RESEARCH ARTICLE

Male-limited evolution suggests no extant intralocus sexual conflict over the sexually dimorphic cuticular hydrocarbons of *Drosophila melanogaster*

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Abstract

Sexually dimorphic traits are likely to have evolved through sexually antagonistic selection. However, recent empirical data suggest that intralocus sexual conflict often persists, even when traits have diverged between males and females. This implies that evolved dimorphism is often incomplete in resolving intralocus conflict, providing a mechanism for the maintenance of genetic variance in fitness-related traits. We used experimental evolution in *Drosophila melanogaster* to directly test for ongoing conflict over a suite of sexually dimorphic cuticular hydrocarbons (CHCs) that are likely targets of sex-specific selection. Using a set of experimental populations in which the transmission of genetic material had been restricted to males for 82 generations, we show that CHCs did not evolve, providing experimental evidence for the absence of current intralocus sexual conflict over these traits. The absence of ongoing conflict could indicate that CHCs have never been the target of sexually antagonistic selection, although this would require the existing dimorphism to have evolved via completely sex-linked mutations or as a result of former, but now absent, pleiotropic effects of the underlying loci on another trait under sexually antagonistic selection. An alternative interpretation, and which we believe to be more likely, is that the extensive CHC sexual dimorphism is the result of past intralocus sexual conflict that has been fully resolved, implying that these traits have evolved genetic independence between the sexes and that genetic variation in them is therefore maintained by alternative mechanisms. This latter interpretation is consistent with the known roles of CHCs in sexual communication in this species and with previous studies suggesting the genetic independence of CHCs between males and females. Nevertheless, direct estimates of sexually antagonistic selection will be important to fully resolve these alternatives.

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Introduction

Pheromones and other signalling chemicals have long been recognized as important media for communication between individuals, from microbes to plants and animals. Among many examples, moths are known to have extremely sensitive chemical mate-attraction systems and social insects use pheromones to create food trails, signal alarm and to regulate caste development and behaviour. Recent technological developments have allowed ever-finer resolution of the nature x and abundance of pheromones and their effects, revealing extraordinary complexity in some cases. The epicuticle of *Drosophila* provides an example: once characterized as a

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waxy layer involved in waterproofing and with a composition that was fixed within an individual upon emergence as an adult, it is now also recognized as a complex and dynamic organ of communication (Ferveur 2005; Kent *et al.* 2008).

Although a recent study (Everaerts *et al.* 2010) detected 59 different cuticular compounds in male and female *Drosophila melanogaster*, the best known and most abundant of these are the cuticular hydrocarbons (CHCs, or CHs, depending upon author). CHCs are long chain, nonvolatile carbon compounds that are likely targets of both natural and sexual selection. With respect to natural selection, CHCs have waterproofing properties (Nelson 1993) and their variation has been associated with desiccation resistance in *Drosophila* and other insects (Toolson and Kupersimbron 1989; Gibbs 1998; Howard and Blomquist 2005). In *D. melanogaster* in particular, natural selection on CHCs in relation to desiccation has been demonstrated via laboratory experimental evolution (Gibbs *et al.* 1997; Kwan and Rundle 2010) and through the analysis of clinal variation in nature (Rouault *et al.* 2000).

CHCs also play the role of contact pheromones in male–female chemical communication and are involved in *D. melanogaster* species recognition (Coyne *et al.* 1994; Billeter *et al.* 2009), intraspecific group recognition (Fang *et al.* 2002), and sex recognition (Savarit and Ferveur 2002; Billeter *et al.* 2009). Rybak *et al.* (2002) demonstrated that male chemical signals act in synergy with acoustic signals to stimulate females, and Grillet *et al.* (2006) investigated the influence of a specific CHC, 7-tricosene (i.e. (Z)-7-$C_{23:1}$), on male mating success using mutants and a phenotypic manipulation known as 'perfuming'. Thus, although direct estimates of selection gradients on males arising from female choice have not been performed in an integrated, multivariate framework, CHCs are likely targets of sexual selection via mate preferences in *D. melanogaster*, as has been shown in two other *Drosophila* species: *D. serrata* (Chenoweth and Blows 2003, 2005) and *D. bunnanda* (Van Homrigh *et al.* 2007).

CHC profiles in *D. melanogaster* are highly sexually dimorphic, differing quantitatively in the relative concentrations of various shared CHCs and qualitatively in the chemical identity of some of them (figure 1; Foley *et al.* 2007). This dimorphism suggests a history of sex-specific selection and possible sexual conflict. This is because persistent sexually antagonistic selection is expected to favour the evolution of mechanisms for the resolution of conflict, including sex-specific gene regulation, sex linkage, gene duplication followed by sex limitation, parental imprinting, and sex-specific maternal effects (Bonduriansky and Chenoweth 2009; Svensson *et al.* 2009). These mechanisms serve to increase the independence of trait expression in males and females, lowering the intersex genetic correlation for the trait and thereby permitting the evolution of sexual dimorphism. Sexually dimorphic traits are therefore commonly recognized as strong candidates of past, and possibly current, sexually antagonistic selection (Bedhomme and Chippindale 2007; Bonduriansky and Chenoweth 2009; Cox and Calsbeek 2009).

Consistent with the expected response to persistent sexual conflict, several mutations have been identified in *D. melanogaster* that appear to affect the synthesis of one or several CHCs in a sex-specific manner (Fang *et al.* 2002; Ferveur and Jallon 1993; Dallerac *et al.* 2000; Wicker-Thomas and Jallon 2000), indicating some independent genetic control between males and females. A recent quantitative genetic analysis (Foley *et al.* 2007) also identified 25 QTL influencing CHC abundance in females and 15 in males, yet found no evidence of QTL co-localization for shared hydrocarbons between sexes, suggesting a substantial degree of independent genetic control. However, whether intralocus conflict persists or has been fully resolved cannot be determined from the existence of dimorphism alone (Bedhomme and Chippindale 2007; Bonduriansky and Chenoweth 2009; Cox and Calsbeek 2009). Both the inherent complexity of the epicuticle and the limitations of QTL analysis make it desirable to take an experimental approach.

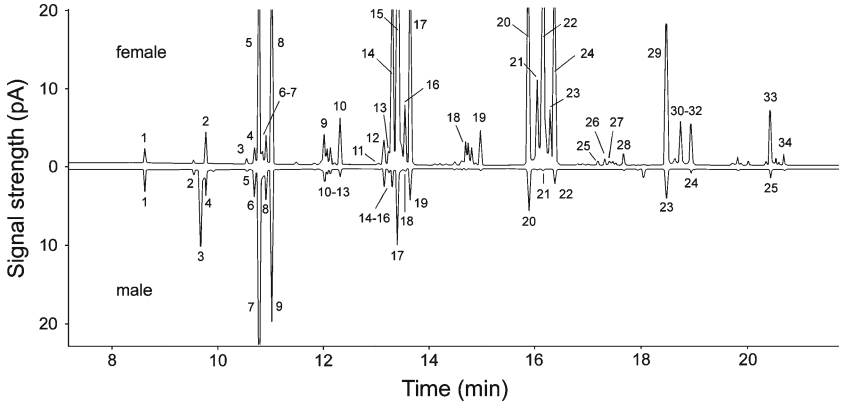

Figure 1. Mirrored gas chromatograph traces of cuticular hydrocarbons of a female (upper) and a male (lower) *Drosophila melanogaster*. Labels indicate the CHCs integrated in the current study. Chemical identities are given in tables 1 and 2.

Table 1. Analysis of the differences between the CHC profiles of ML and C males.

Label	CHC	*F*	*P*	C mean proportion (± s.e.)	ML mean proportion (± s.e.)
M1	C_{21}	2.24	0.231	1.466 (0.036)	1.375 (0.029)
M2	(Z)-9-$C_{22:1}$	0.13	0.742	0.444 (0.009)	0.442 (0.009)
M3	(Z)-7-$C_{22:1}$	0.39	0.575	14.284 (0.641)	14.420 (0.465)
M4	C_{22}	2.32	0.225	2.832 (0.134)	2.988 (0.130)
M5	2-Me-C_{22}	0.19	0.693	0.178 (0.007)	0.168 (0.005)
M6	(Z)-9-$C_{23:1}$	1.39	0.323	2.565 (0.051)	2.456 (0.041)
M7	(Z)-7-$C_{23:1}$	1	0.391	34.518 (0.351)	35.003 (0.322)
M8	**(Z)-5-$C_{23:1}$**	**13.04**	**0.037**	**2.577 (0.031)**	**2.522 (0.048)**
M9	C_{23}	0.02	0.900	11.153 (0.222)	11.080 (0.189)
M10	(Z)-9-$C_{24:1}$	0.68	0.471	1.224 (0.029)	1.277 (0.021)
M11	(Z)-7-$C_{24:1}$	4.33	0.129	0.427 (0.005)	0.444 (0.005)
M12	(Z,Z)-5,9-$C_{24:2}$	0	0.964	0.664 (0.012)	0.666 (0.010)
M13	C_{24}	0.28	0.633	0.601 (0.015)	0.614 (0.012)
M14	2-Me-C_{24}	0.01	0.917	2.184 (0.077)	2.184(0.067)
M15	(Z,Z)-7,11-$C_{25:2}$	0.4	0.574	0.422 (0.033)	0.399 (0.030)
M16	(Z)-9-$C_{25:1}$	0.42	0.562	1.591(0.046)	1.652 (0.041)
M17	(Z)-7-$C_{25:1}$	0.51	0.528	8.169 (0.201)	8.282 (0.186)
M18	(Z)-5-$C_{25:1}$	2.11	0.242	0.228 (0.009)	0.221 (0.009)
M19	C_{25}	0.39	0.578	2.489 (0.070)	2.552 (0.068)
M20	2-Me-C_{26}	0.16	0.713	5.471 (0.158)	5.214 (0.113)
M21	(Z)-7-$C_{27:1}$	0.5	0.530	0.279 (0.031)	0.229 (0.008)
M22	C_{27}	0.16	0.713	1.322 (0.038)	1.316 (0.028)
M23	2-Me-C_{28}	1.04	0.384	3.760 (0.099)	3.467 (0.067)
M24	C_{29}	0.25	0.650	0.406 (0.015)	0.377 (0.011)
M25	2-Me-C_{30}	8.5	0.062	0.747 (0.018)	0.651 (0.016)

Results of mixed linear models (equation 1) testing for differences in proportionate CHCs between the ML and C genotypes when expressed in males. *P* values derive from *F* tests with 1,3 degrees of freedom in each case, uncorrected for multiple comparisons. Values in bold indicates the compound which is significantly different between ML and C before multiple test correction. The last two columns give the mean proportion (± s.e.) of each compound in each selection treatment.

We set out to assess the extent to which intralocus sexual conflict occurs for CHCs in an outbred laboratory population of *D. melanogaster*. For this, we used a manipulative evolution experiment to look for changes in CHCs in replicate populations (derived from a common ancestor) in which the potential constraint on male CHC evolution imposed by antagonistic selection on females was removed using the male-limited (ML) evolution technique developed by Rice (1996, 1998). ML evolution utilizes special genetic constructs in *D. melanogaster* females to limit the transmission of genomic haplotypes (the X and all major autosomes) from father to son as if they were single, ML chromosomes. The effect is to entirely remove any female-specific selection and, consequently, any sexual conflict. Our previously described experiment (Prasad *et al.* 2007) involved four populations in which genomes were restricted to males, each paired to its own control population. The ML selection regime led to higher fitness males, relative to controls, with an associated decrease in the fitness of daughters experimentally expressing the ML-evolved chromosomes (Prasad *et al.* 2007). Male fitness gains were achieved by an increase in male mating success (Bedhomme *et al.* 2008) and not through improved sperm competition (Jiang *et al.* 2011). Indeed, the expression of ML genomes in males and females affected courtship behaviour (Bedhomme *et al.* 2008): males expressing the ML genomes showed a two-fold reduction in the intensity of courtship compared to control males but acquired the same number of matings, whereas females expressing the ML genomes elicited less intense courtship from males than control females did. These data point to aspects of the phenotype that make ML males more attractive to females.

Previous analyses revealed a pattern of pervasive 'masculinization' (i.e., shift of the phenotypes towards the male side of the extent sexual dimorphism) for key life-history traits (Prasad *et al.* 2007) and morphometric characters (Abbott *et al.* 2010) in the ML populations. Changes in CHCs also seem likely contributors to the evolved response. *Drosophila* laboratory stocks have been shown to contain considerable genetic variation for CHCs, as evidenced by their responsiveness to experimental evolution (Higgie *et al.* 2000; Rundle *et al.* 2005; Higgie and Blows 2008; Chenoweth *et al.* 2008), and CHC evolution was recently demonstrated in response to desiccation selection in the stock population used to found the ML selection treatment (Kwan and Rundle 2010). If female-specific selection on this partially shared phenotype is inhibiting males from achieving their sex-specific optimum, the ML treatment would remove this constraint and allow CHCs to evolve in response. Under this scenario, males should evolve a more masculinized, and likely more attractive, profile and females expressing ML haplotypes should also show a shift in CHCs in the male direction of extant sexual dimorphism. Such a response

Table 2. Analysis of the differences between the CHC profiles of ML and C females.

Label	CHC	*F*	*P*	C mean proportion (± s.e.)	ML mean proportion (± s.e.)
F1	(Z)-9-$C_{21:1}$	1.17	0.358	0.324 (0.007)	0.316 (0.016)
F2	C_{22}	0.17	0.705	0.586 (0.012)	0.605 (0.040)
F3	(Z,Z)-7,11-$C_{23:2}$	0.27	0.641	0.685 (0.026)	0.628 (0.021)
F4	(Z)-9-$C_{23:1}$	0.43	0.559	0.290 (0.008)	0.291 (0.026)
F5	(Z)-7-$C_{23:1}$	0	0.995	2.562 (0.165)	2.721 (0.299)
F6	(Z,Z)-5,9-$C_{23:2}$	1.17	0.358	0.259 (0.009)	0.268 (0.015)
F7	(Z)-5-$C_{23:1}$	0	0.976	0.253 (0.016)	0.265 (0.025)
F8	C_{23}	0.95	0.401	6.427 (0.096)	6.237 (0.136)
F9	(Z)-7-$C_{24:1}$	0.02	0.885	0.230 (0.012)	0.243 (0.015)
F10	C_{24}	0.34	0.600	0.959 (0.021)	0.925 (0.020)
F11	(Z,Z)-9,13-$C_{25:2}$	0.45	0.551	0.350 (0.022)	0.320 (0.023)
F12	(Z,Z)-7,11-$C_{25:2}$	0.38	0.583	2.760 (0.096)	2.585 (0.088)
F13	2-Me-C_{24}	0.22	0.674	0.309 (0.050)	0.312 (0.055)
F14	(Z)-9-$C_{25:1}$	0.18	0.702	4.177 (0.252)	4.028 (0.149)
F15	(Z)-7-$C_{25:1}$ + (Z,Z)-5,9-$C_{25:2}$	1.24	0.348	6.526 (0.547)	5.421 (0.337)
F16	(Z)-5-$C_{25:1}$	0.02	0.898	0.511 (0.034)	0.572 (0.109)
F17	C_{25}	0.73	0.455	5.779 (0.116)	5.495 (0.107)
F18	(Z,Z)-7,11-$C_{26:2}$	0.79	0.440	0.427 (0.015)	0.461 (0.063)
F19	C_{26}	0.8	0.437	0.841 (0.019)	0.843 (0.050)
F20	(Z,Z)-7,11-$C_{27:2}$	0	0.987	25.853 (0.824)	25.855 (0.760)
F21	**2-Me-C_{26}**	**96.57**	**0.002**	**4.161 (0.305)**	**4.222 (0.093)**
F22	(Z)-7-$C_{27:1}$ + (Z,Z)-5,9-$C_{27:2}$	0.29	0.625	6.708 (0.524)	7.075 (0.481)
F23	(Z)-5-$C_{27:1}$	0.3	0.622	0.571 (0.045)	0.800 (0.188)
F24	C_{27}	0.2	0.6881	6.137 (0.184)	5.996 (0.186)
F25	(Z,Z)-9,13-$C_{28:2}$	0.57	0.504	0.255 (0.012)	0.349 (0.078)
F26	(Z,Z)-7,11-$C_{28:2}$	0.11	0.766	0.537 (0.019)	0.597 (0.053)
F27	2-Me-C_{28}	0.03	0.879	0.398 (0.016)	0.407 (0.016)
F28	C_{28}	0.1	0.772	0.296 (0.011)	0.290 (0.012)
F29	(Z,Z)-7,1-$C_{29:2}$	1.2	0.353	15.431 (0.572)	16.340 (0.554)
F30	(Z)-9-$C_{29:1}$	1.28	0.341	0.597 (0.041)	0.760 (0.140)
F31	(Z)-7-$C_{29:1}$	0.51	0.526	1.028 (0.060)	1.097 (0.060)
F32	C_{29}	0.18	0.701	1.896 (0.114)	1.871 (0.110)
F33	2-Me-C_{30}	0.25	0.649	1.603 (0.101)	1.544 (0.034)
F34	C_{31}	0.2	0.687	0.275 (0.025)	0.261 (0.017)

Results of mixed linear models (equation 1) testing for differences in proportional CHCs between the ML and C genotypes when expressed in females. *P* values derive from *F* tests with 1,3 degrees of freedom in each case, uncorrected for multiple comparisons. Values in bold indicates the compound which is significantly different between ML and C before multiple test correction. The last two columns give the mean proportion (± s.e.) of each compound in each selection treatment.

would directly infer ongoing intralocus sexual conflict over these traits.

Materials and methods

Experimental evolution of the ML and control populations

The derivation of the ML lines and their matching controls (C) is described in detail elsewhere (Prasad *et al.* 2007). Briefly, four large subpopulations were derived from the previously described laboratory-adapted outbred LH_M population (Chippindale and Rice 2001). Each of the four populations was maintained in isolation for 10 generations. From each of these populations, one pair of selected (ML_{1-4}) and control (C_{1-4}) populations was initiated. Each pair of selected and control populations bearing the same numerical subscript was more closely related to one another through their common ancestry and subsequent handling than to other selected or control populations. To initiate a ML population, 1040 haplotypes, consisting of chromosome I (X), II, and III, but not the tiny chromosome IV (more than 99% of the genome in total, hereafter referred to as haplotypes) were sampled using 'clone generator females' carrying a compound X(C(1)DX, *y*, *f*), a Y chromosome from LH_M base population, and a homozygous-viable translocation of the two major autosomes (T(2:3)*rdgc st in ri* p^p*bw*). These chromosomal constructs and the absence of molecular recombination in male *D. melanogaster* mediate the transmission of the haplotypes from father to son. The males carrying a translocation and a wildtype haplotype originally sampled from LH_M were crossed each generation to 'clone generator females'. In this way, these haplotypes were transmitted only from father to son, the grand-maternal haplotypes being discarded in every generation. Efforts were made to standardize the effective population size between selected and

Table 3. Comparison of ML and C profiles for putatively homologous CHCs.

	Selection		Sex		Selection×sex	
CHC	*F*	*P*	*F*	*P*	*F*	*P*
(Z)-9-$C_{21:1}$	0.73	0.4558	67.47	**0.0038 (F>M)**	0.50	0.5304
C_{22}	0.28	0.6354	444.32	**0.0002 (M>F)**	1.72	0.2805
(Z)-9-$C_{23:1}$	0.65	0.4784	3580.29	**<.0001 (M>F)**	0.2	0.6633
(Z)-7-$C_{23:1}$	0.61	0.4905	22278.68	**<.0001 (M>F)**	0.27	0.6388
(Z)-5-$C_{23:1}$	1.16	0.3598	7427.42	**<.0001 (M>F)**	1.72	0.2810
C_{23}	0.39	0.5765	8.87	0.0587	0.73	0.4553
(Z)-7-$C_{24:1}$	0.09	0.7860	0.17	0.7090	0.00	0.9642
C_{24}	0.03	0.8687	1251.61	**0.0001 (F>M)**	0.89	0.4151
(Z,Z)-7,11-$C_{25:2}$	0.08	0.7935	10600.00	**<.0001 (F>M)**	0.07	0.8117
2-Me-C_{24}	0.09	0.7895	138.66	**0.0013 (M>F)**	0.10	0.7707
(Z)-9-$C_{25:1}$	0.34	0.6022	2248.77	**0.0001 (F>M)**	0.34	0.6013
(Z)-5-$C_{25:1}$	0.07	0.8144	1068.68	**0.0001 (F>M)**	0.304	0.8547
C_{25}	0.08	0.7951	2634.35	**<.0001 (F>M)**	0.55	0.5133
2-Me-C_{26}	0.26	0.6433	61.90	**0.0043 (F<M)**	5.93	0.0930
C_{27}	0.01	0.9330	3098.48	**<.0001 (F>M)**	0.07	0.8025
2-Me-C_{28}	0.69	0.4685	962.78	**0.0001 (M>F))**	0.46	0.5449
C_{29}	0.06	0.8245	1291.36	**0.0001 (F<M)**	0.00	0.9777
2-Me-C_{30}	0.02	0.8869	442.10	**0.0002 (F>M)**	0.59	0.4976

Results of mixed linear models (equation 2) testing for differences in proportional values of shared CHCs between the ML and C (selection) treatments, males and females (sex), and their interaction (d.f. = 1,3 for all tests). CHC identities are given in the first column. *P* values are uncorrected for multiple comparisons; values in bold remain significant after multiple test correction. The direction of the sexual dimorphism is indicated in parentheses for the effect of sex.

control populations by maintaining the same number of haploid genomes in each. This is fully possible for autosomes; for sex chromosomes, the ML populations have 33% more X chromosomes segregating than the control populations. Finally, the same maintenance protocol was used for C and ML populations, except that the C populations had normal transmission of genetic material from one generation to the next, via both males and females. All flies were maintained in 40 mL vials containing standard molasses–cornmeal–yeast medium. Offspring were reared at 25°C and 50% relative humidity in a 12:12 h light/dark cycle under moderate densities of approximately 150 larvae per vial.

This experimental protocol completely prevented recombination in the ML populations, which could slow down their rate of adaptation due to hitchhiking, mutation accumulation, and background selection. To prevent this in each generation, 4% of the genomes were passed through a series of crosses in which the ML haplotypes were expressed in females, allowing them to recombine (Prasad *et al.* 2007). Because this 'recombination loop' constantly received new ML-selected chromosomes, females in it were carrying ML chromosomes from the previous generations of selection. These recombined ML haplotypes were then reintroduced into the general ML population.

CHC assay

At generation 82 of experimental evolution, flies were collected to start a series of crosses necessary to generate the individuals for CHC extraction. These individuals were males and females carrying one ML or control haplotype and the translocation of chromosomes 2 and 3 used to evolve the ML populations. The crosses are described in detail elsewhere (Prasad *et al.* 2007). From these crosses, 30 males and 30 females expressing ML and control genomes were collected from each population as virgins 8–9 days post egg laying. These individuals were housed separately by sex in groups of five in vials containing food. Four-to-five days after virgin collection, flies were anaesthetized using CO_2, and CHCs were extracted using a standard protocol (Blows and Allan 1998) by washing flies individually in 100 μL of hexane for 4 min and then vortexing for another minute. Flies were then discarded and the resulting CHC samples were analysed using an Agilent Technologies (Wilmington, USA) 6890 N gas chromatograph fitted with a HP5 column of 50 m × 0.32 mm internal diameter, pulsed splitless inlet, and flame ionization detector using the temperature programme described in Kwan and Rundle (2010). Individual CHC profiles were determined by integration of the area under 25 peaks in males and 34 peaks in females, representing all those that could be reliably identified in every individual of each sex (figure 1). The pattern of peaks corresponded closely to those from two other populations of *D. melanogaster* (Foley *et al.* 2007; Everaerts *et al.* 2010) and chemical identities were assigned with reference to these studies (tables 1 and 2). In two cases in females (F15 and F22; table 2), we could not reliably separate two CHCs identified in past studies and these were therefore pooled in our integration and are identified accordingly.

Relative proportions of CHCs were calculated by dividing the area under each peak by the total area under all peaks for that individual. This corrects for nonbiological sources

of variation among samples in total CHC concentration that arise from their extraction and subsequent chromatography. Such technical error can be large, even with the use of internal standards, favouring the use of proportions (Blows and Allan 1998; Savarit and Ferveur 2002). We therefore refrain from analysing total CHC content as a trait itself to permit its use as a control for this technical error. Although a log-contrast transformation is often used to break the unit-sum constraint associated with such proportional data (Blows and Allan 1998; Aitchison 1986), such a constraint is only an issue in multivariate analysis that include all traits (or all of their principal components). We are unable to perform such analyses (see below), so to avoid unnecessary issues as to the choice of divisor in calculating logcontrasts, we present results from the analyses of the proportions. However, results change little if logcontrasts are used instead (H. Rundle, unpublished data). Proportions were arcsine-square root transformed prior to analyses, although this has no qualitative effect on any of the results.

Because the identity of many of the CHCs are not shared between sexes, the effect of the experimental evolution treatment was tested separately by sex using a mixed linear model for randomized complete block design (Newman *et al.* 1997; Quinn and Keough 2002):

$$\text{Proportionate CHC} = \text{Treat} + \text{B} + \text{Treat} \times \text{B}, \quad (1)$$

in which 'Treat' is a fixed effect denoting the selection regime (ML versus C treatment), B is a random effect representing the blocking of the experimental units (i.e., populations) into four ML-C pairs due to shared ancestry and handling, and Treat×B is the treatment-by-block interaction, also a random effect. This design accounts for the fact that each ML-C pair (i.e., block) represents a single, evolutionary replicate in a test for effects of the selection treatment. Individuals represent subsamples in this design and we therefore performed the analysis on population means for all traits to avoid pseudoreplication (Bergerud 1996; Quinn and Keough 2002). As with all unreplicated randomized complete block designs, there is no test of the interaction because it cannot be estimated separately from the residual error variance (Quinn and Keough 2002). The model was fit using maximum likelihood as implemented in the mixed procedure in SAS v 9.2 (SAS Institute, Cary, USA).

The ideal analysis would have been a multivariate version of the above model that simultaneously considered all CHCs present in a given sex. However, due to the large number of traits measured compared to the modest number of replicate populations, such a model could not be fit due to limiting degrees of freedom. Following Chenoweth *et al.* (2010), we therefore performed univariate analyses on each proportionate CHC separately using the above model (1). A false discovery rate (FDR) correction (Benjamini and Hochberg 1995) was employed on the resulting significance values. As an alternative approach to these univariate tests, we also conducted a multivariate analysis of variance on the population means of all individuals when scored for the first three principal components of the covariance matrix of CHCs, representing the maximum number that could be included due to limiting degrees of freedom.

Finally, to test for sex-specific treatment effects directly, we created a subset of the total data from both sexes that was composed of CHCs that were chemically identical (figure 1; reference Foley *et al.* 2007). Proportions were recalculated by dividing each peak area by the sum of all shared peak areas for each individual, although the results are qualitatively unchanged if the analysis is performed on the raw CHC values (H. Rundle, unpublished results). The analysis employed a nonadditive mixed linear model for a factorial randomized complete block design:

$$\text{Prop. shared CHC} = \text{Treat} + \text{Sex} + \text{Treat} \times \text{Sex} + \text{B} + \text{Treat} \times \text{B} + \text{Sex} \times \text{B} + \text{Treat} \times \text{Sex} \times \text{B}, \quad (2)$$

where 'Sex' is the fixed effect of sex and other terms are as in equation (1). As with all unreplicated versions of such a design, there is no test of the three-way interaction because it cannot be estimated separately from the residual error variance (Quinn and Keough 2002). Analysis was again performed on population means for all traits in each sex. As before, a multivariate analysis of all traits could not be applied so the model was fit separately for each shared trait using maximum likelihood as implemented in the mixed procedure in SAS v 9.2 (SAS Institute, Cary, USA) and an FDR correction was applied to the resulting significance values where appropriate. We also conducted a multivariate analysis of variance on the population means of individuals when scored for the first three principal components of the covariance matrix of CHCs, representing the maximum number that could be included due to limiting degrees of freedom.

Results

After 82 generations of experimental evolution, there was little evidence of any differences in CHC expression between the ML and C populations. In males, only one CHC, (Z)-5-$C_{23:1}$ (i.e., M8), showed a significant treatment effect, decreasing in ML relative to C males. Although this compound, also known as 5-tricosene, has been implicated as an inhibitor of male courtship when expressed in females (Ferveur and Sureau 1996), its difference here did not remain significant after correction for multiple testing (table 1). In females, again only a single CHC, 2-Me-C_{26} (i.e., F21), showed a significant treatment effect, increasing in ML relative to C females (table 2). No effect of this compound on courtship has been previously described and again, this difference did not remain significant after correction for multiple testing. Treatment effects were also absent in separate multivariate analysis of variance using the first three principal components of the phenotypic covariance matrices of

male and female CHCs, accounting for 93.7% and 92.8% of the total phenotypic variance respectively (males, Wilks' lambda = 0.839, $F_{3,1} = 0.06$, $P = 0.97$; females, Wilks' lambda =0.519, $F_{3,1} = 0.31$, $P = 0.83$).

Evidence was also lacking of any sex-specific divergence in the 18 CHCs identified as putatively homologous, including the two compounds identified above as showing some evidence for a response to selection in one of the sexes (i.e., (Z)-5-$C_{23:1}$ in males and 2-Me-C_{26} in females). The treatment×sex interaction was nonsignificant for all traits, even prior to corrections for multiple testing, and the main effect of treatment was also nonsignificant in all cases (table 3). However, males and females did differ in relative concentrations of 16 of the18 CHCs (after multiple test correction), as indicated by the significant main effects of sex, demonstrating substantial quantitative dimorphism for these traits. These results were essentially unchanged in a multivariate analysis of the populations means for males and females when scored for the first three principal components of the phenotypic covariance matrix of shared CHCs (accounting for 98.4% of the total variance). In particular, sex remained significant (Wilks' lambda = 5.8×10^{-5}, $F_{3,1} = 5713$, $P = 0.0097$) and the treatment and sex×treatment effects were again nonsignificant (treatment, Wilks' lambda = 0.559, $F_{3,1} = 0.27$, $P = 0.85$; sex×treatment: Wilks' lambda = 0.791, $F_{3,1} = 0.09$, $P = 0.96$).

Discussion

Drosophila melanogaster's CHC profile is highly sexually dimorphic: some of the compounds are specific to one sex and nearly all shared compounds differ significantly in relative abundance between males and females (figure 1; table 3; Foley *et al.* 2007). Sexual dimorphism is the expected outcome of persistent sexually antagonistic selection and may evolve through a number of mechanisms that permit some degree of independent genetic control in each sex (e.g., sex-specific gene regulation, sex linkage, gene duplication followed by sex limitation, parental imprinting, and sex-specific maternal effects) (Bonduriansky and Chenoweth 2009; Svensson *et al.* 2009). These mechanisms serve to lower the intersex genetic correlation for the trait, partially or completely resolving sexual conflict. However, this resolution often appears to be imperfect or incomplete (Bedhomme and Chippindale 2007; Bonduriansky and Chenoweth 2009; Cox and Calsbeek 2009; Poissant *et al.* 2010), likely because of limits imposed by genetic architecture, changing selection, pleiotropy, and/or insufficient time to resolve the conflict (i.e. the conflict is of recent origin). Sexually dimorphic traits therefore remain strong candidates for the detection of sexually antagonistic selection. We expected ML selection to reveal such conflict in the sexually dimorphic CHCs of *D. melanogaster*, as it had for other dimorphic traits in these populations. However, our main finding is that CHCs did not evolve measurably under this selection treatment. We consider first issues related to the experimental design and its power, and then discuss two evolutionary genetic scenarios for the inferred lack of polymorphic loci segregating alleles with male-benefit sexually antagonistic effects in the LH_M (ancestor) population.

Power and potential experimental artifacts

As with any null result, it is possible that CHCs are actual targets of sexually antagonistic selection but that we failed to detect a significant treatment effect due to limitations of the experimental design. For example, statistical power may have been weak due to limited replication (only four populations per treatment) with respect to a set of traits that are inherently variable or are measured with large error. This does not appear to be the case, however. Replication was sufficient to detect significant divergence in developmental time between control and ML treatments (Prasad *et al.* 2007) and the coefficient of variation of this trait within each of the eight populations averaged 0.44 in each sex. Although the coefficients of variation for CHCs within the populations vary somewhat among traits, across traits and populations they average 0.27 and 0.38 in males and females respectively, similar to or less than that observed for developmental time. With respect to experimental power, calculations are not straightforward when random effects are included in a model, although insight may be gained by treating the fixed effect of interest separately (Quinn and Keough 2002). A one-way analysis of variance of the treatment effect (with four observations per treatment representing the means of the four populations), fit via least-squares, reveals that the smallest difference in proportional CHC concentration that would be declared significant (at $\alpha = 0.05$), given our experimental design and data, averages 0.25% in males and 0.39% in females. In comparison, much larger differences in multiple CHCs evolved in this population in response to desiccation selection (up to 3.6%; see Kwan and Rundle 2010).

It could also be that the restricted level of recombination in the ML populations slowed the response of CHCs to our selection treatment due to genetic hitchhiking of deleterious variation and/or through clonal interference. However, previous work suggests that it takes only a small amount of recombination to eliminate this problem (Rice 1996). In our experiment, 4% of the ML genomes within a population experienced recombination in each generation, mirroring the protocol of a previous ML selection experiment (Rice 1996). Responses to selection were observed in these studies and involved traits ranging from morphological characters (e.g., wing venation; Abbott *et al.* 2010) to fitness itself (Rice 1996; Prasad *et al.* 2007), indicating that recombination was not sufficiently low to prevent adaptation.

Although environmental factors can influence CHC abundances (Ferveur 2005), we also consider it highly unlikely that microenvironmental differences between culture vials

could obscure any but minute evolved differences between the selection treatments given the degree of control exercised in the experiments. Moreover, because CHCs in the same stock population have been shown to respond readily to desiccation selection, energetically-mediated trade-offs are also an unlikely explanation for the absence of response to ML selection. Finally, the absence of difference between ML and C lines could come from parallel evolution in both selection treatments in response to the specific and novel conditions of the experiment. This is unlikely because the maintenance protocol of the ML and C populations was designed not only to minimize the differences between the two evolution treatments but also to minimize changes from the regimen of the ancestral population. The food, the larval density in the vials, the number of adult flies contributing to the next generation, and the egg-laying time were virtually identical in the ancestral, the ML and the C populations. We conclude that adaptation to a novel environment is unlikely to have obscured potential ML treatment effects.

Evolutionary genetic scenarios

Rather than artifacts or a lack of experimental power, we suggest two scenarios that may explain the current absence of intralocus sexual conflict over CHC expression. First, CHCs may have never been the target of sexually antagonistic selection at all. Although sexual dimorphism will often evolve via sexually antagonistic selection because most new alleles are expressed in both sexes, in theory it may also evolve via completely sex-limited mutations (e.g., on the Y chromosome or in previously sex-limited autosomal genes) or as a correlated effect, due to pleiotropy, of sexually antagonistic selection on another trait (Bonduriansky and Chenoweth 2009). However, neither of these appear likely in our case: numerous sex-specific QTL on the X and autosomes have been identified that affect CHC expression in *D. melanogaster* (Foley *et al.* 2007) and the latter scenario would require that these pleiotropic effects on another trait under sex-specific selection have since vanished to explain the current absence of intralocus sexual conflict. Most importantly, for this suite of characters to have entirely avoided intralocus sexual conflict, the 15 previously detected independent CHC loci in males and 25 in females would all have had to involve genes that were already sex-limited prior to being recruited in the CHC synthesis.

The second scenario, which we believe to be more likely, is that the extensive sexual dimorphism in CHCs is the result of past intralocus sexual conflict that has been fully resolved. In other words, genetic independence has evolved secondarily, possibly through gene-duplication events. Although a direct demonstration of sexually antagonistic selection on CHCs is lacking in *D. melanogaster* and would be difficult to estimate for qualitatively dimorphic traits. It nevertheless appears likely given what is known about the roles of CHCs in sexual communication in this species (Rybak *et al.* 2002; Savarit and Ferveur 2002; Grillet *et al.* 2006; Billeter *et al.* 2009) and the fact that sex-specific selection on CHCs arising from mate choice has been shown in another *Drosophila* species (Rundle and Chenoweth 2011; Chenoweth and Blows 2003, 2005).

As noted earlier, a substantial response in CHCs to selection for desiccation resistance was demonstrated in the LH_M population contemporary to the ML selection experiment, indicating the presence of standing genetic variation in a number of these traits (Kwan and Rundle 2010). Whereas natural selection recruited alleles affecting CHCs, sex-limited selection did not. Apparently, genetic variation in male CHCs was not sustained by sexual conflict but by some other form of trade-off.

Our results strongly imply the genetic independence of CHC expression in male and female *D. melanogaster*. This interpretation is consistent with results from other studies suggesting some degree of sex-specific genetic control of these traits (Ferveur and Jallon 1993; Dallerac *et al.* 2000; Wicker-Thomas and Jallon 2000; Fang *et al.* 2002; Foley *et al.* 2007). Also consistent with this, Shirangi *et al.* (2009) report extremely fast evolution of sex-specific regulation of enzyme expression in the CHC synthesis pathway in the *Drosophila* genus. Similar, although somewhat less extensive genetic independence has been explored in detail for the quantitatively sexually dimorphic CHCs found in *D. serrata*, where intersex genetic correlations are reduced and laboratory populations have been shown to respond in sex-specific ways to selection in novel environments (Rundle *et al.* 2005; Chenoweth *et al.* 2008).

If the genetic basis of CHCs is sex-specific, as suggested by this study and others, then the ML evolution procedure could still affect them through the accumulation of mutations in female-specific loci. In this case, the majority of the loci affecting the CHC biosynthesis pathway in females were free to accumulate mutations, unchecked by selection, for 82 generations. The effects of mutation accumulation would be to increase the variance among replicate populations in CHCs when ML-evolved chromosomes were expressed in females, and probably to depress relative abundances. There is no evidence of such increased variance or reduced abundance in ML versus C females in our experiment, possibly due to the large population size maintained or to insufficient time for the effects of such a process to accrue.

Conclusions

We suspected that the evolution of more attractive CHC profiles in ML males was partially responsible for their increased mating success and courtship efficiency relative to control males (Bedhomme *et al.* 2008). This appears not to be the case. Recent morphological analysis of these experimentally evolved populations indicates higher symmetry and masculinized shape-measurements of the ML males (Abbott *et al.* 2010). Moreover, a recent study established that locomotory activity is a sexually antagonistic trait in the ancestral

LH_M laboratory population (Long and Rice 2007); despite a significant positive intersexual genetic correlation, males are selected for higher activity and females for lower activity. Taken together, increased reproductive success in the ML males appears to have resulted from changes in morphology, body size, and/or behaviour, resulting in better courtship performance. These characters all retained substantial gender load—reduced fitness caused by the selection of loci for performance in the other sex—while cuticular hydrocarbons did not. Our data from a manipulative experiment therefore support the findings of Foley *et al.* (2007) that the same CHCs are expressed by different loci in females and males. Although direct evidence of sexually antagonistic selection is lacking and therefore constitutes an important goal of ongoing work, our interpretation of these data is that CHCs are likely strongly sexually antagonistic when shared between the sexes. The necessary implication is that multiple historical evolutionary events have segregated genetic control between the sexes over time such that this baroque multilocus phenotype has, piece by piece and genomewide, been sexually isolated.

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