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LETTER

Cuticular hydrocarbons as a basis for chemosensory self-referencing in crickets: a potentially universal mechanism facilitating polyandry in insects

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Abstract

Females of many species obtain benefits by mating polyandrously, and often prefer novel males over previous mates. However, how do females recognise previous mates, particularly in the face of cognitive constraints? Female crickets appear to have evolved a simple but effective solution: females imbue males with their own cuticular hydrocarbons (CHCs) at mating and utilise chemosensory self-referencing to recognise recent mates. Female CHC profiles exhibited significant additive genetic variation, demonstrating that genetically unique chemical cues are available to support chemosensory self-referencing. CHC profiles of males became more similar to those of females after mating, indicating physical transfer of CHCs between individuals during copulation. Experimental perfuming of males with female CHCs resulted in a female aversion to males bearing chemical cues similar to their own. Chemosensory self-referencing, therefore, could be a widespread mechanism by which females increase the diversity of their mating partners.

Keywords

Chemical communication, crickets, cuticular hydrocarbons, *Gryllodes sigillatus*, mate choice, mate recognition, polyandry, self-referent phenotype matching, sexual selection.

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INTRODUCTION

Polyandry is pervasive across animal mating systems (Zeh & Zeh 2003), and especially so in the insects (Arnqvist & Nilsson 2000). Females of numerous insect species mate repeatedly throughout their lives, invariably with many different males. Such behaviour seems paradoxical because females often mate more frequently than is necessary to ensure fertilisation of their eggs (Ridley 1988), often at the cost of increased time and energy expenditures, as well as the increased risk of injury, disease and predation (Daly 1978). However, a growing body of evidence has revealed that females can increase their fitness by obtaining material benefits from males during mating (Arnqvist & Nilsson 2000) or indirect genetic benefits through paternally derived genes that enhance offspring viability (Jennions & Petrie 2000; Tregenza & Wedell 2002; Bretman *et al.* 2004; Ivy 2007; Slatyer *et al.* 2012). Although direct benefits of multiple mating could in theory be realised by mating repeatedly with the same male, only by mating with different partners can females secure the diverse ejaculates that can maximise indirect genetic benefits. In polyandrous species, therefore, selection should favour mechanisms by which females forego matings with previous partners in favour of novel males. Indeed, a number of empirical studies across an array of animal taxa including pseudoscorpions (Zeh *et al.* 1998), crickets (Bateman 1998; Ivy *et al.* 2005; Gershman 2009), hide beetles (Archer & Elgar 1999), dung flies (Hosken *et al.* 2003) and guppies (Eakley & Houde 2004), have demonstrated a female mating preference for novel males over previous mates.

Although a female preference for novel mates has been well established, the mechanism by which females distinguish between previous mates and novel males remains unknown. How do females recognise previous mates, particularly when cognitive constraints would appear to limit their ability to learn the unique features of each of their mates over their reproductive lifetime? A recent study of female decorated crickets (*Gryllodes sigillatus*) provides a clue. When females were given a choice between a male previously mated to the focal female's inbred sister ('familiar' male) and a male mated to an unrelated female ('novel male'), they preferentially mated with the 'novel' male (Ivy *et al.* 2005). This suggests that the focal female perceived chemical cues left on the male by her inbred sister as her own, and consequently identified the 'familiar' male as a previous mating partner. This kind of chemosensory self-referencing would only require the female to compare these cues with an internal representation of her own chemical phenotype, and avoid mating with any male whose cues match her own phenotype (Hauber & Sherman 2001).

If females rely on self-referent chemical cues to recognise previous mates, then individual females must possess unique chemical signatures that allow discrimination of 'self' from the cues of other individuals they encounter. Cuticular hydrocarbons (CHCs), lipid compounds present on the surface of the insect epicuticle, offer considerable promise in this regard. CHCs often play an integral role in insect chemical communication (Howard & Blomquist 2005), facilitating species recognition, kin recognition and sex recognition in a variety of insect taxa, including crickets (Tregenza & Wedell 1997; Nagamoto *et al.* 2005; Ryan & Sakaluk 2009). There is

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also evidence that CHCs can be transferred between individuals through direct physical contact (Coyne *et al.* 1994; Blows & Allan 1998; Harris & Moore 2005; Everaerts *et al.* 2010). Successful copulation in *G. sigillatus* requires that the female physically mount the male, and that the pair remain in direct physical contact for 2–4 min until the male successfully transfers a spermatophore (Sakaluk 1987) (Fig. 1). Wide variation in CHCs have been documented in crickets at the species and population levels (Mullen *et al.* 2007), as well as within and between the sexes (Warthen & Uebel 1980; Tregenza & Wedell 1997; Mullen *et al.* 2007; Thomas & Simmons 2008). CHCs, therefore, seem likely candidates as the chemical cues facilitating female recognition of, and discrimination against, previous mating partners.

Here, we report results of studies designed to test the hypothesis that CHCs are used by female crickets to facilitate chemosensory self-referencing. Gas chromatography-mass spectrometry (GC-MS) analysis revealed significant genetic variation in female CHC profiles, demonstrating that genetically unique chemical cues are available to support chemosensory self-referencing. Solvent-free, solid-phase microextraction (SPME) showed that CHC profiles of males became more similar to those of females after mating, indicating physical transfer of CHCs between individuals during copulation. Experimental perfuming of males with female CHCs resulted in a female aversion to mating with males bearing cues similar to their own. However, outbred females showed no aversion to mating with sibling males, suggesting that discrimination against previous mates is not simply an incidental consequence of a mechanism that evolved to facilitate inbreeding avoidance. Given the pervasiveness of CHCs as recognition cues among arthropods, chemosensory self-referencing via CHCs could be a ubiquitous mechanism by which females across a broad range of animal mating systems increase the diversity of their mating partners.

METHODS AND MATERIALS

GC-MS analyses of female CHC extracts

Nine inbred lines were generated by subjecting randomly selected *G. sigillatus* individuals from a large, panmictic laboratory population to 16 generations of full-sib mating (see Appendix S1 in Supporting Information for additional details on rearing conditions). Seven days after adult eclosion, crickets were freeze-killed at −80 °C for 10 min. Cuticular hydrocarbons were extracted by whole-body immersion in 2 mL of hexane (Fisher H303-4) for 10 min (see Appendix S1).

Figure 1 A mating pair of decorated crickets, *Gryllodes sigillatus*. The female's (above) ventral side remains in direct physical contact with the male's (below) dorsum for 2–4 min during copulation. Photograph by David Funk.

Samples were analysed on a 6890 series GC (Agilent Technologies, Cheshire, UK) using a DB5-HT column (30 m × 0.25 mm ID × 0.1 μm film thickness) with a flame ionisation detector. Samples were injected via an Agilent 7683B autoinjector at a volume of 1 μL each (see Appendix S1). Differences in hydrocarbon peaks obtained by the gas chromatograph were analysed for 31 females from each of the nine genetic lines.

To identify peaks corresponding to individual CHCs, an additional four females from each line were analysed on an Agilent Technologies 7890A GC attached to a 5975B inert MSD using the temperature program outlined in Appendix S1. Data were analysed using MSD Chemstation software (version E.02.00.493) (Agilent Technologies, Cheshire, UK). Methyl branched alkanes were identified by their mass spectra (Nelson *et al.* 1972), and the identities of the peaks were confirmed using retention indices (Francis & Veland 1981). The positions of double bonds in unsaturated hydrocarbons were determined by interpreting the mass spectra of the dimethyl disulphide derivatives (DMDS, Francis & Veland 1981). For two of the alkadienes, the levels of DMDS derivatives were too low, and these were identified based on their mass spectra using the methods of Howard *et al.* (2003). The identification of the positions of the double bonds of alkatrienes was beyond the scope of our study. To quantify CHC peaks, the relative abundance was measured using ion 57 as the target ion for saturated, and ion 55 for unsaturated compounds.

To analyse differences in CHC profiles across genetic lines, we used a multivariate approach modified after Dietmann *et al.* (2005) and Herzner *et al.* (2006). The standardised peak areas at each retention time ($\log_{10}$ transformed, corresponding to the relative abundances of CHCs) were analysed by multivariate analysis of variance (MANOVA) to test for an effect of genetic line on thirteen CHCs. We then used discriminant analysis using the CANDISC procedure in SAS to investigate what combinations of CHC peaks discriminate females into genetic lines. We examined the strength of the relationships between the CHC peaks and the discriminant functions by interpreting factor loadings > |0.25| as contributing significantly to the axis of variation represented by a discriminant function. We used the DISCRIM procedure in SAS to determine the extent to which the CHC profiles of individuals could correctly predict the membership of each female to a genetic line. The proportion of individuals misclassified was estimated using Lachenbruch's jackknife procedure (CROSSVALIDATE option in SAS), in which each observation is classified based on the discriminant function derived from analysis of the remaining $n-1$ observations (Stevens 2002; SAS Institute Inc. 2006).

Genetic analyses of female CHCs

The heritability of each cuticular hydrocarbon compound was calculated as the intraclass correlation from an ANOVA on inbred lines using the protocol established by David *et al.* (2005). Genetic correlations and standard errors were estimated using the jackknife procedure of Roff & Preziosi (1994); see Appendix S1.

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SPME analysis of mated males

Experimental subjects were from inbred lines B (males) and H (females, see Appendix S1). Upon adult eclosion, individuals were isolated and housed in same sex-groups to ensure virginity. On days 9–11 post-eclosion, we randomly sampled experimental individuals via SPME approximately 2 h prior to the dark cycle (time 1). For SPME sampling, we sampled each sex on the portions of the body that come into direct physical with the opposite sex during copulation (Fig. 1). Individual males were sampled by lightly rubbing the SPME fibre across the dorsal surface of the abdomen and wings, equally for a total of 1 min. Females were sampled by lightly rubbing the ventral surface of the abdomen with the fibre for 1 min. Males and females were then held in a darkened room and allowed 2 h of recovery, after which males and females were paired and allowed to mate. Control males were treated in the same manner, but were not paired with a female and therefore, remained unmated. After successful mating, experimental males were immediately separated from females and resampled via SMPE using the same method as described above (time 2). Control males were resampled at the same time.

SPME fibres used were 7 μm Polydimethylsiloxane (Supelco, see Appendix S1). Once the SPME sample had been taken, the fibre was injected into an Agilent 7890A GC coupled to an Agilent 5975B mass spectrometer (see Appendix S1). To analyse differences in CHCs detected by SPME, we calculated the relative abundance of each CHC peak using a log-contrast transformation (Blows & Allan 1998). The area of each CHC peak was divided by the total peak area of all CHCs for each individual. These ratios were then divided by an arbitrarily chosen peak (peak 1, 7-MeC_{33}) and log transformed. Differences in hydrocarbon peaks obtained by the gas chromatograph were analysed for 61 virgin females, 61 males before and after mating, and 24 control males sampled at time 1 and time 2, with approximately 4 h between sampling. We used Repeated Measures MANOVA on each CHC peak to test for significant differences between virgin and mated males due to mating (Bonferroni adjusted $\alpha = 0.0029$). We performed Principal Component Analysis (PCA) on the relative abundance of CHCs obtained for both mated males and control males at sample time two. We then used MANOVA on the principal component scores to detect significant differences between mated and control males.

Perfuming with CHCs and its effect on mate choice

Experimental females were from inbred lines B and H (see Appendix S1). Upon adult eclosion, individuals were housed in same sex-groups for 6–12 days to ensure their virginity. On day 7–13 post-eclosion, experimental individuals were randomly assigned to a mating combination consisting of one focal female and two males. Males within a mating combination were from the same genetic line as each other to control for any differences in female choice due to the genetic background of males. To avoid any possible effects of inbreeding avoidance on female choice, males used in mate choice trials were never from the same line as the focal female. A male from each sibling dyad was randomly assigned as either the 'familiar male' or the 'novel male.' One of each male in a dyad was randomly selected to be marked with a small dot of correction fluid 48-h prior to mate choice trials to facilitate subsequent identification. Twenty-four hours prior to mate choice trials, focal females were mated with a randomly selected male from our outbred colony and ampulla retention time was standardised to 30 min for all females to equalise mating experience. We used 57 females from line B (mated to males from line H), and 45 individuals from line H (mated to males from line B) as focal individuals ($n = 102$).

'Familiar' CHC perfumes were created using hexane-extracted CHCs from three virgin females of the same age and from the same genetic line as the focal female, which were subsequently applied to the 'familiar' male. To create the 'novel' CHC perfumes, hexane-extracted CHCs from another three randomly selected virgin females from one of three genetic lines (lines G, D or E) different from that of the focal female and both males were applied to the 'novel male' (see Appendix S1). Two hours prior to mating trials, males were confined in the glass tubes containing the residual CHCs and were vortexed in a tube rack on medium-low speed for 1 min (Thomas & Simmons 2009). Each individual male was then vortexed separately on low speed for an additional 10 s to ensure CHC transfer by contact of the male's body with the walls of the tube. All males were allowed to recover from the perfuming treatment for 2 h before being used in mate choice trials.

Mate choice trials were established on day 8–14, post-eclosion. All trials were conducted blind to treatment under red lighting approximately 2 h into the dark cycle. Experimental females were paired with both the 'novel' and 'familiar' male in clear plastic shoeboxes (34.2 cm × 20.9 cm × 11.8 cm). As females do not mate with males that do not produce courtship song (Adamo & Hoy 1994), only those trials in which both males actively courted the female were included in experimental analyses. The identity of the male with which the female copulated (indicated by successful transfer of a spermatophore) was recorded, along with the time of successful copulation relative to the time when males first began courtship of the female. We used a χ^2 test for equal proportions to determine if there was a female mating preference for novel or familiar males and χ^2 test of independence to determine if female mating preferences differed with the inbred line of origin for females (line B or H) or males from which CHCs were extracted (lines G, D or E).

Inbreeding avoidance

As there may be incidental selection against inbreeding avoidance in our inbred genetic lines, we tested for inbreeding avoidance in outbred females established from wild *G. sigillatus* collected at Phoenix, AZ prior to the experiment. F_1 crickets (produced by different females collected in the wild) were used in a full-sib/half-sib mating design in which 13 randomly selected males were each mated to two females to establish 26 F_2 family lines of known parentage (see Appendix S1). One of each male in a mating dyad was randomly marked with a small dot of correction fluid 48-h prior to mate choice trials to facilitate subsequent identification. On day seven post-eclosion, experimental females were randomly assigned to mating trials in one of two treatments and allowed to choose between: (1) a full-sib male and an unrelated male (from a family line other than the one from which the focal female originated), or (2) a half-sib male and an unrelated male. For each focal female family (within sire), we performed five replicates of each mate-choice treatment ($n = 130$).

All mating trials were observed blind to treatment under red light illumination approximately 3 h into the dark cycle. Matings were

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conducted in plastic shoeboxes lined with paper towel. The identity of the male with which the female copulated (as indicated by the successful transfer of a spermatophore) was recorded, along with the time of successful copulation relative to the time when males first began courtship of the female. Only those trials in which both males actively courted the female were included in the experimental analyses. We used χ^2 contingency analysis to determine if there was a female mating preference for related or unrelated males, and whether female mate choice differed among treatments.

RESULTS

GC-MS analyses of female CHC extracts

We analysed cuticular hexane extracts for 287 females from nine inbred lines and identified a total of 15 different CHCs (Fig. 2). Mass spectrometric characterisation of female cuticular hydrocarbons revealed a diverse mixture of compounds ranging from 33 to 41 hydrocarbons in length, and consisting of branched alkanes, alkenes, alkadienes and alkatrienes (Table 1). Consistent with our predictions, we found significant differences in female cuticular hydrocarbons due to genetic line (MANOVA, Pillai's trace = 4.83, $F_{104,2112} = 30.89$, $P < 0.0001$). Discriminant analysis yielded four discriminant functions that explained 91.41% of the between-group variation in CHCs (Table S1). The predictive model correctly discriminated females to genetic line with 100% success for six of the nine genetic lines, and between 84 and 97% of the females in the remaining three lines, with a total misclassification rate of only 2.87%. Only 11% correct classifications would be expected for each of the nine genetic lines by chance alone.

As might be expected in genetic lines derived from randomly selected individuals from a larger panmictic colony, some lines were more similar in their hydrocarbon profiles than others (Fig. 3).

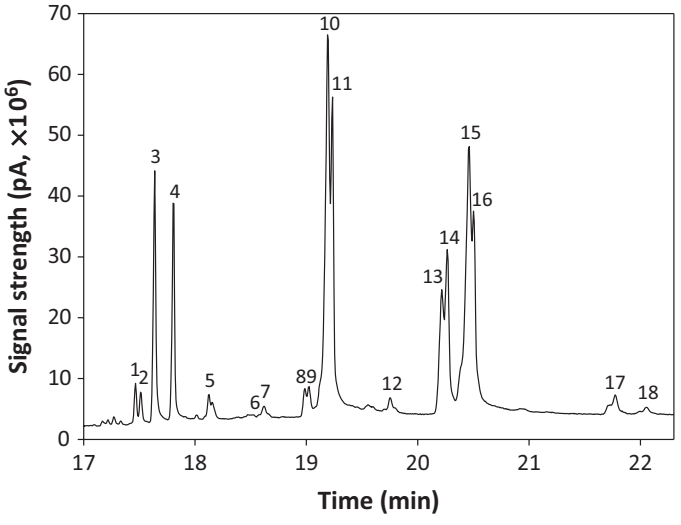

Figure 2 A typical GC profile from epicuticular extracts of a female decorated cricket derived using SPME analysis. The *x*-axis shows the retention time (min) and the *y*-axis shows the GC signal strength (picoamperes). We found 18 distinct hydrocarbon peaks using SPME, which are characterised by their mass spectra in Table 1. Fifteen of these peaks were quantified using GC-flame ionisation detector and GC-MS and used in our genetic analysis.

Table 1 Chemical characterisation of CHCs. DMDS: diagnostic ions after derivitisation with dimethyl disulphide

Peak	RI	Compound	Formula	Diagnostic ions
1†§	3338	7-MeC_{33}		464 (M-15), 394
2†	3347	5-MeC_{33}		464 (M-15), 422
3†	3376	3-MeC_{33}		464 (M-15), 450
4†	3406	3,7-diMeC_{33}		478 (M-15), 464, 394, 127
5†	3490	7-C_{35}ene		DMDS: 585 (M+), 145, 440
6†‡	3551	3,13-diMeC_{36}		506, 352, 211
7†‡	3573	5,9-diMeC_{36}		478,155
8	3664	5,9-C_{37}diene		517(M+), 123, 135, 432, 444*
9	3672	3,9-C_{37}diene		517(M+), 123, 135, 458, 474*
10†¶	3684	9,31-C_{37}diene		DMDS: 705 (M+), 131, 173
11†¶	3691	7,31-C_{37}diene		DMDS: 705 (M+), 131, 145
12†	3776	9,31-C_{38}diene		DMDS: 719 (M+), 145, 173
13†	3842	Alkatriene	$C_{39}H_{74}$	543 (M+)
14†	3849	Alkatriene	$C_{39}H_{74}$	543 (M+)
15†	3885	9,31-C_{39}diene		DMDS: 733 (M+), 159, 173
16†	3893	7,31-C_{39}diene		DMDS: 733 (M+), 145, 159
17†	4033	Alkatriene	$C_{41}H_{78}$	571 (M+)
18†	4051	9,31-C_{41}diene		761 (M+), 145, 173

*Identification based on Howard *et al.* (2003) as DMDS derivatives were too small.

†Peaks identified in GC-MS analysis of solvent extracts.

‡Peaks not included in the genetic analyses of solvent extracts due to insufficient quantities for all samples.

§Peak used to standardise the remaining 17 peaks for the analysis of SPME data.

¶These peaks were combined for the GC-MS analysis of solvent extracts, but were resolved into two separate peaks in the SPME analysis.

Examination of the factor loadings for each of the CHC peaks onto the four discriminant functions (DF) showed that all peaks contributed significantly to group separation with the exception of 9,31-C_{39}diene (Table S1). According to the discriminant analysis, much of the contribution to between-group variation in CHCs (for DF 1 and 2, cumulative 68.71%) was predominantly due to alkatrienes (peaks 14 and 17) and alkenes 7-C_{35}ene, 9,31-C_{37}diene, and 9,31-C_{38}diene.

Genetic analyses of female CHCs

Heritabilities of standardised peak areas of the 13 CHC compounds were uniformly high and statistically significant, with an average heritability (± 1 S.E.) of 0.978 ± 0.008 (Table S2). There were statistically significant genetic correlations between many of the 13 cuticular hydrocarbon compounds (Table S2). All of the branched alkanes 3-MeC_{33}, 5-MeC_{33}, 7-MeC_{33}, and 3,7-diMeC_{33} were highly, positively correlated with one another. Moreover, with the exception of one alkatriene (peak 13), these branched alkanes were also predominately positively genetically correlated with all other CHC peaks (Table S2). We found significant positive correlations between many of the alkene compounds (including 7-C_{35}ene and 9,31-C_{37}diene, 7-C_{35}ene and 9,31-C_{38}diene, and 9,31-C_{37}diene and 9,31-C_{38}diene). One alkatriene (peak 13) was significantly negatively correlated with the other alkatriene peaks (peaks 14 and 17).

SPME analysis of mated males

SPME analysis detected a total of 18 CHC peaks for both males and females (Table 1). Comparisons of the SPME data for mean

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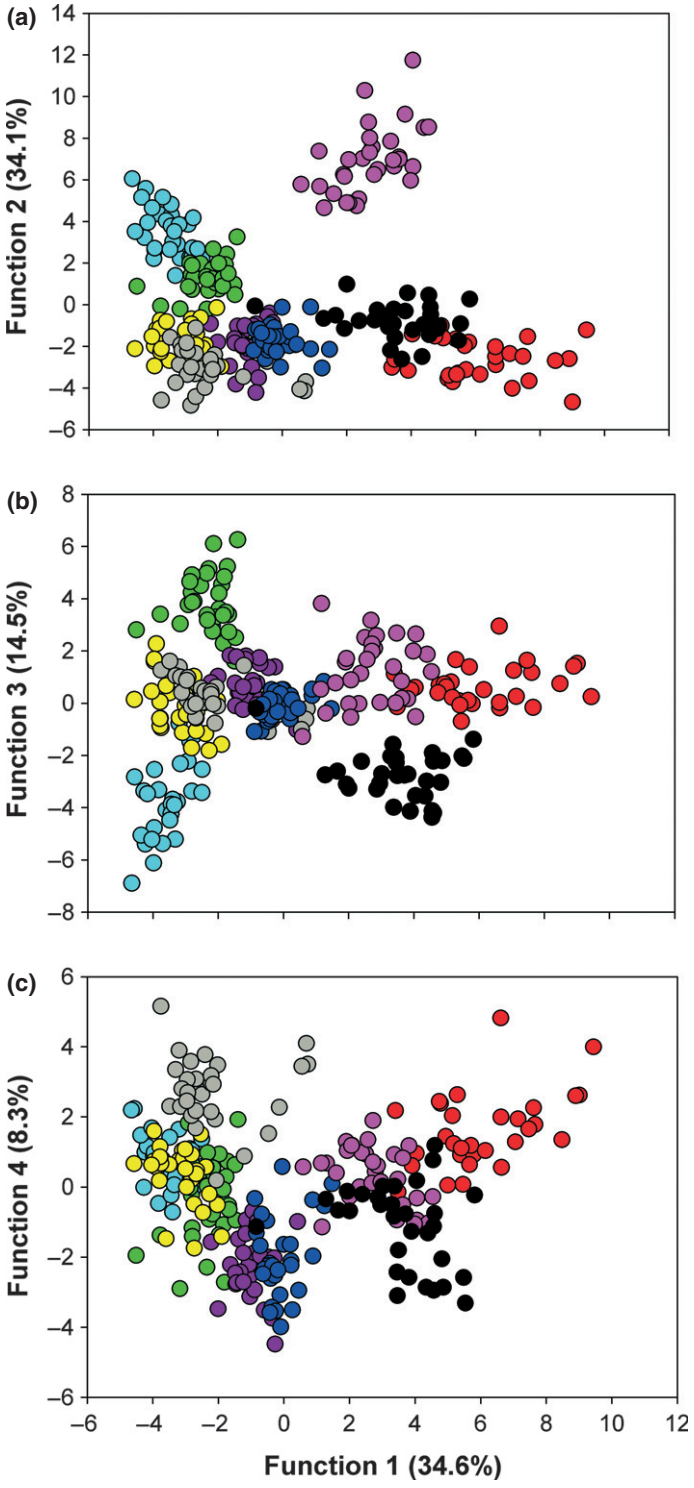

Figure 3 Discriminant analysis of cuticular hydrocarbon extracts of females from nine genetic lines. Each colour represents a genetic line: Line A = cyan, Line B = red, Line C = green, Line D = purple, Line E = yellow, Line F = grey, Line G = dark blue, Line H = pink, Line I = black. Plots show discriminant functions 4 (a), 3 (b) and 2 (c), plotted against function 1. Despite some overlap, females discriminate significantly into genetic lines based on the relative amounts of the 13 cuticular hydrocarbon peaks (n = 287).

relative concentration of each male CHC peak before and after mating revealed significant differences between virgin and mated males in 12 of the 17 CHCs analysed (Repeated Measures MANOVA, Bonferroni adjusted α = 0.0029; Table 2). Examination of the means for each of these 12 CHCs shows that the chemical profile of mated males becomes more similar to that of females after mating for all of these compounds except 9,31-C_{41}diene (Fig. 4). Compounds that were more abundant in females tended to increase in mated males, whereas compounds that were less abundant in females showed an overall decrease in males after mating.

Table 2 Results of Repeated Measures MANOVA for SPME. We analysed each of the 17 CHC peaks for males from SPME samples taken before (virgin) and after (mated) mating. Significant effects of mating are shown in bold (Bonferroni adjusted α = 0.0029). Although 18 CHC peaks were detected, Peak 1 was used to standardise the remaining peaks and therefore only 17 peaks were analysed

Peak #	Description	Pillai's Trace	F	df	P
2	5-MeC_{33}	0.367	34.74	1, 60	**0.0001**
3	3-MeC_{33}	0.000	0.02	1, 60	0.8992
4	3,7-diMeC_{33}	0.004	0.26	1, 60	0.26
5	7-C_{35}ene	0.368	34.97	1, 60	**0.0001**
6	3,13-diMeC_{36}	0.078	5.1	1, 60	0.0276
7	5,9-diMeC_{36}	0.405	40.86	1, 60	**0.0001**
8	5,9-C_{37}diene	0.276	22.92	1, 60	**0.0001**
9	3,9-C_{37}diene	0.477	54.81	1, 60	**0.0001**
10	9,31-C_{37}diene	0.521	65.34	1, 60	**0.0001**
11	7,31-C_{37}diene	0.364	34.31	1, 60	**0.0001**
12	9,31-C_{38}diene	0.309	26.78	1, 60	**0.0001**
13	Alkatriene $C_{39}H_{74}$	0.316	27.76	1, 60	**0.0001**
14	Alkatriene $C_{39}H_{74}$	0.510	62.48	1, 60	**0.0001**
15	9,31-C_{39}diene	0.164	11.77	1, 60	**0.0011**
16	7,31-C_{39}diene	0.430	45.28	1, 60	**0.0001**
17	Alkatriene $C_{41}H_{78}$	0.088	5.77	1, 60	0.0194
18	9,31-C_{41}diene	0.052	3.27	1, 60	0.0755

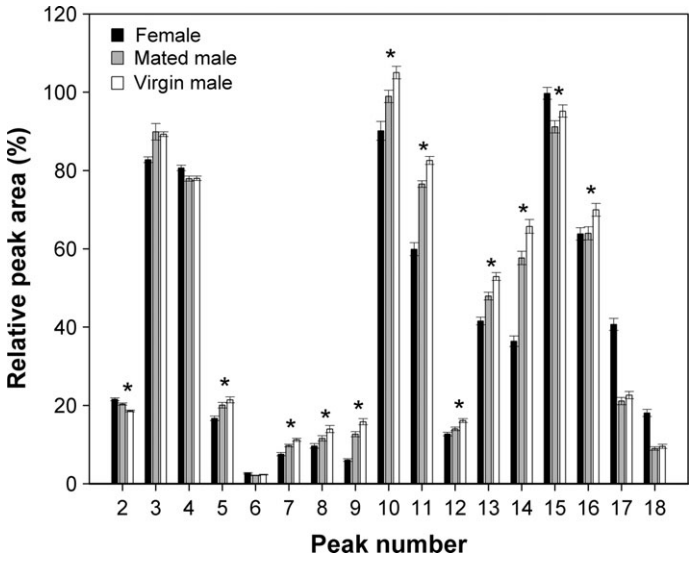

Figure 4 Cuticular hydrocarbons detected by SPME before and after mating. Mean (± S.E.) relative peak area (in per cent) for each cuticular hydrocarbons peak for virgin females, mated males and virgin males detected via SPME. Asterisks indicate peaks that showed statistically significant differences between mated and virgin males (Repeated Measures MANOVA, Bonferroni adjusted α = 0.0029; see Table 2 for statistical summary). Peak 1 (7-MeC_{33}) was used to standardise the remaining 17 peaks and therefore is not included in the analysis.

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Principal component analysis of the relative abundance of CHCs for mated and control males (time 2) returned three principal components (PC) with eigenvalues > 1. These three PCs cumulatively explained 75.8% of the variance in CHCs (PC1: 51.8%, PC2: 12.9%; PC3: 11.1%). We then compared the principal component scores generated from the PCA for mated and control males sampled during the same time period. We found a significant difference in CHC profiles between mated males and control males (MANOVA, Pillai's trace = 3.25, $F_{3,81} = 30.89$, $P < 0.026$). The canonical structure of the MANOVA revealed that the first 2 PCs made the most significant contribution to between-group variation in CHCs (standardised canonical coefficients: PC1 = 0.868, PC2 = 0.589, PC3 = 0.089). To interpret which of the original CHCs contributed to each principal component, we examined the correlations between the relative peak abundance for each CHC and the three principal components (Table S3). We used the criteria suggested by Mardia *et al.* (1979) in which correlations above 0.7 times the highest correlation within a PC were considered to contribute significantly to that PC.

For PC1, several of the larger molecular weight alkenes (9,31-C_{38}diene, 7,31-C_{39}diene, 9,31-C_{39}diene, 9,31-C_{41}diene) and alkatrienes (peaks 13, 14 & 17) weighted positively on this factor (Table S3). Examination of the PC scores for individual males reveals that mated males tended to have less of these compounds relative to control males (Fig. S1). These results correspond to our comparison of males sampled before and after mating. All but two of these compounds showed significant differences between virgins and mated males, and three of these compounds (9,31-C_{38}diene, 7,31-C_{39}diene; alkatriene, peak 14) were shown to decrease after mating. CHCs that weighted negatively on PC1 included smaller molecules such as 7-C_{35}ene, 5,9-C_{37}diene, and 5-MeC_{33} (Table S3). Mated males tended to have more of these compounds than control males (Fig. S1).

2, 5,9-diMeC_{36}, 9,31-C_{37}diene, and 7,31-C_{37}diene weighted positively on PC2, and mated males tended to have less of these compounds relative to control males (Table S3, Fig. S1). These results also correspond to the comparison of males before and after mating, as all three of these CHCs were shown to significantly decrease in males after mating. Principal component three did not appear to contribute to the separation of mated and control males in any biologically meaningful way. It is interesting to note that five of the CHC compounds identified as major factors in the PCA above (alkatrienes, peaks 14 & 17, and 7-C_{35}ene, 9,31-C_{37}diene, and 9,31-C_{38}diene) were also found to be major discriminant factors explaining genetic variance among inbred lines in the analysis of female CHCs.

Perfuming with CHCs and its effect on mate choice

In mate-choice trials, focal females mated significantly more often with 'novel' males bearing the CHC cues of unrelated, inbred females than with 'familiar' males bearing the cues of inbred sisters (62 'novel' vs. 40 'familiar'; χ^2 test for equal proportions: $\chi^2_1 = 4.75$, $P = 0.029$). There was no significant difference in mate preference for familiar or novel males due to focal female genetic line (line B or H) (χ^2 test of independence: $\chi^2_1 = 0.31$, $P = 0.581$). Moreover, female mate preference for familiar or novel males did not differ with regard to the genetic line from which male CHCs were extracted (lines D, E or G) (χ^2 test of independence: $\chi^2_2 = 0.92$, $P = 0.632$).

Inbreeding avoidance

In mate-choice trials, there was no significant preference for related or unrelated males due to mate-choice treatment (74 related males vs. 56 unrelated males; χ^2 test for equal proportions: $\chi^2_1 = 2.49$, $P = 0.114$). For the full-sib treatment, females showed no significant mating preference for related or unrelated males (39 full-sib vs. 26 unrelated; χ^2 test for equal proportions: $\chi^2_1 = 2.60$, $P = 0.107$). For the half-sib treatment, there was no significant female preference for related males relative to unrelated males (35 half-sib vs. 30 unrelated; χ^2 test for equal proportions: $\chi^2_1 = 0.39$, $P = 0.535$).

DISCUSSION

Our results support the hypothesis that cuticular hydrocarbons facilitate chemosensory self-referencing in crickets. Female CHC profiles exhibited significant additive genetic variation, demonstrating that genetically unique chemical cues are available to support chemosensory self-referencing. Hydrocarbon compounds of similar chemical nature are likely to share a genetic basis, given that many of these compounds are known to share common biochemical pathways during insect lipid production (Howard & Blomquist 2005; Van Homrigh *et al.* 2007). The substantial genetic covariance between many of the CHCs observed in the present study suggests that these compounds are likely to be inherited together, making them more reliable as cues of unique genetic identity for female recognition of 'self' (Falconer & Mackay 1996).

Several features of CHCs combine to make them excellent candidates as insect recognition cues used to distinguish between individuals: chemical stability, low volatility (due to long carbon chain) and a diversity of structures allowing for significant variability in lipid composition (Howard & Blomquist 2005). Insect hydrocarbons typically range from 11 to 43 carbons in length (Howard & Blomquist 2005), whereas those isolated from *G. sigillatus* females ranged from 33 to 41 carbons in length. The long-chain nature of these large lipid compounds suggests that they are more likely to remain stable for longer periods of time after their transfer to males during copulation. We know from previous work that these compounds allow behavioural discrimination of previous mates for at least 24–28 h after initial copulation (Ivy *et al.* 2005).

If females transfer their own CHCs to males during mating thereby facilitating later recognition through chemosensory self-referencing, we predicted that the CHC profiles of males would become more similar to the CHC profiles of females after mating. Consistent with these predictions, SPME analysis of males before and after mating revealed that the chemical profiles of mated males became more similar to those of females after mating for 11 of 17 CHCs analysed. Compounds that were more abundant in females tended to increase in mated males, whereas compounds that were less abundant in females showed a relative decrease in males after mating. This pattern of CHC change is similar to that found in *Drosophila melanogaster* after mating, when males and females show reciprocal variation in CHCs due to mechanical transfer of compounds between males and females during copulation (Scott 1986; Scott *et al.* 1988; Everaerts *et al.* 2010).

In our perfuming trials, we found that external application of female CHCs to males directly affected female mating preferences. In mate-choice trials, focal females given the opportunity to mate with a 'familiar' male bearing the cues of inbred sisters, or a 'novel'

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male bearing the cues of unrelated females, mated significantly more often with 'novel' males. These results show that CHC extracts from the female epicuticle contain sufficient information to facilitate chemosensory self-referencing in decorated crickets. There is also evidence in other species that CHCs are transferred from one individual to another via direct physical contact. Female cockroaches, *Nauphoeta cinerea*, discriminate against potentially sperm-limited males that have had multiple female mating partners, and this effect was also observed when epicuticular rubbings from multiple females were applied to virgin males (Harris & Moore 2005). Male field crickets, *Teleogryllus oceanicus*, respond to the perceived risk of sperm competition by adjusting their ejaculate allocation in response to the number of distinct CHC extracts from individual males present on females (Thomas & Simmons 2009).

In our final experiment, we found no evidence of inbreeding avoidance by females in mate-choice trials. This result suggests that discrimination against previous mates is not simply an incidental consequence of a mechanism that evolved to facilitate inbreeding avoidance. Instead, chemosensory self-referencing based on CHCs likely has evolved as a mechanism to facilitate recognition and discrimination against previous mates.

Earlier studies demonstrating female recognition of previous mates in arthropods have often invoked learning and memory of individual male traits as the basis for mate recognition (Johnson 1977; Linsenmair 1985; Caldwell 1992). Although such a mechanism might be important for mate recognition in monogamous species (Linsenmair 1985), or species with complex social structures (Steiger *et al.* 2008), it may not be as reliable a method of individual mate recognition for polyandrous species such as *Gryllodes sigillatus*. Instead, the kind of simple self-referencing employed by female *G. sigillatus* permit them to identify previous mates without any specialised cognitive abilities. Rather than a female learning the traits of her various partners over the course of her reproductive lifetime, she need only learn her own CHC profile and then assess a male for traces of her own cuticular hydrocarbons (Hauber & Sherman 2001). Alternatively, females may use a form of online-processing that does not require a 'self' recognition template, but simply a comparison of her phenotype to that of a male during any interaction (Hauber & Sherman 2001).

Any benefits to self-referencing will depend on the frequency with which females encounter previous mates. Although the probability that a female will encounter a previous mate in nature is unknown, lifetime measures of male and female mating success of marked individuals in a large outdoor enclosure (Sakaluk *et al.* 2002) suggest that it is can be quite high, at least within 24–48 h of mating. Sakaluk *et al.* (2002) reported that individuals of both sexes typically aggregate in large clusters in only a small minority of the shelters that are available, and that they remain in these shelters most of the day.

The application of DNA profiling techniques to free-living animals in nature has revolutionised our understanding of animal systems, revealing that polyandry is pervasive across all major animal taxa (Zeh & Zeh 2003). However, the proximate mechanisms by which cognitively simple animals such as crickets maximise their opportunities for polyandry remain virtually unknown. The results presented here demonstrate unequivocally that cuticular hydrocarbons provide the proximate basis for chemosensory self-referencing, mediating the female preference for novel mates demonstrated in crickets. Given the pervasiveness of CHCs as recognition cues among arthropods, chemosensory self-referencing via CHCs could be a ubiquitous mechanism by which females across a broad range of animal mating systems increase the diversity of their mating partners.

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AUTHORSHIP

CBW, JH and SKS designed research; CBW, SS, CGH, GDO, CM, JH and SKS collected data; CBW and JH analysed data; CBW, JH and SKS wrote the manuscript.

REFERENCES

Adamo, S.A. & Hoy, R.R. (1994). Mating behaviour of the field cricket *Gryllus bimaculatus* and its dependence on social and environmental cues. *Anim. Behav.*, 47, 857–868.

Archer, M.S. & Elgar, M.A. (1999). Female preference for multiple partners: sperm competition in the hide beetle, *Dermestes maculatus* (DeGeer). *Anim. Behav.*, 58, 669–675.

Arnqvist, G. & Nilsson, T. (2000). The evolution of polyandry: multiple mating and female fitness in insects. *Anim. Behav.*, 60, 145–164.

Bateman, P.W. (1998). Mate preference for novel partners in the cricket *Gryllus bimaculatus*. *Ecol. Entomol.*, 23, 473–475.

Blows, M.W. & Allan, R.A. (1998). Levels of mate recognition within and between two *Drosophila* species and their hybrids. *Am. Nat.*, 152, 826–837.

Bretman, A., Wedell, N. & Tregenza, T. (2004). Molecular evidence of post-copulatory inbreeding avoidance in the field cricket *Gryllus bimaculatus*. *Proc. R. Soc. Lond. B*, 271, 159–164.

Caldwell, R.L. (1992). Recognition, signaling and reduced aggression between former mates in a stomatopod. *Anim. Behav.*, 44, 11–19.

Coyne, J.A., Crittenden, A.P. & Mah, K. (1994). Genetics of a pheromonal difference contributing to reproductive isolation in *Drosophila*. *Science*, 265, 1461–1464.

Daly, M. (1978). The cost of mating. *Am. Nat.*, 138, 771–774.

Dietmann, V., Liebig, J., Hölldobler, B. & Peeters, C. (2005). Changes in the cuticular hydrocarbons of incipient reproductives correlate with triggering of worker policing in the bulldog ant, *Myrmecia gulcosa*. *Behav. Ecol. Sociobiol.*, 58, 486–496.

Eakley, A.L. & Houde, A.E. (2004). Possible role of female discrimination against 'redundant' males in the evolution of colour pattern polymorphism in guppies. *Proc. R. Soc. Lond. B (Suppl)*, 271, S299–S301.

Everaerts, C., Farine, J.-P., Cobb, M. & Ferveur, J.-F. (2010). *Drosophila* cuticular hydrocarbons revisited: mating status alters cuticular profiles. *PLoS ONE*, 5, 1–12.

Falconer, D.S. & Mackay, T.F.C. (1996). *Introduction to Quantitative Genetics*. Longman, Harlow, UK.

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Francis, G. W. & Veland, K. (1981). Alkylthiolation for the determination of double-bond positions in linear alkenes. *J. Chromatogr.*, 219, 379–384.
Gershman, S.N. (2009). Postcopulatory female choice increases the fertilization success of novel males in the field cricket, *Gryllus vocalis*. *Evolution*, 63, 67–72.
Harris, W.E. & Moore, P.J. (2005). Female mate preference and sexual conflict: females prefer males that have had fewer consorts. *Am. Nat.*, 165, S64–S71.
Hauber, M.E. & Sherman, P.W. (2001). Self-referent phenotype matching: theoretical considerations and empirical evidence. *Trends Neurosci.*, 24, 609–616.
Herzner, G., Schmitt, T., Heckel, F., Schreier, P. & Strohm, E. (2006). Brothers smell similar: variation in the sex pheromone of male European Beewolves *Philanthus triangulum* (Hymenoptera:Crabronidae) and its implications for inbreeding avoidance. *Biol. J. Linn. Soc.*, 89, 433–442.
Hosken, D.J., Martin, O.Y., Born, J. & Huber, F. (2003). Sexual conflict in *Sepsis cynipsea*: female reluctance, fertility and mate choice. *J. Evol. Biol.*, 16, 485–490.
Howard, R.W. & Blomquist, G.J. (2005). Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annu. Rev. Entomol.*, 50, 371–393.
Howard, R.W., Jackson, L.L., Banse, H. & Blows, M.W. (2003). Cuticular hydrocarbons of *Drosophila birchii* and *D. serrata*: identification and role in mate choice in *D. serrata*. *J. Chem. Ecol.*, 29, 961–976.
Ivy, T.M. (2007). Good genes, genetic compatibility and the evolution of polyandry: use of the diallel cross to address competing hypotheses. *J. Evol. Biol.*, 20, 479–487.
Ivy, T.M., Weddle, C.B. & Sakaluk, S.K. (2005). Females use self-referent cues to avoid mating with previous mates. *Proc. Roy. Soc. B*, 272, 2475–2478.
Jennions, M.D. & Petrie, M. (2000). Why do females mate multiply? A review of the genetic benefits *Biol. Rev.*, 75, 21–64.
Johnson, V.R. Jr, (1977). Individual recognition in the banded shrimp *Stenopus hispidus* (Olivier). *Anim. Behav.*, 25, 418–428.
Linsenmair, K.E. (1985). Individual and family recognition in subsocial arthropods, in particular in the desert isopod *Hemilepistus reaumuri*. *Fortschr. Zool.*, 31, 411–436.
Mardia, K.V., Kent, J.T. & Bibby, J.M. (1979). *Multivariate Analysis*. Academic Press, London.
Mullen, S.P., Mendelsen, T.C., Schal, C. & Shaw, K.L. (2007). Rapid evolution of cuticular hydrocarbons in a species radiation of acoustically diverse Hawaiian crickets (Gryllidae: Trigonidiinae: *Laupala*). *Evolution*, 61, 223–231.
Nagamoto, J., Aonuma, H. & Hisada, M. (2005). Discrimination of conspecific individuals via cuticular pheromones by males of the cricket *Gryllus bimaculatus*. *Zool. Sci.*, 22, 1079–1088.
Nelson, D.R., Sukkestad, D.R. & Zaylskie, R.G. (1972). Mass spectra of methyl-branched hydrocarbons from eggs of the tobacco hornworm. *J. Lipid Res.*, 13, 413–421.
Ridley, M. (1988). Mating frequency and fecundity in insects. *Biol. Rev.*, 63, 509–549.
Roff, D.A. & Preziosi, R. (1994). The estimation of the genetic correlation: the use of the jackknife. *Heredity*, 73, 544–548.
Ryan, K.M. & Sakaluk, S.K. (2009). Dulling the senses: the role of the antennae in mate recognition, copulation and mate guarding in decorated crickets. *Anim. Behav.*, 77, 1345–1350.
Sakaluk, S.K. (1987). Reproductive behaviour of the decorated cricket, *Gryllodes supplicans* (Orthoptera: Gryllidae): calling schedules, spatial distribution, and mating. *Behaviour*, 100, 202–225.
Sakaluk, S.K., Schaus, J.M., Eggert, A.-K., Snedden, W.A. & Brady, P.L. (2002). Polyandry and fitness of offspring reared under varying nutritional stress in decorated crickets. *Evolution*, 56, 1999–2007.
SAS Institute Inc. (2006). *SAS OnlineDoc® 9.1.3*. SAS Institute, Cary, NC.
Scott, D. (1986). Sexual mimicry regulates the attractiveness of mated *Drosophila melanogaster* females. *Proc. Natl Acad. Sci. USA*, 83, 8429–8433.
Scott, D., Richmond, R.C. & Carlson, D.A. (1988). Pheromones exchanged during mating: a mechanism for mate assessment in *Drosophila*. *Anim. Behav.*, 36, 1164–1173.
Slatyer, R.A., Mautz, B.S., Backwell, P.R.Y. & Jennions, M.D. (2012). Estimating genetic benefits of polyandry from experimental studies: a meta-analysis. *Biol. Rev.*, 87, 1–33.
Steiger, S., Franz, R., Eggert, A.-K. & Müller, J.K. (2008). The Coolidge effect, individual recognition and selection for distinctive cuticular signatures in a burying beetle. *Proc. Roy. Soc. B*, 275, 1831–1838.
Stevens, J. (2002). *Applied Multivariate Statistics for the Social Sciences*. Lawrence Erlbaum Associates, Mahwah, NJ.
Thomas, M.L. & Simmons, L.W. (2008). Sexual dimorphism in cuticular hydrocarbons of the Australian field cricket *Teleogryllus oceanicus* (Orthoptera: Gryllidae). *J. Insect Physiol.*, 54, 1081–1089.
Thomas, M.L. & Simmons, L.W. (2009). Male-derived cuticular hydrocarbons signal sperm competition intensity and affect ejaculate expenditure in crickets. *Proc. Roy. Soc. B*, 276, 383–388.
Tregenza, T. & Wedell, N. (1997). Definitive evidence for cuticular pheromones in a cricket. *Anim. Behav.*, 54, 979–984.
Tregenza, T. & Wedell, N. (2002). Polyandrous females avoid costs of inbreeding. *Nature*, 415, 71–73.
Van Homrigh, A., Higgie, M., McGuigan, K. & Blows, M.W. (2007). The depletion of genetic variance by sexual selection. *Curr. Biol.*, 17, 528–532.
Warthen, J.D. Jr, & Uebel, E.C. (1980). Comparison of the unsaturated cuticular hydrocarbons of male and female house crickets, *Acheta domesticus* (L.) (Orthoptera: Gryllidae). *Insect Biochem.*, 10, 435–439.
Zeh, J.A. & Zeh, D.W. (2003). Toward a new sexual selection paradigm: polyandry, conflict, and incompatibility. *Ethology*, 109, 929–950.
Zeh, J.A., Newcomer, S.D. & Zeh, D.W. (1998). Polyandrous females discriminate against previous mates. *Proc. Natl Acad. Sci. USA*, 95, 13732–13736.

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