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# Sexual selection and experimental evolution of chemical signals in *Drosophila pseudoobscura*

J. HUNT*, R. R. SNOOK†, C. MITCHELL*, H. S. CRUDGINGTON† & A. J. MOORE*‡

*Centre for Ecology and Conservation, College of Life and Environmental Sciences, University of Exeter, Cornwall Campus, Penryn, UK
†Department of Animal and Plant Sciences, University of Sheffield, Sheffield, UK
‡Department of Genetics, University of Georgia, Athens, GA, USA

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## Abstract

Our expectations for the evolution of chemical signals in response to sexual selection are uncertain. How are chemical signals elaborated? Does sexual selection result in complexity of the composition or in altered quantities of expression? We addressed this in *Drosophila pseudoobscura* by examining male and female cuticular hydrocarbons (CHs) after 82 generations of elevated (E) sexual selection or relaxed sexual selection through monogamy (M). The CH profile consisted of 18 different components. We extracted three eigenvectors using principal component analysis that explained 72% of the variation. principal component (PC)1 described the amount of CHs produced, PC2 the trade-off between short- and long-chain CHs and PC3 the trade-off between apparently arbitrary CHs. In both sexes, the amount of CHs produced was greater in flies from the E treatment. PC3 was also higher, indicating that sexual selection also influenced the evolution of CH composition. The sexes differed in all three PCs, indicating substantial sexual dimorphism in this species, although the magnitude of this dimorphism was not increased as a result of our experimental evolution. Collectively, our work provides direct evidence that sexual selection plays an important role in the evolution of CHs in *D. pseudoobscura* and that both increased quantity and overall composition are targeted.

## Introduction

Sexually selected characters come in a diversity of forms, but common features include signalling by stimulating the sensory system of the receiver (West-Eberhard, 1984; Grafen, 1990; Rowe, 1999; Greenfield, 2002) and elaboration of structure or signal (Emlen, 2008; Jones & Ratterman, 2009). Sexually selected signals can be acoustic, visual or olfactory (Darwin, 1871; West-Eberhard, 1984). Most contemporary studies of sexual selection have focused on visual or acoustic signals (Andersson, 1994; Greenfield, 2002; Emlen, 2008; Jones & Ratterman, 2009). Nevertheless, both male–male competition (Moore, 1997; Moore *et al.*, 1997; Martín *et al.*, 2007) and mate choice (Baker & Cardé, 1979; Greenfield, 1981; Arnold & Houck, 1982; Löfstedt, 1993; Svensson, 1996; Moore & Moore, 1999; Chenoweth & Blows, 2003, 2005) have been shown to influence the evolution of pheromones. The relative lack of attention to sexual selection on chemical signals is not surprising given that pheromones were identified just over 60 years ago, and the tools and techniques for identification and analysis are still improving (Wyatt, 2003). Nevertheless, chemical communication is the most primitive form of communication, and it is likely that sexually selected chemical signals are ubiquitous (Wyatt, 2003; d'Ettorre *et al.*, 2008). Some authors have suggested that chemical communication is the last major frontier in the study of animal communication (Wyatt, 2003; Cardé & Millar, 2004; Howard & Blomquist, 2005; Johansson & Jones, 2007; Blomquist & Bagnères, 2010).

A ubiquitous group of chemicals in terrestrial arthropods, known as cuticular hydrocarbons (CHs), are common targets for sexual selection (d'Ettorre *et al.*, 2008).

*Correspondence:* Allen J. Moore, Department of Genetics, University of Georgia, Athens, GA 30602, USA.
Tel.: +1 706 542 4423; fax: +1 706 542 3910;
e-mails: a.j.moore@exeter.ac.uk; ajmoore@uga.edu

CHs are a complex mixture of organic compounds that are deposited as a waxy layer on the surface of the cuticle. Although it is likely that CHs initially evolved to restrict evaporative water loss (Howard & Blomquist, 2005), in insects these compounds often also serve as multicomponent chemical signals that function in a wide range of different social contexts, including discrimination among individuals (Steiger *et al.*, 2008; Guerrieri *et al.*, 2009; vanZweden & d'Ettorre, 2010), species recognition (Noor & Coyne, 1996; Higgie *et al.*, 2000; Blows, 2002; Blows & Higgie, 2002; Ferveur, 2005; Gleason *et al.*, 2005), informing the formation and maintenance of social groups (Greene, 2010; Leibig, 2010), influencing task decisions (Greene & Gordon, 2003) and the overall regulation of social interactions (Kent *et al.*, 2007, 2008; d'Ettorre *et al.*, 2008; Krupp *et al.*, 2008; Leibig, 2010). CHs can be influenced by both mechanisms of sexual selection (inter- and intrasexual selection), influencing mate choice (Hine *et al.*, 2002; Thomas & Simmons, 2009a, 2010) and dominance hierarchies (Roux *et al.*, 2002; Thomas & Simmons, 2009b).

The most complete evidence for the role of CHs in sexual selection comes from studies of two species of *Drosophila*, *D. melanogaster* and *D. serrata*. In *D. melanogaster*, CHs influence sexual recognition and mating with single CH components playing a critical role (Ferveur & Sureau, 1996; Grillet *et al.*, 2006; Kurtovic *et al.*, 2007; Ferveur & Cobb, 2010). As in most sexually selected traits in *D. melanogaster*, CHs are dynamic as well as multicomponent, and the level of expression of CH components changes during courtship and mating (Yew *et al.*, 2009), as well as with age (Everaerts *et al.*, 2010). CH expression by males reflects time of day, social interactions and social group composition (Kent *et al.*, 2007, 2008; Krupp *et al.*, 2008). In *D. serrata*, there is mutual mate choice for CHs, leading to stabilizing sexual selection on females but directional selection on males (Chenoweth & Blows, 2005). Rundle *et al.* (2005) showed that natural selection promoting adaptation to novel food sources produced an evolutionary response in female CHs, as well as female mating preferences, but had a lesser effect on male CHs. Sex-specific responses of CHs suggest that the CHs of male and female *D. serrata* are relatively free to evolve independently, which may be facilitated by the low inter-sexual genetic correlations that exist for CHs in this species (Chenoweth & Blows, 2003). In addition, male *D. serrata* appear to assess female CHs during courtship and rapidly adjust their own profile, suggesting that, as in *D. melanogaster*, the CH profile is a dynamic trait (Petfield *et al.*, 2005). Despite this wealth of knowledge, very little is known about sexual selection on CHs in other *Drosophila* species. For example, *D. pseudoobscura* has served as a model to investigate sexual selection (Markow *et al.*, 1996; Crudgington *et al.*, 2005, 2009; Snook *et al.*, 2005; Anderson *et al.*, 2007; Bacigalupe *et al.*, 2007, 2008), odour is involved in mate choice (Leonard *et al.*, 1974; Leonard & Ehrman, 1976), and CHs influence the interspecific mate recognition (Noor & Coyne, 1996). Yet little is known about the role (if any) of CHs in sexual selection in *D. pseudoobscura*.

Investigating sexual selection on CHs is tractable in *D. pseudoobscura*. Individual components of the CH profile have been identified (Blomquist *et al.*, 1985), although this analysis is limited because samples were of mixed age and sex and from a single isofemale line. There is evidence that CHs play an important role in reducing transcuticular water loss in *D. pseudoobscura*. Toolson (1982) showed that the rate of water loss was greatest with higher proportions of relatively short-chain methyl-branched alkanes and alkadienes and lowest with higher proportions of longer-chain alkanes and alkadienes. More than 50% of this variance in water loss is explained by variation in one CH component, *n*-pentacosadiene. Toolson & Kuper-Simbrón (1989) found qualitatively similar results when they followed the evolutionary progression of CHs in a single isofemale line over a 3-year period, as it adapted to different temperature regimes (17 or 24°C). Given this background, we hypothesized that CHs influence mating success and would therefore respond to sexual selection. It is also of interest to see how sexual selection might influence a trait where natural selection plays a defined role.

In this study, we measure the evolutionary response of male and female CHs in *D. pseudoobscura* after 82 generations of experimental evolution under conditions of relaxed (monogamous, M) and elevated (promiscuous, E) sexual selection. Previous work on these experimental populations found differences in behaviour, reproductive traits and fecundity between these lines (Crudgington *et al.*, 2005, 2010), although this enforced regime of sexual conflict did not result in either pre- or post-zygotic reproductive isolation (Bacigalupe *et al.*, 2007). Additionally, courtship song evolved rapidly with E males initiating song and singing faster than M males (Snook *et al.*, 2005). Although mean mating speed and copulation duration do not differ across treatments, the phenotypic trade-off between these traits is more pronounced in M males (Bacigalupe *et al.*, 2008). Furthermore, although E and M males do not differ in the number or size of sperm produced or relative testis mass, E males have larger accessory glands, greater mating capacity and sire the most offspring during sperm competition (Crudgington *et al.*, 2009; Snook *et al.*, 2010). Given the differences in the opportunity for sexual selection between our treatments, as well as the evolutionary divergence of other traits influencing courtship and mating, we predicted that the quantity and/or composition of CHs would consistently differ between our experimental treatments if CHs are sexually selected. Our results suggest that sexual selection could play an important role in the evolution of CHs in *D. pseudoobscura*.

## Materials and methods

### Experimental relaxation and elevation of sexual selection

We altered the level of female promiscuity in our experimental evolution lines by enforcing two different social contexts that influence sexual selection intensity (M, monogamous, and E, promiscuous mating), with four replicates of each treatment. In M lines, a single male and female were housed together ($n = 80$ each generation), whereas in the E lines, six males were housed with one female ($n = 40$ each generation). The four replicate lines of each treatment were established from an originally wild-caught population. For each generation, within each selection treatment (M or E), females and males were randomly allocated to vials. Within each replicate line of the two treatments, 5-day-old virgin males and females were housed together for 10 days (changing to a second vial after 5 days), after which adults were discarded. Subsequent virgin progeny were collected and used to propagate the next generation (for full details, see Crudgington *et al.*, 2005).

For this study, we sampled the CHs of males and females from each replicate of each treatment after 82 generations of experimental evolution. In total, we sampled the CHs of 15 flies of each sex per replicate line for each treatment ($n = 240$ flies). Flies were 5-day-old virgins when CHs were extracted, and prior to extraction, they were housed in 40-ml vials (containing 5 mL of '*Drosophila* quick mix medium', Blades Biological, UK) according to sex and replicate line at the same density (15 flies per vial), with a total of three vials of each sex per replicate line. For each sex per replicate line, we sampled five flies at random from each vial for the analysis of CHs. This was performed to standardize any effects that either mating or social interactions may have on CH expression.

### Cuticular hydrocarbon extraction and gas chromatography

Cuticular hydrocarbons were extracted from individual flies using HPLC-grade hexane. Flies were completely submerged in 50 $\mu$L of hexane containing 100 ng of pentadecane as an internal standard. The samples were left to soak for 4 min and then vortexed for a further minute before the fly was removed.

We injected 1 $\mu$L of sample of this extract into a GC-MS (Agilent 7890A GC coupled with an Agilent 5975B Mass spectrometer and CTC autosampler) fitted with a DB-1ms column of 30 m × 0.25 mm internal diameter using helium as a carrier gas. We set the inlet at 250 °C and the injection in pulsed splitless mode. We optimized the separation of the extract using a column profile which began at 70 °C for 1 min, rising at 20 °C/min to 180 °C, then 4 °C/min to 220 °C and finally 15 °C/min to 320 °C where it was held for 2 min. We set the MS transfer line at 280 °C. The electron-impact mass spectra (EI-MS) were recorded with an ionization voltage of 70 eV and a source temperature of 230 °C.

We calculated the abundance of each CH using MSD CHEMSTATION software (version E.02.00.493; Agilent Technologies) as the area under the peak on the chromatograph, using ion 57 as the target ion (Fig. 1). Our analysis identified 18 unique CHs (Table 1). The molecular formula of each CH is based on the mass spectrum and molecular ion, indicating whether a compound is saturated or has one or two double bonds. The majority of CHs were identified using NIST library matches. A few saturated CHs were identified based on having the same formula as those in the study by Blomquist *et al.* (1985), and a few unsaturated CHs were identified based on those in the study by Noor & Coyne (1996). We calculated Kováts retention index for CHs using the formula in the study by Majlát *et al.* (1974) and determined the position of double bonds of alkadienes using the protocol outlined in the study by Howard *et al.* (2003). It is not known whether the unnamed unsaturated CHs in Table 1 are straight-chain hydrocarbons or exhibit a degree of branching.

### Body size

In *D. pseudoobscura*, females are bigger than males, although the size has not changed in our experimental lines, and there are no differences among replicates (Snook *et al.*, 2010). Nevertheless, we measured the body size of 5 flies of each sex per replicate in each of the sexual selection treatments ($n = 80$) to determine whether changes in body size might explain the evolution of CHs. We measured the length of the first

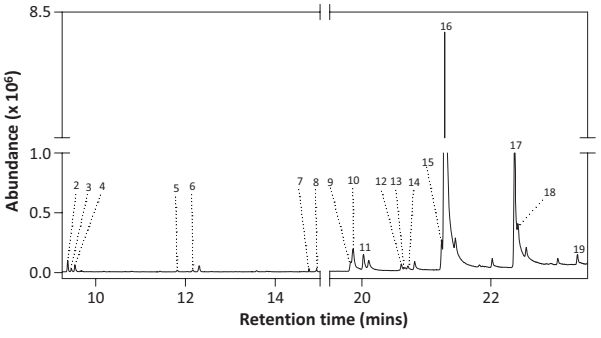


**Fig. 1** A chromatograph of a typical cuticular hydrocarbons (CH) profile of a male *Drosophila pseudoobscura*. All peaks were present in each sex, but in different relative amounts. Peak 1 is not shown but in the internal standard. The numbers correspond to the compounds listed in Table 1. The break in the retention time between 15 and 19.5 min reflects a time period where no CH components were recovered.

**Table 1** The identification of the 18 cuticular hydrocarbon compounds in male and female *Drosophila pseudoobscura* (see Fig. 1) and their relative contribution, expressed as the mean percentage (±SE) of the total abundance of all peaks, to show the degree of sexual dimorphism in cuticular hydrocarbons (CHs). The peak numbers correspond with those presented on the chromatogram in Fig. 1 RT is the retention time (measured in minutes) for a given CH component, and KRI is the Kováts retention index calculated according to Majlát *et al.* (1974) in comparison against *n*-alkane standards ($C_7$–$C_{40}$).

| Peak | RT | Formula | KRI | Identification | % in males | % in females |
|---|---|---|---|---|---|---|
| 2 | 9.380 | $C_{15}H_{28}O_2$ | 1680 | Methyl tetradecenoate* | 0.14 ± 0.01 | 0.10 ± 0.00 |
| 3 | 9.465 | $C_{17}H_{36}$ | 1697 | Alkane | 0.49 ± 0.02 | 0.30 ± 0.01 |
| 4 | 9.539 | $C_{15}H_{30}O_2$ | 1704 | Methyl tetradecanoate* | 0.11 ± 0.01 | 0.08 ± 0.01 |
| 5 | 11.816 | $C_{17}H_{32}O_2$ | 1881 | 9-Hexadecenoic acid* | 0.05 ± 0.00 | 0.04 ± 0.00 |
| 6 | 12.170 | $C_{17}H_{34}O_2$ | 1905 | Hexadecanoic acid* | 0.09 ± 0.01 | 0.07 ± 0.00 |
| 7 | 14.755 | $C_{19}H_{34}O_2$ | 2067 | 9,12-Octadecadienoic acid* | 0.05 ± 0.00 | 0.04 ± 0.00 |
| 8 | 14.924 | $C_{19}H_{36}O_2$ | 2076 | 9-Octadecenoic acid* | 0.06 ± 0.00 | 0.04 ± 0.00 |
| 9 | 19.843 | $C_{25}H_{52}$ | 2462 | 2-Methyl tetracosane† | 2.20 ± 0.04 | 1.57 ± 0.03 |
| 10 | 19.865 | $C_{25}H_{48}$ | 2467 | 5,9 Pentacosadiene‡ | 15.76 ± 0.77 | 1.77 ± 0.09 |
| 11 | 20.029 | $C_{25}H_{50}$ | 2487 | 8-Pentacosene§ | 3.14 ± 0.08 | 1.78 ± 0.05 |
| 12 | 20.611 | $C_{26}H_{50}$ | 2567 | 5,9-Hexacosadiene§ | 0.44 ± 0.01 | 0.41 ± 0.01 |
| 13 | 20.675 | $C_{26}H_{50}$ | 2576 | 3,9-Hexacosadiene§ | 0.26 ± 0.01 | 0.21 ± 0.01 |
| 14 | 20.712 | $C_{26}H_{52}$ | 2583 | 9-Hexacosene§ | 0.61 ± 0.02 | 0.38 ± 0.01 |
| 15 | 21.241 | $C_{27}H_{56}$ | 2663 | 2-Methylhexacosane† | 6.21 ± 0.18 | 4.85 ± 0.14 |
| 16 | 21.289 | $C_{27}H_{52}$ | 2671 | (Z-Z)-5,9 Heptacosadiene‡ | 36.58 ± 0.78 | 47.67 ± 0.43 |
| 17 | 22.369 | $C_{29}H_{60}$ | 2862 | 2-Methyl octacosane† | 23.59 ± 0.34 | 27.58 ± 0.32 |
| 18 | 22.412 | $C_{29}H_{56}$ | 2872 | 5,9-Nonacosadiene§ | 8.32 ± 0.17 | 10.41 ± 0.16 |
| 19 | 23.344 | $C_{31}H_{64}$ | 3062 | 2-Methyltriacontane† | 1.91 ± 0.05 | 2.71 ± 0.06 |

*Methyl esters.
†Identification based on Blomquist *et al.* (1985).
‡Identification based on Noor & Coyne (1996).
§Position of double bonds determined using the methods outlined in the study by Howard *et al.* (2003).

posterior cell of the right wing, taken from the anterior cross-vein (the junction of the longitudinal vein III) to the distal tip (border of the wing) of vein III, as an index of body size. We placed the flies in 100% ethanol and removed the wing with fine forceps. We then mounted the wing on a slide with 25 $\mu$L of Hoyer's solution and a coverslip, which was then allowed to dry at room temperature for 24 h. We captured an image of the wing using a PixeLINK digital camera (PL-A662) attached to a Leica MZ6 dissecting microscope (Leica Microsystems GmbH, Wetzlar, Germany) and measured the length of the wing vein using IMAGEJ software (version 1.43, http://rsbweb.nih.gov/ij).

### Statistical analysis

Prior to analysis, we divided the abundance of each CH peak by the abundance of the internal standard in the sample, and the resulting value was $\log_{10}$-transformed (to create a log contrast for each CH peak) to achieve a normal distribution. We used principal component (PC) analysis to reduce the dimensionality of this data set, and the PCs were extracted on males and females together (using the correlation matrix) to ensure that PC scores were directly comparable across the sexes. PC analysis was necessary due to the occurrence of strong correlations between many of the individual CHs in our data set (65% of correlations exceeded 0.60). We calculated mean PC scores for each sex and replicate line per treatment and analysed these scores using a multivariate analysis of covariance (MANCOVA) that included treatment and sex as main effects in the model, as well as their interaction, and body size as a covariate. We then used univariate ANCOVAs to determine which PCs contributed to the overall multivariate effect. We found that model reduction (i.e. removal of treatment × sex) did not alter the results, so we present the complete model. As a further check for the contributions of body size to differences in CHs, we calculated the mean wing length for each sex and replicate line per treatment to examine whether there were any differences in body size between sexual selection treatments and across the sexes using a 2-factor ANOVA that included treatment and sex as main effects, as well as their interaction. The main question addressed here was whether sex differences in body size depended on the line examined. All analyses were conducted in JMP (version 8, SAS Institute Inc., Cary, NC, USA).

## Results

Our principal component analysis revealed three PCs with eigenvalues exceeding one, which collectively explained 72% of the variation in the CHs of

*D. pseudoobscura* (Table 2). PC1 explained 43% of the variation in CHs and was positively loaded to each CH peak (Table 2), suggesting that this vector describes the absolute amount of CH produced. PC2 explained a further 16% of the variation in CHs and was positively loaded to seven compounds (peaks 2, 4, 5, 6, 7, 8 and 10) and negatively loaded to five compounds (peaks 12 16, 17, 18 and 19) (Table 2). Based on the retention times of these peaks (Table 1), this vector describes the trade-off between long- and short-chain CHs (Table 2), with the majority of the latter consisting of methyl esters (Table 1). PC3 explains the remaining 13% of the variation in CHs and was positively loaded to five compounds (peaks 3, 9, 10, 11 and 14) and negatively loaded to six compounds (peaks 5, 6, 7 17, 18 and 19) (Table 2). Consequently, this vector also describes a trade-off between CHs components, but this is unrelated to carbon chain length or functional grouping.

Our MANCOVA revealed a significant overall effect of sexual selection treatment (M or E) and sex on the mean PC scores describing the variation in CHs, but there was not a significant interaction between these main effects nor was there a significant effect of body size on CHs (Table 3). This latter finding demonstrates that the differences we observe in CHs across our treatments and the sexes are not simply the result of variation in size. Furthermore, while the body size of females are larger ($F_{1,12} = 67.016$, $P = 0.0001$), the increase in the amount of CHs produced was not due to either females or males from the E treatment being larger than M (treatment: $F_{1,12} = 1.099$, $P = 0.315$; treatment x sex: $F_{1,12} = 0.078$, $P = 0.785$).

Univariate ANCOVAs showed that the overall multivariate effect of sexual selection treatment on CHs was due to changes in PC1 and PC3, but not PC2, across treatments (Fig. 2, Table 3). In both sexes, PC1 was higher in the E treatment, indicating that elevated levels of sexual selection have resulted in the evolution of increased CH production (Fig. 2a, Table 3). Likewise, PC3 was higher in the E treatment, suggesting that elevated levels of sexual selection also influenced the evolution of CH composition (Table 3), with flies in this treatment producing higher levels of peaks 3, 9, 10, 11 and 14 and lower levels of peaks 5, 6, 7, 17, 18 and 19 relative to those from the M treatment (Fig. 2c).

Univariate ANCOVAs showed that the overall multivariate effect of sex on CHs was due to changes in all three PCs, indicating substantial sexual dimorphism in both the amount and composition of CHs produced in this species (Table 3). Irrespective of the sexual selection treatment, females produced more CHs than males (Fig. 2a), likely reflecting their larger size than males. Compared with males, females also produced more long- and less short-chain CHs (Fig. 2b), as well as

**Table 2** Principal component (PC) analysis of cuticular hydrocarbons (CHs) in *Drosophila pseudoobscura* identified in Table 1 and Fig. 1 We began numbering with peak 2, as peak 1 was the internal standard. We have retained PCs with an eigenvalue greater than one in our subsequent analyses, and we interpret factor loadings > 0.25 as biologically significant (in boldface) (Tabachnick & Fidell, 1989).

| | PC1 | PC2 | PC3 |
|---|---|---|---|
| Eigenvalue | 7.735 | 2.931 | 2.251 |
| % variance | 42.971 | 16.285 | 12.505 |
| Loading | | | |
| Peak 2 | **0.570** | **0.529** | −0.133 |
| Peak 3 | **0.450** | 0.212 | **0.382** |
| Peak 4 | **0.372** | **0.676** | −0.182 |
| Peak 5 | **0.309** | **0.586** | **−0.332** |
| Peak 6 | **0.388** | **0.601** | **−0.257** |
| Peak 7 | **0.294** | **0.526** | **−0.263** |
| Peak 8 | 0.204 | **0.725** | −0.152 |
| Peak 9 | **0.860** | −0.131 | **0.275** |
| Peak 10 | 0.167 | **0.365** | **0.827** |
| Peak 11 | **0.761** | 0.144 | **0.521** |
| Peak 12 | **0.848** | **−0.272** | 0.056 |
| Peak 13 | **0.781** | −0.109 | 0.138 |
| Peak 14 | **0.786** | −0.001 | **0.417** |
| Peak 15 | **0.855** | −0.179 | 0.154 |
| Peak 16 | **0.889** | **−0.265** | −0.197 |
| Peak 17 | **0.843** | **−0.302** | **−0.343** |
| Peak 18 | **0.831** | **−0.265** | **−0.340** |
| Peak 19 | **0.693** | **−0.351** | **−0.513** |

**Table 3** Multivariate analysis of covariance (MANCOVA) examining the effect of sexual selection treatment and sex, plus their interaction, and body size on the cuticular hydrocarbons (CH) profile of experimental lines of *Drosophila pseudoobscura*. We also provide univariate ANOVAs for each principal component to aid the interpretation of the overall multivariate effect.

| | MANCOVA | | |
|---|---|---|---|
| | Wilks' $\lambda$ | $F_{3,9}$ | $P$ |
| Treatment (A) | 0.333 | 6.022 | 0.016 |
| Sex (B) | 0.093 | 29.373 | 0.0001 |
| A × B | 0.863 | 0.475 | 0.707 |
| Body size | 0.619 | 1.847 | 0.209 |
| | Univariate ANCOVAS | | |
| | | $F_{1,11}$ | $P$ |
| Treatment (A) | PC1 | 5.330 | 0.041 |
| | PC2 | 0.012 | 0.915 |
| | PC3 | 17.445 | 0.002 |
| Sex (B) | PC1 | 9.148 | 0.012 |
| | PC2 | 5.598 | 0.037 |
| | PC3 | 14.469 | 0.003 |
| A × B | PC1 | 0.028 | 0.870 |
| | PC2 | 0.005 | 0.943 |
| | PC3 | 0.889 | 0.366 |
| Body size | PC1 | 0.314 | 0.587 |
| | PC2 | 0.853 | 0.375 |
| | PC3 | 4.352 | 0.061 |

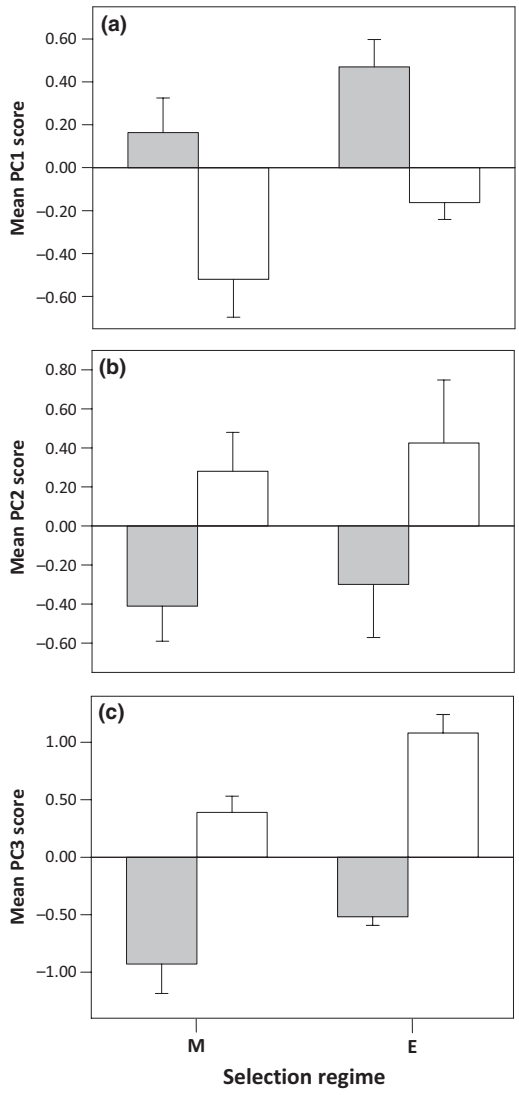


**Fig. 2** Mean (±SE) principal component (PC) scores for male (white bars) and female (grey bars) *Drosophila pseudoobscura* from the monogamous (M) and elevated promiscuity (E) sexual selection lines. (a) PC1, (b) PC2 and (c) PC3.

more of peaks 5, 6, 7, 17, 18 and 19 and less of peaks 3, 9, 10, 11 and 14 (Fig. 2c).

## Discussion

Signalling is at the heart of sexual selection (West-Eberhard, 1984; Grafen, 1990; Andersson, 1994; Rowe, 1999; Greenfield, 2002), and chemical signals influencing social interactions are ubiquitous (Wyatt, 2003; Cardé & Millar, 2004; Howard & Blomquist, 2005; Johansson & Jones, 2007; d'Ettorre *et al.*, 2008; Blomquist & Bagnères, 2010). Yet we are only beginning to grasp the complexity of chemical signals and the role they play in sexual selection. Here, we measured the evolutionary response of male and female CHs in *D. pseudoobscura* after 82 generations of experimental evolution under conditions of relaxed and enhanced sexual selection. We predicted that if CHs were a chemical signal that plays an important role in sexual selection, we would observe differences in the quantity and/or composition of CHs between our sexual selection treatments and between sexes. In agreement with this prediction, we found consistent evolutionary responses in both the amount and composition of CHs produced by the sexes across our sexual selection treatments. Qualitative aspects of the CHs important in desiccation resistance, presumably under natural selection, showed less change. Thus, sexual selection appears to target CHs likely to have an important function in sexual signalling in *D. pseudoobscura*.

We identified an evolutionary response in two principle components reflecting variation in CHs: PC1, which reflects a quantitative change, and PC3, which reflects a qualitative change in components. PC1 was positively loaded by each CH component and therefore reflected the absolute amount of CHs produced. In both sexes, PC1 scores were higher in E than in M lines, suggesting that a history of more intense sexual selection has resulted in the evolution of increased CH production. This result would be consistent with CHs playing an important role in sexual signalling if an overall increase in CHs is easier to detect or generates a greater degree of stimulation to chemosensory receptors (e.g. Inoshita *et al.*, 2011). Alternatively, in *D. melanogaster*, CHs are linked to the production of fatty acid precursors (Pennanec'h *et al.*, 1997), which are also known to play an important role in egg (e.g. Parra-Peralbo & Culi, 2011) and sperm (e.g. Chao & Xun, 2012) production in this species. It is therefore possible that our selection regime favoured was for homoeostatic or physiological traits influencing gamete production, which also indirectly caused changes in CHs across lines. The exact nature of the change we observe is difficult to determine without further experiments. It could be that either M lines or E lines changed or both did, but only manipulative experiments and comparison to an unselected population will begin to answer this. Nevertheless, given the elevated sexual selection in our experimental evolution lines, it is parsimonious to suggest that the signalling function increased in flies from our E lines. In contrast, PC3 described an aspect of CH composition characterized by trade-offs between specific CH components (Table 1). We found that both E males and E females, but particularly E males, produced more CHs and a CH composition consisting of higher levels of peaks 3, 9, 10, 11 and 14 and lower levels of peaks 5, 6, 7, 17, 18 and 19 (see Table 1 for chemical identity of these peaks) relative to flies evolving under enforced monogamy. There is no obvious functional characterization for PC3 based on the identity of these CHs (i.e. there is

no consistent categorization for the whole), although the largest change was in *5,9*-pentacosadiene, which is implicated in reducing evaporative water loss (Toolson, 1982). This trades-off with the longest chain hydrocarbons, perhaps indicating an increase in signalling at the cost of increased evaporative water loss.

We did not observe an evolutionary response across treatments for PC2, which has a clearer potential functional interpretation. This eigenvector described the trade-off between short- and long-chain CHs, a trade-off that has been previously shown to reduce evaporative water loss across the cuticle in *D. pseudoobscura* (Toolson, 1982; Toolson & Kuper-Simbrón, 1989). This function is likely to be under stabilizing natural selection. When reared at 24 °C, flies produce a greater proportion of long-chain CHs, which reduces water loss compared to flies reared at 17 °C (Toolson, 1982). One of the major CH components, *n*-pentacosadiene, that explained over 50% of the variance in water loss in *D. pseudoobscura* (Toolson, 1982) was heavily weighted to PC2 in our study (identified as 5,9 pentacosadiene in Table 1). More generally, an increase in the relative abundance of long-chain CHs appears a common adaptation to desiccation in *Drosophila* (Gibbs *et al.*, 1997; Savarit & Ferveur, 2002). A study by Frentiu & Chenoweth (2010) showed the expression of short- and long-chain CHs varied along a latitudinal and temperature gradient on the east coast of Australia for *D. serrata* and *D. melanogaster*. Consequently, given that we minimized any environmental differences across treatments and replicates, this lack of an evolutionary response that we observe in PC2 is likely to reflect continued stabilizing natural selection.

Field populations of *D. pseudoobscura* are known to be polyandrous (Cobbs, 1977; Pruzan-Hotchkiss *et al.*, 1981), and the E treatment was set up to elevate competition for mates relative to the natural mating system of this species. The M line relaxes sexual selection. Therefore, two nonmutually exclusive explanations to explain the observed changes in CHs between our experimental populations exist. First, we can hypothesize that the CH evolution pattern observed is a consequence of reduced CH production in the M treatment. Such reduction would be expected if CHs were costly to produce (e.g. Chase *et al.*, 2002; Kent *et al.*, 2007) or maintain (e.g. Roux *et al.*, 2002; Smith *et al.*, 2009), as any benefits of sexually selected signalling with CHs should be greatly reduced under enforced monogamy. This explanation reflects the potential conflict between natural selection and sexual selection on CHs, where the dual functions of protection and signalling occur. Second, the pattern may be explained by increased production of specific CHs in the E populations. Such an increase may occur if the effectiveness of the specific combinations of CHs influenced by sexual selection reflects their relative amounts. Despite advances in molecular and neural characterization of pheromone perception in *Drosophila* (Ramdya & Benton, 2010), the mechanism that could facilitate such a change is not yet clear. One possibility is that sensory neurons measure the difference in chemical signals between two receptors, in much the same way that eyes perceive colour (the visual equivalent of CH blends) (Solomon & Lennie, 2007). These experimental lines may be used to shed some light into how perception influences the evolution of signal composition as distinguishing the possibilities will require further research.

Although our study (as with Toolson, 1982; Toolson & Kuper-Simbrón, 1989) suggests that there are no sex-specific CH compounds expressed in *D. pseudoobscura*, we nevertheless found extensive sexual dimorphism in both the quantity and composition of CHs produced. Females produced, on average, more CHs than males (Fig. 2a), which is not surprising given that females are the larger sex in this species. Compared to males, females were characterized by more negative PC2 (naturally selected CHs) scores, indicating the production of relatively more long- and less short-chain CHs (Fig. 2b). These findings suggest that females are more likely to be resistant to the effects of desiccation than males due to less surface area, overall more CHs and relatively more long-chain CHs. A similar pattern of sex-specific CH expression was also shown in populations of *D. melanogaster* selected for resistance to desiccation with females producing more longer-chain CHs and having greater desiccation resistance than males (Gibbs *et al.*, 1997). Given the other changes we see, it may be possible to investigate the trade-offs between natural and sexual selection acting on CH in this species.

We also found that females produced more of peaks 5, 6, 7, 17, 18 and 19 and less of peaks 3, 9, 10, 11 and 14 than males. At this stage, we have no clear reason why the sexes differ in the expression of these particular CH components. However, given that CHs presumably under natural selection (i.e. PC2) did not differ between treatments, we hypothesize that these CH differences between the treatments – and particularly those of E males – reflect their role in sexual selection. Future research should address the functional significance of these sexually dimorphic differences to uncover the role of these CHs in sexual selection.

Despite showing clear sexual dimorphism in all three PCs, the lack of a significant treatment by sex interaction demonstrates that the magnitude of sexual dimorphism did not differ across our sexual selection treatments. This suggests that sexual dimorphism in CH expression was pre-existing in our base population. In fact, Toolson & Kuper-Simbrón (1989) show sexual dimorphism in a number of CH components in field-collected flies, although they examined fewer CH components. Thus, our treatment did not result in the evolution of greater sexual dimorphism. This may

reflect genetic correlations or constraints on further evolution, or may simply reflect too few generations for further (statistically significant) sexual dimorphism to evolve. Genetic studies are required for a resolution of this point.

In conclusion, both quantity and composition of chemical signals can evolve in response to sexual selection. Using experimental evolution, we cannot determine which mechanism of sexual selection, male–male competition or female mate choice or both, might be involved. Nevertheless, we found a consistent evolutionary change in both the amount and composition of CHs in *D. pseudoobscura* in response to experimental manipulation of the intensity of sexual selection but no change in sexual dimorphism. Although additional work is needed to identify the exact mechanism (s) that have produced these evolutionary responses, our work adds to the modest but growing list of empirical studies showing that chemical signals are likely to play an important role in sexual selection.

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