010). Risk models emulate the conditions facing a species with a low level of sperm competition and the probability (or risk) that a female will mate either once or twice (Parker & Pizzarri, 2010). Assuming males have information on their mating role, risk models predict that the ESS is for males to increase ejaculate expenditure with risk (Ball & Parker, 1998; Parker, 1990; Parker et al., 1997). In contrast, intensity models simulate much stronger sperm competition in a species where males frequently experience sperm competition from

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more than one ejaculate (Parker & Pizzarri, 2010). Intensity models predict that if males can adequately assess the number of competing ejaculates, the male ESS is to decrease ejaculate expenditure with increasing intensity of sperm competition above the level of one competing ejaculate (Parker et al., 1996). While there is strong empirical support for an increase in ejaculate size with the risk of sperm competition, there is little support for a decrease in ejaculate size with the intensity of sperm competition, although it should be noted that far fewer empirical tests of the latter exist (Kelly & Jennions, 2011). Unfortunately, the same rigorous theoretical and empirical attention has not been given to how the risk and/or intensity of sperm competition influences non-sperm components of the ejaculate. This is surprising given that non-sperm components of the ejaculate, such as mating plugs (e.g., Sutter & Lindholm, 2016), accessory gland proteins (e.g., Chapman et al., 2000), and nuptial food gifts manufactured by the male (e.g., Wedell, 1991), are known to play a key role in sperm competition.

The effectiveness of a male in strategically adjusting his ejaculation to the risk and intensity of sperm competition depends critically on his ability to accurately assess the presence and number of mating rivals (Bretman et al., 2011). Indeed, many sperm competition games models explicitly assume that males have some degree of information (either partial or perfect) about female mating status (Parker & Pizzarri, 2010). Despite this, the exact cue(s) that males use to assess their risk and/or intensity of sperm competition remains elusive for many species (Bretman et al., 2011). Some of the best examples, however, come from insects where males have been shown to assess the risk of sperm competition using visual (e.g., Orr & Rutowski, 1991; Polak et al., 2001) and acoustic (e.g., Bailey et al., 2010; Gray & Simmons, 2013; Rebar & Greenfield, 2017) cues and adjust either their behavior or ejaculate accordingly. More recently, there has been a growing appreciation that cuticular hydrocarbons (CHCs) can also provide males with important chemical cues to assess the risk and intensity of sperm competition. Cuticular hydrocarbons are long-chained hydrocarbons (mainly alkanes, alkenes, and branched alkanes) that form a waxy coating on the exoskeleton of virtually all insects and can be physically transferred from one individual to another during tactile courtship and mating (reviewed by Weddle et al., 2013a). Males from a diverse range of insect species can use differences in CHCs to discriminate between mated and unmated females to minimize the risk of sperm competition (reviewed by Thomas, 2011). Moreover, male broad-horned flour beetles (*Gnatocerus cornutus*; Lane et al., 2015) and field crickets (*Teleogryllus oceanicus*; Thomas & Simmons, 2009) can even use the number of different rival males' CHCs present in the female to gauge and adjust their ejaculate to the intensity of sperm competition. Given how ubiquitous CHCs are in insects, it is likely that they play a far more important role as chemical cues of the risk and intensity of sperm competition than is currently appreciated.

In decorated cricket (*Gryllodes sigillatus*), females are highly promiscuous, often mating with several different males each night (Sakaluk et al., 1987, 2002). Males produce a long-range advertisement call to attract a female, and once contact has been established, the male produces a distinct close-range courtship call and stereotypical lateral movements (reviewed in Weddle et al., 2013). If the female chooses to mate, she climbs onto his back and he secures her subgenital plate with his epiphallus (Sakai et al., 1991). The male then transfers an externally attached spermatophore to the female that consists of two parts: a sperm-containing ampulla and a larger gelatinous spermatophylax (Sakaluk, 1984). After transfer, the female dismounts the male and immediately detaches the spermatophylax from the ampulla with her mandibles and commences feeding on it (Sakaluk, 1984). While the female feeds on the spermatophylax, sperm are evacuated into her reproductive tract from the ampulla. After consuming the spermatophylax, the female immediately removes and consumes the ampulla, thereby terminating sperm transfer (Sakaluk, 1984). Females vary considerably in the length of time that they feed on the spermatophylax, and the longer the female is delayed from removing the ampulla, the more sperm is transferred into her sperm storage organ (Sakaluk, 1984). Females take significantly longer to consume a larger spermatophylax (Sakaluk, 1985) and are less likely to prematurely discard nuptial gifts (which occurs in up to 25% of matings; Sakaluk, 1987) when it has a more attractive combination of free amino acids (Gershman et al., 2012). As sperm competition follows a simple sperm lottery model in *G. sigillatus* (Eggert et al., 2003; Sakaluk & Eggert, 1996), the increase in sperm transfer resulting from spermatophylax consumption benefits the male through a greater share of paternity (Calos & Sakaluk, 1998; Sakaluk, 1986). In contrast, consuming spermatophylax does not provide any direct benefits to the female, such as increased survival or lifetime reproductive output (Will & Sakaluk, 1994; Kasuya & Sato, 1998; Ivy & Sakaluk, 2005; but see Ivy et al., 1999 for a hydration benefit), and it actively hinders her from exerting post-copulatory mate choice via the premature removal of the ampulla of unattractive males (Ivy & Sakaluk, 2007). The genes expressed in males to produce spermatophylaxes that manipulate the female to feed for longer are linked to genes in females that make them more susceptible to feeding on spermatophylaxes for longer (i.e., less able to exert post-copulatory choice) (Gershman et al., 2013). Consequently, it has been argued that sexual conflict has been a key driver of nuptial gift evolution in *G. sigillatus* (Gershman et al., 2012, 2013; Rapkin et al., 2016; Sakaluk, 2000; Sakaluk et al., 2006).

Previous work on this species has shown that males are able to assess the risk and intensity of sperm competition and strategically adjust their ejaculate accordingly, although the exact response of males appears variable across studies (Gage & Barnard, 1996; Schaus & Sakaluk, 2001). Gage and Barnard (1996) showed that sperm number increased with both the risk (no rival versus one rival male) and intensity (one versus seven rival males) but there was no change in the mass of the ampulla or the spermatophylax. The findings of Schaus and Sakaluk (2001), however, are more difficult to interpret. This study used a repeated-measures design where the order in that each male experienced each density treatment (no rival versus one or six rivals) was randomized. While the main effect of density treatment was not significant, the interaction between this treatment and order was significant. This occurred because males in the no rival treatment produced more sperm when they experienced this treatment first than when they experienced it in the second or third position in the order, whereas there was little effect of the order on the sperm produced by males in the one or six rival treatments. Importantly, as both studies manipulated the risk and intensity of sperm competition by varying the number of rival males, it is not possible to isolate the exact cue(s) that regulate these effects.

It is possible that CHCs provide this cue, as they are known to play an important role in the mating system of *G. sigillatus* (Ivy et al., 2005; Steiger et al., 2015; Weddle et al., 2013).

Female mating preferences are significantly influenced by the CHC profile of males in *G. sigillatus*, with females preferring a specific combination of male CHCs to others (Steiger et al., 2015). Cuticular hydrocarbons are physically transferred from the female to the male at mating and influence female mating decisions in this species (Ivy et al., 2005; Weddle et al., 2013). More specifically, females can recognize their own CHC profile on a previous male mating partner (a process known as chemosensory self-referencing; Hauber & Sherman, 2001) and avoid remating with this male (Ivy et al., 2005; Weddle et al., 2013). Subsequent work has shown that this process is regulated by a form of "online processing" where females continually assess and compare their own CHC profile to CHCs present in the male after mating rather than relying on an early-learned or innate reference template (Capodeanu-Nägler et al., 2014). Chemosensory self-referencing is therefore a finely tuned mechanism that helps maintain polyandry in *G. sigillatus*. Females receive indirect benefits from polyandry in this species, with polyandrous females producing more offspring that survive to sexual maturation than females that repeatedly mate with the same male (Ivy & Sakaluk, 2005). However, even though males invariably also transfer their CHCs to females at mating, it is not known whether these transferred CHCs provide a reliable cue of the number of different male mating partners encountered by a female (and therefore the risk and intensity of sperm competition) or if males can assess these cues and adjust their ejaculate expenditure accordingly.

Here we examine whether male decorated crickets can use the CHCs transferred to their potential mating partner by rival males to assess the risk and intensity of sperm competition and adjust the properties of his ejaculate accordingly. We address this question with two experiments. In our first experiment, we examine whether the physical transfer of CHCs from males to females can provide reliable cues of the risk and intensity of sperm competition. We tested this by extracting the CHCs from one, three, or five males and perfuming unmated females with these extracts. We then quantified the CHC profile of these perfumed unmated females, as well as a sample of unperfumed and unmated males and females for comparison. If the CHCs transferred to a female by rival males provide a reliable cue of the risk and intensity of sperm competition, then we should be able to distinguish statistically between these treatments based on their CHC profiles, with the pattern of divergence across treatments reflecting the number of rival males. In our second experiment, we examine the ability of males to assess these chemical cues and strategically adjust their ejaculation in response to the risk and/or intensity of sperm competition. To test this, we mated males to females that had been perfumed with the CHCs of either one, three, or five males, or to an unperfumed female (as a control). We collected the ejaculates produced in these matings and quantified three key ejaculate components (sperm number, spermatophylax mass, and spermatophylax attractiveness) that are likely to bias the outcome of sperm competition in *G. sigillatus*. If males can assess these chemical cues, we predict that males will produce more sperm and/or larger, more attractive spermatophylaxes when the risk of sperm competition increases (from no rival males to one rival male). Conversely, we predict that males will produce less sperm and/or smaller, less attractive spermatophylaxes when the intensity of sperm competition increases.

Methods

Outbred stock population

Gryllodes sigillatus used in this study were descended from approximately 500 adult crickets collected in Las Cruces, New Mexico, USA in 2001. These founding animals were used to initiate a laboratory culture allowed to breed panmictically. Crickets were distributed across twelve 15-L transparent plastic containers and housed in an environmental chamber (Climatron, Thermoline Scientific) at 32 ± 1 °C on a 14 hr:10 hr light:dark cycle. Crickets were provided ad libitum with cat food pellets (Friskies 7, Nestle Purina PetCare, Rhodes, NSW, Australia), rodent chow (Specialty Feeds, Glen Forrest, WA, Australia), and water in 60-ml glass tubes plugged with cotton wool, and egg cartons for shelter. When adults were detected, moistened cotton wool was provided in a 90-mm Petri dish as an oviposition substrate. Hatching nymphs were collected *en masse* and approximately 500 nymphs were allocated at random to each container to establish the next generation. This process ensures gene flow in each generation to promote the maintenance of genetic variation in this outbred stock population.

Experimental animals

A total of 3,000 nymphs were collected on the day they hatched from oviposition pads (taken from our outbred stock population) and placed in individual containers (5 × 5 × 5 cm) with a piece of egg carton for shelter, water bottle (2.5-ml test tube plugged with cotton wool) and a dry cat biscuit. The food and water were replaced, and containers were cleaned weekly. Final instar nymphs were checked daily for eclosion to adulthood. On the day of eclosion, male and female crickets were assigned at random to either Experiment 1 or 2. All crickets were 10 days post-eclosion and unmated when used in these experiments. These crickets were maintained in individual containers to prevent physical contact with (and the subsequent transfer of CHCs from) other crickets.

Experiment 1: CHC transfer as a reliable cue of the risk and intensity of sperm competition

Experimental design

A total of 450 males were euthanized at –20 °C for 15 min and then allocated at random to one of three groups, consisting of one, three, or five males. We extracted the CHCs from the male crickets in these groups by whole-body immersion in a 5-ml glass vial containing 3 ml of hexane and 0.3 g of solid glass beads (Sigma-Aldrich, Z273619, 1 mm diameter) for 5 min. When CHCs were extracted from 3 and 5 males, crickets were immersed sequentially in the same 3 ml of hexane, each for a 5-min period. After CHCs were extracted, the hexane was evaporated in a fume hood using an N_2 gas sample condenser (Glas-Col, 109A11-80481, IN, USA).

Next, we allocated an unmated female at random to each of these extraction vials. The female was placed in the extraction vial and vortexed (using a single tube vortex mixer, VM1 model, RATEK, Victoria, Australia) on the lowest speed setting (200 rpm) for 1 min. During vortexing, the glass beads become mobile, increasing the contact area for the physical transfer of male CHCs to the female. Immediately after vortexing, each female was transferred to a new 5-ml glass vial

and euthanized at –20 °C for 15 min. We then extracted the CHCs present in each female in 3 ml of hexane containing 10 ppm dodecane as an internal standard for 5 min. For comparison, we also extracted CHCs from a random sample of unmated males and females (without perfuming) using this procedure. Furthermore, to ensure that glass beads did not introduce contaminants to our CHC samples, we also completed this extraction process in glass vials that only contained 0.3 g of glass beads (and no cricket, to serve as a control). A total of 2 ml of each extract was transferred to an individual glass autosampler vial (Chromacol, UK) that was capped with a lid containing a Teflon septum, sealed with Parafilm M, and stored at –20 °C until use.

Collectively, this procedure resulted in six CHC treatments: CHCs extracted from females perfumed with the CHCs from (i) one, (ii) three, or (iii) five males, as well as CHCs extracted from (iv) unperfumed males, (v) unperfumed females, and (vi) glass beads only (control). In total, we replicated this procedure for 50 crickets in each of treatments (i)–(v). For treatment (vi), however, we only used 10 replicates.

Chemical analysis of CHCs

We ran our CHC extracts from each treatment using a gas chromatography–mass spectrometry (GC–MS) methodology previously optimized for *G. sigillatus* (Steiger et al., 2015; Weddle et al., 2012). In brief, CHC extracts were run on an Agilent 7890A Gas Chromatograph coupled to an Agilent 5975B insert Mass Spectrometer. A 1 µl volume of each CHC sample was injected using an Agilent CTC PAL Auto sampler chilled to 5 °C onto a DB5-HT column (30 m × 0.25 mm ID × 0.1 µm film thickness) using hydrogen as the carrier gas at a constant flow rate of 1.2 ml min^{-1}. The optimal temperature profile for maximal separation of CHCs was as follows: hold at 100 °C for 1 min, ramp from 100 °C to 350 °C at 7.5 °C min^{-1} and the hold at 350 °C for 4 min (total run time per sample = 38.33 min). The inlet and MS transfer line were set at 230 °C and 150 °C, respectively, and the injection was run in the pulsed splitless mode. The area under each CHC peak (15 in total) was quantified using MSD Chemstation software (version E.02.00.493; Agilent Technologies, Cheshire, UK) with ions 55 and 57 set as the target ions for unsaturated and saturated compounds, respectively.

Prior to statistical analysis, the area under each CHC peak was divided by the area of the internal standard (dodecane) to control for drift in the sensitivity of the GC–MS over time and to ensure our data were not subject to unit-sum-constraint. This proportional value was then $\log_{10}$ transformed to ensure the normality of each peak in our dataset.

Experiment 2: Male ejaculate responses to the risk and intensity of sperm competition

Experimental design

To ensure that all males were sexually mature and to eliminate any possible first mating effects on ejaculate characteristics (reviewed by Torres-Vila & Jennions, 2005), we mated each of 160 experimental unmated males to an unmated female taken at random from our stock population. All matings were conducted in clear plastic arenas (30 × 18.5 × 11.5 cm) under red lighting in a constant temperature room set to 28 ± 1 °C. For each mating trial, the female was introduced into the plastic arena and given 2 min to acclimate before the male was introduced. The mating was considered successful if the male transferred a spermatophore to the female. Immediately after spermatophore transfer, the male was separated from the female and placed in his own individual container.

After this initial mating, we extracted the CHCs from (i) one, (ii) three, or (iii) five males, or from (iv) glass beads only (as a control) and used these to perfume 160 experimental unmated females (n = 40 experimental females per treatment). Cuticular hydrocarbons were extracted and experimental females perfumed following the protocols outlined in Experiment 1. Immediately after perfuming, experimental females were returned to their individual containers and the beads used to perfume them were transferred into a second, individual container (5 × 5 × 5 cm). Experimental males were then allocated at random to these containers and housed with beads for 3 hr so that they were exposed to the chemical cues when developing their next spermatophore (Schaus & Sakaluk, 2001). After 3 hr, we paired each experimental male with the experimental female that had been perfumed with the respective beads to mate. All second matings were conducted under the same conditions and following the same protocol as the initial matings. Immediately following this second mating, the spermatophore was detached from the female with a pair of fine forceps. The ampulla and spermatophyax were then separated and stored in different 0.5-ml Eppendorf tubes at –80 °C prior to quantifying the number of sperm in the ampulla and the dry weight, amino acid composition, and multivariate attractiveness of the spermatophylax. We measured the pronotum width of each experimental male using a dissection microscope (Leica MZ6) fitted with an eyepiece graticule. In total, we collected the spermatophore of 40 males in each treatment (total n = 160 spermatophores).

Quantifying sperm number

To estimate the number of sperm produced by each male, the ampulla was placed in 75 µl of distilled water and sheared with micro scissors. The ampulla was sheared further by pushing the suspension through a 25 G needle attached to a 1-ml syringe until the sample was cloudy. The sperm heads were stained with dilute Hoescht stain (1:100) and stirred vigorously for 30 s to prevent agglutination. For each sperm sample, three separate 10 µl aliquots were pipetted into counting chambers of a FastRead slide (http://www.fastread.co.uk/) and the number of sperm per aliquot was counted at 100× magnification (Olympus BX60 compound microscope used under phase contrast with UV and white light settings). Prior to our experiment, we confirmed that this sperm extraction and counting procedure was repeatable (intraclass correlation coefficient = 0.731, 95% confidence intervals = 0.611, 0.850; Wolak et al., 2012) by mating a sample of 40 unmated males and females paired at random from our stock population. We used the average of three sperm counts to estimate the total number of sperm produced by each male in our subsequent analysis.

Quantifying the weight, amino acid composition, and multivariate attractiveness of the spermatophylax

Each spermatophylax was freeze-dried for 24 hr using a Labconco Freeze-drier (Labconco, Kansis City, MO, USA) and then weighed using an electronic balance (Ohaus Explorer Professional model EP214C, NJ, USA). Freeze-dried spermatophylaxes were ground using a pestle in an Eppendorf that contained 150 µl of ethanol.

Amino acids were extracted from each spermatophylax using an EZ:Faast reagent kit for free amino acid analysis (Phenomenex, Torrance, CA, USA) (Gershman et al., 2012, 2013). A total of 100 µl of sample was pipetted into a sample vial containing 100 µl of internal standard solution (Norvaline 0.2 mM, N-propanol 10%). This sample was drawn through a sorbent pipette tip using a 1.5-ml syringe. Two hundred microliters of washing solution (N-propanol) were added to the sample vial and drawn through the sorbent tip. When all the liquid had passed through the sorbent tip into the syringe, air was drawn through to drain the sorbent tip, and the liquid in the syringe was discarded, leaving the sorbent tip in the sample vial. Two hundred microliters of eluting medium (a 3:2 mixture of NaOH and N-propanol) was added to the sample vial. Using a 0.6-mm syringe with the piston halfway up the barrel, the eluting medium was drawn into the sorbent tip until the liquid reached the filter at the top of the sorbent particles. The sorbent particles and liquid were then ejected from the tip into the vial. In total, 50 µl of chloroform was then added to each vial using a Drummond Dialamatic Microdispenser (Drummond Scientific, Broomall, PA, USA). The liquid in the vial was then emulsified by repeatedly vortexing for 8 s. The vial was left for 1 min to permit the reaction to proceed and the liquid to separate into two layers. The sample was then re-emulsified by vortexing for 5 s and the reaction allowed to proceed for a further minute. In total, 100 µl of iso-octane was then added to each vial using the microdispenser and the sample vortexed for 5 s. The sample was left for a further minute for the reaction to proceed. About 100 µl of hydrochloric acid (1 N) was then added to each vial and the sample vortexed for 5 s. The sample was then allowed to separate, and the top layer was pipetted into an autosampler vial for analysis on GC–MS.

We injected 0.2 µl of the extracted amino acid sample into the GC–MS fitted with a Zebron ZB-AAA column of 10 m × 0.25 mm ID × 2 µm, using helium as the carrier gas at a flow rate of 1 ml min^{-1}. The inlet was set at 325 °C and the injection was executed in pulse splitless mode. Separation of the sample was achieved using the method supplied with the EZ:Faast kit, which used a column profile starting at 110 °C, rising at 20 °C min^{-1} to 320 °C where it was held for 1 min (total run time per sample = 11.5 min). The MS transfer line was set at 300 °C. Data were analyzed using MSD Chemstation software and amino acids were quantified based on standard solutions provided in the kit. A range of standard solutions varying in concentration was prepared and calibration curves were constructed for each amino acid. This enabled us to measure the absolute quantity of each amino acid (measured in nanomoles per milliliter of internal standard) present in each spermatophylax.

We measured the following 22 free amino acids: alanine, glycine, α-aminobutyric acid, valine, leucine, isoleucine, threonine, serine, proline, asparagine, aspartic acid, methionine, 4-hydroxyproline, glutamic acid, phenylalanine, glutamine, orthinine, glycyl-proline, lysine, histidine, tyrosine, and tryptophan. However, three of the amino acids (α-aminobutyric acid, orthinine, and glycyl-proline) were not present in all samples and were therefore excluded from further analyses. As the quantity of each amino acid was measured in absolute amounts and the weight of the spermatophylax varied across males and our CHC perfuming treatments, it was necessary to correct the amount of each amino acid to the weight of the spermatophylax being compared. We therefore divided the amount of each amino acid by the dry weight of the spermatophylax so that our data for each amino acid is expressed in units of nanomoles per milliliter of internal standard per gram of spermatophylax (nmol/ml/mg). The data for each amino acid were $\log_{10}$ transformed prior to further analyses to ensure normality.

Previously, we used multivariate selection analysis to locate the combination of amino acids that significantly influence whether a female would prematurely discard the spermatophylax after mating (Gershman et al., 2012). We used the results of this study to assign a multivariate "attractiveness" score to each spermatophylax based on its amino acid composition, as done in previous studies (Duffield et al., 2015; Gershman et al., 2013; Rapkin et al., 2016). As this selection analysis was based on principal components (PCs) derived from the 19 amino acids, it was necessary to first project the amino acid composition of spermatophylaxes from our current study into the same multivariate space described by the selection analysis. This was achieved by substituting the amount of each amino acid present in the spermatophylaxes of males from our current study into the linear equation (i.e., eigenvector) describing each PC in the selection analysis. These equations are presented for each of the three PCs in Table 1 of Gershman et al. (2012) (see Supplementary Table S1). The equation that best describes the effects of amino acid composition on spermatophylax attractiveness (w, measured as the acceptance or rejection of the spermatophylax by a female) is given by the vector of linear selection gradients:

$$w = (-0.034PC1) + (-0.177PC2) + (-0.181PC3)$$

where PC1, PC2, and PC3 represent the three PCs describing the amino acid composition of the spermatophylax in our selection analysis. Using this equation, we calculated a multivariate attractiveness score for each spermatophylax produced by a male in our experiment. These attractiveness scores were used in our subsequent analyses examining how males adjust the amino acid composition of the spermatophylax in relation to the perceived risk of sperm competition. Importantly, higher attractiveness scores reflect spermatophylaxes that are more likely to be consumed by a female and therefore promote the greater transfer of sperm.

Statistical analysis

We used discriminant function analysis (DFA) to determine if the CHC profile of females perfumed with the extracts of one, three, or five males, as well as unperfumed and unmated males and females, could be distinguished. As we had multiple treatments, we compared the discriminant function scores taken from our DFA for each discriminant function using analysis of variance (ANOVA) and Bonferroni post-hoc tests to determine exactly which treatments differed. We calculated the percentage of cross-validated group cases correctly classified to determine how well our DFA distinguished between our treatments. We calculated this percentage of correctly classified cases when the DFA was run on all five treatments and when a DFA was run including only the three perfumed female treatments (i.e., treatments i, ii, and iii). Our DFA and subsequently univariate tests were conducted using IBM SPSS (version 25).

Sperm number, spermatophylax weight, and spermatophylax attractiveness all deviated significantly from a normal distribution and this could not be adequately remedied through data transformation. We therefore examined differences in the

effect of our perfuming treatment and male pronotum width on these ejaculate characteristics using a permutation-based multivariate analysis of covariance (MANCOVA). In this model, we included perfuming treatment as a fixed effect, male pronotum width as a covariate and sperm number, spermatophylax dry weight, and spermatophylax attractiveness as response variables. In short, the permutation procedure starts by running the above MANCOVA model using the original data to extract an *F*-ratio (F^o). The data are then shuffled at random across treatments (so there is no systematic difference between treatments) to extract a second *F*-ratio (F^p) from this randomized data set. This process is then iterated 9,999 times and a significance value for each term in the model is determined as (Anderson, 2017):

$$P = \frac{(\text{count } F^p \geq F^o) + 1}{(\text{total count } F^p) + 1}$$

Our permutation-based MANCOVA was conducted using the "*vegan*" package in R (v3.6.2; R Core Team, 2019), using Euclidean distance as the method to create the resemblance matrix (Anderson, 2017).

We followed our multivariate model with a series of permutation-based univariate analysis of covariance (ANCOVA) models run for each ejaculate component to determine which of these ejaculate components (s) contribute to any overall multivariate effects observed. In these univariate ANCOVAs, we used the same model structure as our multivariate model, as well as the same permutation procedure (with 9,999 iterations) implemented in the "*vegan*" package in R. As our perfuming treatment had more than two levels, it was also necessary to conduct post-hoc tests to determine which treatments differed in our ANCOVA models run for each ejaculate component. As these models included a covariate, we performed pairwise contrasts between the estimated marginal means of our treatments for each ejaculate component using a bootstrapping method implemented in the "*emmeans*" (v1.7.2; Lenth, 2022) and "*car*" (v3.0-10; Fox & Weisberg, 2019) packages in R. This approach explicitly incorporates all sources of correlation, from both the existing data structure and multiple contrasts, when adjusting *p*-values. The R code for this procedure is provided in the Online Supplement (Supplementary Text S1).

Results

Experiment 1: CHC transfer as a reliable cue of the risk and intensity of sperm competition

Extracts taken from glass beads alone did not contain any CHCs indicating that any patterns observed in the remaining treatments cannot be attributed to contaminants present on the glass beads. Discriminant function analysis on the remaining five treatments revealed three significant discriminant functions that collectively explained 99.21% of the variance in CHC profiles (Table 1). Discriminant function (DF) 1 explained 62.10% of this variance and was positively loaded to each CHC compound (Table 1). Consequently, this vector describes the absolute abundance of CHCs. Analysis of variance showed that DF1 scores differed significantly across treatments ($F_{4,245} = 138.28$, $p = 0.0001$). Post-hoc analysis revealed that the order of increasing CHC abundance across treatments was: unperfumed males ≤ unperfumed females < females perfumed with CHCs extracted from one male < females perfumed with CHCs extracted from three males < females perfumed with CHCs extracted from five males (Figure 1A and B).

Table 1. Discriminant function analysis (DFA) examining whether cuticular hydrocarbon (CHC) profiles can be statistically differentiated across our five perfuming treatments. Function loadings that are |0.25| or greater are viewed as biologically significant. CHCs are named where known (based on Rapkin et al., 2016) and unnamed CHCs (denoted by an asterisk) are described by their basic chemical structure. Two unnamed Alkatrienes with the same chemical structure ($C_{39}H_{74}$) have been combined into a single peak due to poor separation (peak 11) following Weddle et al. (2013).

	DF1	DF2	DF3
Eigenvalues	2.26	1.20	0.15
% of variance explained	62.10%	33.11%	4.00%
Wilks' lambda	0.12	0.39	0.85
χ^2	510.36	228.10	39.25
df	60	42	26
p	0.0001	0.0001	0.046

		Factor loadings		
Peak	CHC compound	DF1	DF2	DF3
1	7-MeC_{33}	**0.56**	−0.12	**0.25**
2	5-MeC_{33}	**0.55**	−0.09	0.17
3	3-MeC_{33}	**0.64**	−0.02	0.15
4	3,7-diMeC_{33}	**0.64**	−0.13	0.10
5	7-C_{35}ene	**0.43**	−0.03	0.10
6	3,13-diMeC_{36}	**0.37**	**−0.30**	**0.29**
7	5,9-diMeC_{36}	**0.30**	**−0.30**	0.23
8	9,31-C_{37}diene	**0.42**	0.04	0.09
9	7,31-C_{37}diene	**0.44**	0.08	0.07
10	9,31-C_{38}diene	**0.30**	0.07	**0.30**
11	Alkatriene ($C_{39}H_{74}$)*	**0.30**	0.01	0.13
12	9,31-C_{39}diene	**0.61**	0.01	**0.28**
13	7,31-C_{39}diene	**0.51**	0.18	0.18
14	Alkatriene ($C_{41}H_{78}$)*	**0.39**	0.12	−0.06
15	9,31-C_{41}diene	**0.48**	**0.31**	−0.01

DF2 explained a further 33.11% of the variation in CHCs and was negatively loaded to two CHC compounds (3,13-diMeC_{36} and 5,9-diMeC_{36}) and positively loaded to one CHC compound (9,31-C_{41}diene) (Table 1). Based on the retention time of these compounds, this vector describes the trade-off between these short- and long-chained CHCs. Analysis of variance showed that DF 2 scores differed significantly across treatments ($F_{4,245} = 73.73$, $p = 0.0001$). Post-hoc analysis revealed that the order of increasing CHC chain length across treatments was: unperfumed males < females perfumed with CHCs extracted from five males < females perfumed with CHCs extracted from three males < females perfumed with CHCs extracted from one male < unperfumed females (Figure 1A and C).

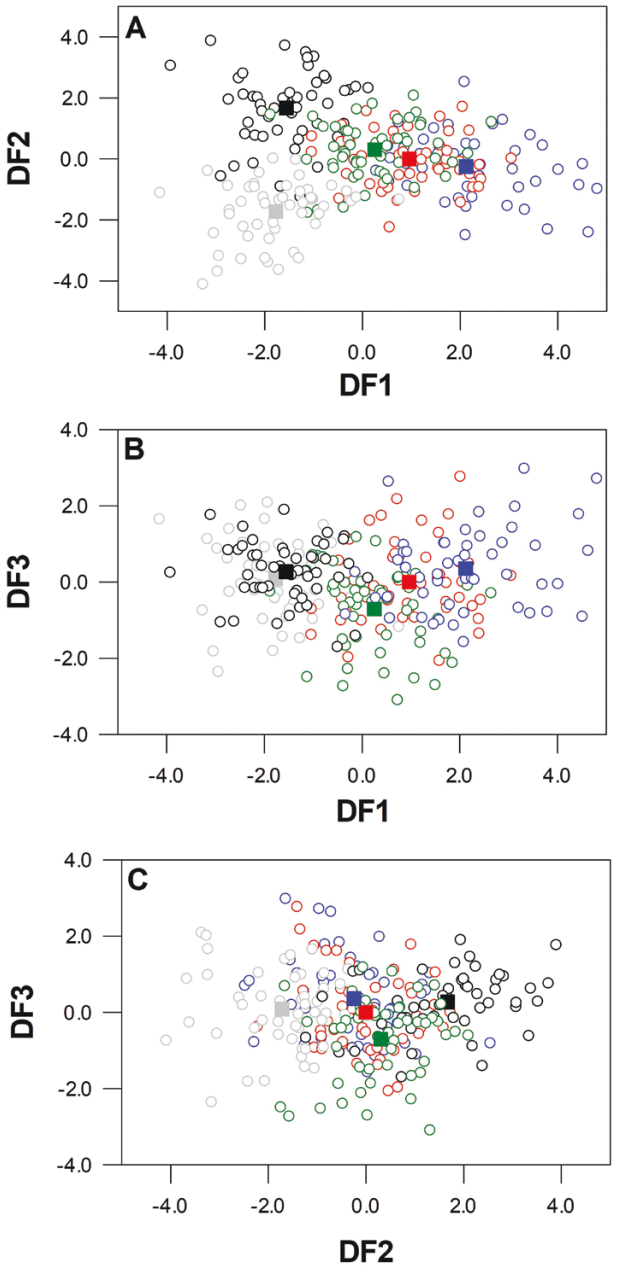

Figure 1. Discriminant function (DF) plot showing the divergence of cuticular hydrocarbon (CHC) profiles across our five perfuming treatments along the three significant discriminant functions: (A) DF1 versus DF2, (B) DF1 versus DF3, and (C) DF2 versus DF3. In each plot, grey open symbols represent unperfumed and unmated males, black open symbols represent unperfumed unmated females, green open symbols represent unmated females perfumed with CHCs extracted from one male, red open symbols represent unmated females perfumed with CHCs extracted from three males, and blue symbols represent unmated females perfumed with CHCs extracted from five males. In each plot, the closed squares (using the same color scheme) represent the group centroid for each perfuming treatment.

DF3 explained the remaining 4.00% of the variation in CHCs and was positively loaded to four CHC compounds (7-MeC_{33}, 3,13-diMeC_{36}, 9,31-C_{38}diene, and 9,31-C_{39}diene) (Table 1). Therefore, this vector describes an increase in these specific CHC compounds. Analysis of variance showed that DF3 scores differed significantly across treatments ($F_{4,245}$ = 8.90, p = 0.0001). Post-hoc analysis revealed that the order of increasing abundance of these specific CHC compounds across treatments was: females perfumed with CHCs extracted from one male < females perfumed with CHCs extracted from three males ≤ unperfumed males ≤ unperfumed females ≤ females perfumed with CHCs extracted from five males (Figure 1B and C).

Importantly, our DFA showed that 71.2% of cross-validated grouped cases were correctly classified. This percentage dropped slightly to 65.3% of correctly classified cases if we ran our DFA including only the three treatments involving perfuming (i.e., unperfumed males and females were excluded from the analysis). Collectively, this provides clear potential for males to assess the risk and intensity of sperm competition based on the transfer of CHCs from rival males to their female mating partners.

Experiment 2: Male ejaculate responses to the risk and intensity of sperm competition

Our permutation-based MANCOVA model revealed significant multivariate effects of our perfuming treatment and male pronotum width on male ejaculate components (Table 2). Univariate permutation-based ANCOVAs showed that the overall multivariate effect of male pronotum width was driven by the fact that spermatophylax weight (R^2 = 0.65, $\beta \pm SE$: 0.62 ± 0.04, t_{159} = 17.07, p = 0.0001) and spermatophylax attractiveness (R^2 = 0.15, $\beta \pm SE$: = 0.49 ± 0.10, t_{159} = 5.31, p = 0.0001) both increased with pronotum width, whereas sperm count did not (t_{159} = 1.43, p = 0.15) (Table 2). The overall multivariate effect of perfuming treatment was driven by significant effects on all three ejaculate components (Table 2). However, post-hoc analysis based on estimated marginal means revealed that each ejaculate component responded differently to our perfuming treatment (Figure 2, Supplementary Table S2).

Males that mated with a female perfumed with the CHCs of one male produced significantly more sperm than males that mated with an unperfumed female (Figure 2A, Supplementary Table S2A). However, sperm number decreased significantly when females were perfumed with the CHCs of three males and further decreased when females were perfumed with the CHCs of five males (Figure 2A, Supplementary Table S2A). Males mated to females perfumed with the CHCs of three and five males also produced significantly fewer sperm than males mated to an unperfumed female (Figure 2A, Supplementary Table S2A). This pattern indicates that males can adjust their sperm number in response to both the risk and intensity of sperm competition.

Males that mated with a female perfumed with the CHCs of one male produced significantly heavier spermatophylaxes than males that mated with an unperfumed female (Figure 2B, Supplementary Table S2B). Although males that mated with a female perfumed with the CHCs of three or five males produced lighter spermatophylaxes than males that mated with a female perfumed with the CHCs of one male, the difference in spermatophylax mass between these treatments was not significant (Figure 2B, Supplementary Table S2B). Furthermore, only males that mated with a female perfumed with the CHCs of five males produced spermatophylaxes that were significantly lighter than males mating with an unperfumed female (Figure 2B, Supplementary Table S2B). This pattern indicates that males can adjust the weight of their spermatophylax to the risk of sperm competition, but that their response to the intensity of sperm competition is less certain.

Males that mated with a female perfumed with the CHCs of one male produced significantly more attractive spermatophylaxes than males that mated with an unperfumed female (Figure 2C, Supplementary Table S2C). Although

Table 2. Permutation-based multivariate analysis of covariance (MANCOVA) examining the effects of CHC perfuming treatment and male pronotum width (PW) on ejaculate components (sperm count, spermatophylax dry weight, and spermatophylax attractiveness) in *G. sigillatus*. This overall multivariate model was followed by a series of permutation-based univariate analysis of covariance (ANCOVA) models to determine which ejaculate component(s) contributed to any overall multivariate effects. As we had five different perfuming treatments, pairwise post-hoc analyses based on estimated marginal means were used to determine which treatments differed for each ejaculate component (presented in Figure 2 and Supplementary Table S2).

		Permutation-based MANCOVA				
	Model terms	R^2	SS	df	F^P	*p*
	Perfuming treatment	0.33	157.92	3,159	45.98	0.0001
	Pronotum width	0.30	141.64	1,159	123.72	0.0001
	Permutation-based ANCOVAs					
Model terms	**Ejaculate component**	R^2	SS	*df*	F^P	*P*
Perfuming treatment	Sperm count	0.78	123.81	3,159	183.86	0.0001
	SPHYLAX weight	0.15	24.43	3,159	70.10	0.0001
	SPHYLAX attractiveness	0.06	9.68	3,159	4.01	0.01
Pronotum width	Sperm count	0.00	0.40	1,159	1.76	0.19
	SPHYLAX weight	0.73	116.57	1,159	1,003.64	0.0001
	SPHYLAX attractiveness	0.16	24.67	1,159	30.68	0.0001

Abbreviations: SS = sums of squares, F^P = permutation-based *F* value, SPHYLAX = spermatophylax.

the attractiveness of spermatophylaxes produced by males when mating with a female perfumed with the CHCs of three and five males was significantly lower than when mating to a female perfumed with the CHCs of only one male, spermatophylax attractiveness did not differ between these two treatments (Figure 2C, Supplementary Table S2C). Moreover, the attractiveness of spermatophylaxes produced by males in both treatments did not differ significantly from those produced by males that mated with an unperfumed female (Figure 2C, Supplementary Table S2C). This pattern indicates that males adjust the attractiveness of their spermatophylax to the risk of sperm competition but not to the intensity of sperm competition.

Discussion

In this study, we examined whether male decorated crickets could use the CHCs transferred to their potential mating partners by rival males to assess the risk and intensity of sperm competition and to adjust the properties of their ejaculates accordingly. We found that unmated females perfumed with CHCs extracted from one, three, or five males were chemically distinct from each other, as well as from unperfumed unmated males and females, indicating the potential for CHCs transferred from previous male mating partners to provide a reliable cue for the risk and intensity of sperm competition. We then showed that males can adjust their ejaculate in response to these CHC cues, albeit to different degrees for sperm and non-sperm components of the ejaculate. Sperm number, spermatophylax weight, and spermatophylax attractiveness all increased with the risk of sperm competition. Although sperm number decreased with the intensity of sperm competition, spermatophylax mass only decreased at the highest intensity of sperm competition (the CHCs of five rival males), and spermatophylax attractiveness did not change over this range. Collectively, our findings demonstrate that the transfer of male CHCs provides a reliable and usable cue for the risk and intensity of sperm competition in *G. sigillatus* but that not all components of the ejaculate are equally responsive to these cues.

Almost all sperm competition studies examining the potential for CHCs to serve as a cue for the risk and intensity of sperm competition have used perfuming without characterizing the resulting changes to the CHC profile of the female (Thomas, 2011). Indeed, only a single-sperm competition study has documented the change in female CHCs with perfuming, showing that the CHC profile of female broad-horned beetles (*Gnatocerus cornutus*) changed with perfuming but did not become more like males or mated females in composition (Lane et al., 2015). We also show that perfuming unmated female *G. sigillatus* with male CHC extracts significantly altered their chemical profile, but we found that these changes provided a much clearer cue for the risk and intensity of sperm competition. In our study, the CHC profile of unmated females when perfumed with the CHCs from one, three, or five rival males, as well as unperfumed and unmated males and females, was captured in three main dimensions of CHC variation. The first two dimensions provide information on both the risk and intensity of sperm competition. DF1 describes the total abundance of CHCs and was lowest for unperfumed males and females and increased with the number of male CHC extracts used to perfume the female. DF2 describes the trade-off between short- and long-chained CHCs, with the largest difference being between unperfumed males and females; moreover, the profile of perfumed females became more like unperfumed males as the number of male extracts used for perfuming increased from one to five. In contrast, DF3 is only likely to provide information on the risk of sperm competition. DF3 describes a change in four specific CHC compounds, but only females perfumed with the extract from a single male deviated from the other treatment groups. Importantly, the fact that crickets could be correctly allocated to their respective treatment groups 71.2%

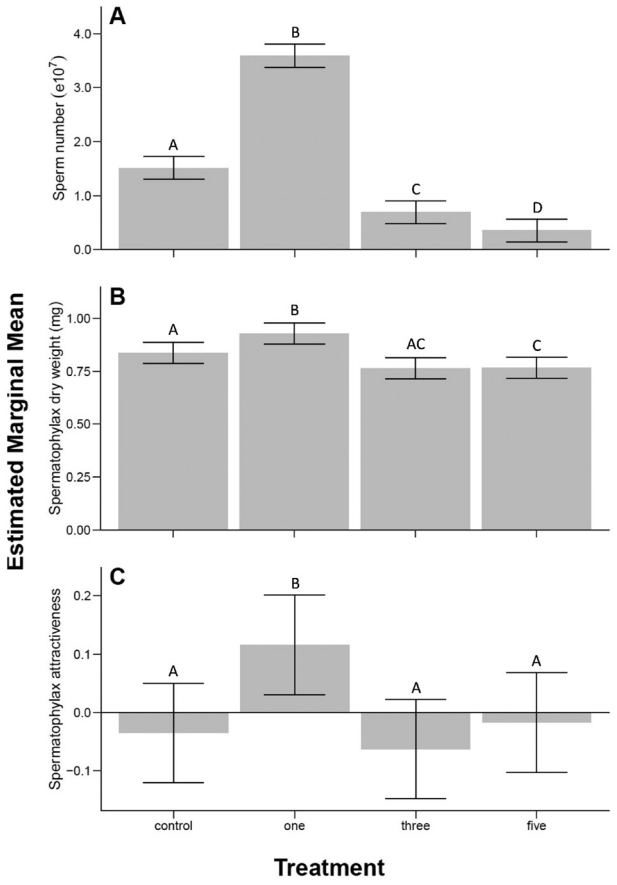

Figure 2. Estimated margin means (and 95% confidence intervals) for (A) sperm count, (B) spermatophylax dry weight, and (C) spermatophylax attractiveness in response to our perfuming treatments in male decorated crickets. Estimated marginal means were used because our analysis contained pronotum width as a covariate and two of our ejaculate components were significantly influenced by this covariate (Table 2). Estimated marginal means were estimated for each ejaculate component per perfuming treatment at the mean of the covariate. Treatments with different letters are significantly different at $p < 0.05$.

of the time indicates that these changes in CHCs with perfuming provide a reliable cue of the risk and intensity of sperm competition in *G. sigillatus*.

Most sperm competition models assume that males have some prior knowledge (either partial or perfect) about female mating status (Parker & Pizzari, 2010). However, that the physical transfer of CHCs provides a reliable cue for the risk and intensity of sperm competition in *G. sigillatus* does not necessarily mean that males are able to use this information to strategically adjust their ejaculate. If males can assess these cues, theory predicts that they should increase the number of sperm in their ejaculate with the risk of sperm competition (e.g., Ball & Parker, 1998; Parker, 1990; Parker et al., 1997) but decrease sperm number with the intensity of sperm competition (e.g., Parker et al., 1996). Consistent with these predictions, we found that males mating with a female perfumed with the CHCs of a single male produced more sperm than males mating with an unperfumed female. Moreover, as the number of male CHCs used to perfume a female increased above a single male, the number of sperm produced by the competing male decreased and were fewer than males mated to an unperfumed female. While meta-analysis has provided general empirical support for an increase in sperm number with the risk of sperm competition, there is little support for the predicted decrease in sperm number with the intensity of sperm competition (Kelly & Jennions, 2011). Our work is therefore novel in demonstrating that male *G. sigillatus* can strategically adjust their sperm number to both the risk and intensity of sperm competition and that CHCs are the chemical cues they use to regulate these responses.

While CHCs have long been implicated as important chemical cues in sperm competition (Thomas, 2011), surprisingly few studies have directly tested this by altering the CHC profile of females (Lane et al., 2015; Thomas & Simmons, 2009). Even these few studies have shown that males can respond in different ways. For example, sperm viability decreased in male field crickets (*T. oceanicus*) with the intensity of sperm competition (from 1 to 15 male CHC extracts), but not with the risk of sperm competition (Thomas & Simmons, 2009). Conversely, male broad-horned flour beetles (*G. cornutus*) significantly increased sperm number when mating with females perfumed with the CHC extracts of three males compared to unperfumed females, although it is unclear whether males were responding to the risk or intensity of sperm competition (Lane et al., 2015). It is possible that the strong effects of sperm competition risk and intensity on sperm number that we observe is amplified by the 3-hr pre-exposure to perfumed beads in our experiment. In the wild, it is likely that males are constantly sampling females and accumulating information on sperm competition risk and intensity before a given mating. We would therefore argue that our pre-exposure to perfumed beads captures some of this complexity, although further experiments (possibly varying bead exposure time) are clearly needed to test this. Nevertheless, the patterns of sperm allocation we observe are likely to have important implications for the outcome of sperm competition in *G. sigillatus*. As sperm competition follows a simple lottery in *G. sigillatus* (Eggert et al., 2003; Sakaluk & Eggert, 1996), the increase in sperm number observed with the risk of sperm competition should increase a male's share of paternity. Indeed, our finding that sperm number is ~ 2.4 times higher when mating with a female perfumed with the CHCs of a single male versus an unperfumed female suggests that the paternity benefits of this increased sperm allocation are likely to be considerable. The reduction in sperm number with the intensity of sperm competition should also enable males to avoid costly sperm allocation when the reproductive benefit is expected to be low. The costs of sperm production are poorly understood in *G. sigillatus*, as well as for most insect species (Wedell, 2002), and more work is clearly needed before this pattern of sperm allocation is fully appreciated.

In many insect species, the outcome of sperm competition is also biased by a range of non-sperm components of the ejaculate, including nuptial gifts manufactured by the male (e.g., Wedell, 1991). Yet, theoretical models and empirical studies of sperm competition frequently overlook these important ejaculate components. Here we show that the spermatophylax in *G. sigillatus*, a nuptial gift produced by males that the female consumes during mating and that prolongs sperm transfer (Sakaluk, 1984), also responds to the risk and intensity of sperm competition but in different ways to sperm number. Males produced a heavier and more attractive spermatophylax with an increased risk of sperm competition. However, spermatophylax mass only decreased when mating to a female perfumed with the CHCs of five males and there

was no change in spermatophylax attractiveness when mating to a female perfumed with the CHCs of three or five males relative to an unperfumed female. As female feeding time and subsequent sperm transfer increases with both the weight (Sakaluk, 1985) and attractiveness (Gershman et al., 2012) of the spermatophylax, the increase in these spermatophylax properties with the risk of sperm competition reinforces the pattern observed for sperm number and should therefore further improve a male's share of paternity. However, the fact that spermatophylax weight and attractiveness did not decline with the intensity of sperm competition is surprising given that producing a spermatophylax is costly for male *G. sigillatus*. For example, spermatophylax mass (Gershman et al., 2010; Kerr et al., 2010) and an attractive free amino acid composition (Duffield et al., 2015) are traded against immunity and lifespan, respectively, and both spermatophylax properties are only maximized on high calorie diets (Rapkin et al., 2016).

This raises the obvious question: why do males adjust sperm number and spermatophylax properties differently to the intensity of sperm competition? The obvious answer is that spermatophylax size and free amino acid composition take longer to adjust than sperm number and are therefore less responsive to the intensity of sperm competition. However, this does not explain why males can increase both sperm number and spermatophylax properties in response to the risk of sperm competition when measured over the same timeframe. This suggests that it may be easier for males to increase the size and free amino acid composition of the spermatophylax than it is to decrease them. Moreover, the fact that these spermatophylax properties were very similar to those observed in control males raises the possibility that this represents the minimum viable spermatophylax size and free amino acid combination in *G. sigillatus*. That is, producing a smaller spermatophylax or one containing a lower combination of free amino acids than this has a disproportionately large cost to male fitness. However, more empirical work is needed to test whether this can explain the inability of male *G. sigillatus* to adjust spermatophylax properties to the intensity of sperm competition.

In conclusion, our work provides clear evidence that the physical transfer of CHCs to females provides males with a reliable cue of the risk and intensity of sperm competition that enables them to adjust sperm and non-sperm components of their ejaculate accordingly, albeit in different ways. Given that CHCs are ubiquitous in terrestrial arthropods and readily transferred between individuals through physical contact (Everaerts et al., 2010; Weddle et al., 2013; Yew et al., 2008, 2009, 2011), it is likely that the role of CHCs as usable cues in sperm competition is far more widespread than currently appreciated. However, testing the generality of this conclusion requires far more empirical studies.

That said, our findings raise an obvious question: do CHCs remain sufficiently reliable as cues over time and different ecological conditions or are additional cues needed? Unfortunately, very little is known about the stability and reliability of CHCs when transferred between individuals. Our work shows that CHCs transferred to females remain stable and reliable as cues of sperm competition, at least in the short timeframe and benign laboratory conditions of our experiment. However, the reliability of chemical cues (including CHCs) is likely to vary considerably under the different environmental conditions experienced in the field and it has been proposed that this may be selected for the evolution of multiple cues (Bro-Jørgensen, 2009). For example, in lotic environments where chemical cues are rapidly dispersed (and are therefore less reliable), a range of decapod species (blue crab, Teytaud, 1971; horseshoe crab, Saunders et al., 2010; and crayfish, Acquistapace et al., 2002) require a combination of chemical and visual cues to successfully locate and attract a mate. The social environment is also likely to play a key role in the reliability of CHCs, especially as cues of sperm competition. In species that aggregate, the reliability of cues of sperm competition may be reduced due to the constant transfer of CHCs, necessitating the use of additional cues (Liu et al., 2020). Indeed, male *Drosophila* (Bretman et al., 2011; Maguire et al., 2015) and moths (Liu et al., 2020) are better able to detect rivals and adjust their ejaculates when receiving multiple cues (i.e., chemical, tactile, and/or acoustic) compared to a single cue. In nature, *G. sigillatus* are gregarious and frequently aggregate at high density under shelters (Sakaluk, 1987; Sakaluk et al., 2002). Males show strong site fidelity, whereas females typically move between aggregations each night to mate, with most mating at least once a night but some mating up to twice (Sakaluk, 1987; Sakaluk et al., 2002). This is likely to reduce the effectiveness of CHCs as reliable cues of the risk and/or intensity of sperm competition and it is possible that additional cues are also used. For example, acoustic cues are used to gauge the risk and intensity of sperm competition in other cricket species (e.g., Gray & Simmons, 2013; Rebar & Greenfield, 2017). Further studies are clearly needed for *G. sigillatus* to determine the relative importance of chemical, acoustic, tactile, and visual cues for the detection of rival males and whether males can strategically allocate their ejaculate components accordingly.

Supplementary material

Supplementary material is available online at *Evolution*.

Data availability

The data from this study are available from Dryad, https://doi.org/10.5061/dryad.83bk3jb1g

Author contributions

C.M.H., J.H., S.K.: Conceptualization; K.D., C.M.H., J.H., S.K.: Methodology; J.R., J.H., C.M.H.: Formal analysis and investigation; C.M.H., J.H.: Writing—original draft preparation; S.K., K.D., J.R.: Writing—review and editing; J.H.: Funding acquisition; C.M.H., J.H.: Resources.

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