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REDUCED GENETIC VARIANCE AMONG HIGH FITNESS INDIVIDUALS: INFERRING STABILIZING SELECTION ON MALE SEXUAL DISPLAYS IN *DROSOPHILA SERRATA*

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Directional selection is prevalent in nature, yet phenotypes tend to remain relatively constant, suggesting a limit to trait evolution. However, the genetic basis of this limit is unresolved. Given widespread pleiotropy, opposing selection on a trait may arise from the effects of the underlying alleles on other traits under selection, generating net stabilizing selection on trait genetic variance. These pleiotropic costs of trait exaggeration may arise through any number of other traits, making them hard to detect in phenotypic analyses. Stabilizing selection can be inferred, however, if genetic variance is greater among low- compared to high-fitness individuals. We extend a recently suggested approach to provide a direct test of a difference in genetic variance for a suite of cuticular hydrocarbons (CHCs) in *Drosophila serrata*. Despite strong directional sexual selection on these traits, genetic variance differed between high- and low-fitness individuals and was greater among the low-fitness males for seven of eight CHCs, significantly more than expected by chance. Univariate tests of a difference in genetic variance were nonsignificant but likely have low power. Our results suggest that further CHC exaggeration in *D. serrata* in response to sexual selection is limited by pleiotropic costs mediated through other traits.

KEY WORDS: **Cuticular hydrocarbons, evolutionary limit, fitness optimum, opposing selection, pleiotropy, sexual selection.**

Studies of the evolution of quantitative traits in nature have yielded two general observations: directional selection on heritable traits is common and often quite strong (Endler 1986; Hoekstra et al. 2001; Kingsolver et al. 2001; Hereford et al. 2004), but in the absence of environmental change, a sustained evolutionary response is rare (Svensson and Gosden 2007; Kingsolver and Diamond 2011). Rather, investigations over various time scales indicate that phenotypic trait means tend to remain relatively constant, with change best described by a model of stabilizing selection around a slowly moving optimum (Estes and Arnold 2007). The failure of directional selection to produce a prolonged response suggests that these traits have reached some form of an evolutionary limit. The genetic nature of this limit, however, remains a largely unresolved issue.

Directional selection erodes genetic variance in target traits, making a simple lack of genetic variance in the direction of selection a possible evolutionary limit. Empirical data, however, suggest that this is not a general explanation: variation exists for the majority of traits studied (Lynch and Walsh 1998; Barton and Partridge 2000; but see Hoffmann et al. 2003), with those most closely linked to fitness often exhibiting the highest levels (Houle 1992). Although multivariate genetic constraints arising from the genetic covariance structure among suites of traits may often decrease the genetic variance available to selection (Barton

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and Partridge 2000; Blows and Hoffmann 2005), such covariances appear insufficient to produce absolute constraints (Beldade et al. 2002; Conner et al. 2003; Hine et al. 2011). Furthermore, the covariance structure of a set of traits can also increase genetic variance in the direction of selection, and it appears that genetic architecture may facilitate an evolutionary response as often as constraining it (Agrawal and Stinchcombe 2009). Overdominance of loci responding to selection may also impose an evolutionary limit by restricting the additive genetic variance available to selection (Falconer and Mackay 1996). However, there is currently little evidence to suggest that overdominance is a general cause of selective limits (Eisen 1980; Falconer and Mackay 1996; Lynch and Walsh 1998).

It has long been recognized that trait responses may eventually be halted by opposing natural selection (Fisher 1930), providing an alternative mechanism for an evolutionary limit. Consistent with this, artificial selection experiments often observe plateaus in trait responses despite the presence of additive genetic variance. Traits have also often been observed to regress toward their former values when artificial selection is relaxed (Reeve and Robertson 1953; Enfield 1980; Falconer and Mackay 1996; Hine et al. 2011), suggesting an important role for opposing natural selection in generating evolutionary limits. Whether the genetic limits arising from artificial selection experiments are representative of those occurring more generally in unmanipulated populations is not known, in part because ascertaining the nature of these limits in the absence of a selection experiment has proven challenging.

Sexual display traits provide a striking example of how directional selection often fails to generate sustained evolutionary change (Kruuk et al. 2002) and such traits, therefore, provide an ideal system in which to study the genetic basis of evolutionary limits. Directional sexual selection is often strong in nature (Kingsolver et al. 2001), is thought to be persistent, and commonly targets traits that are associated with high levels of genetic variance (Pomiankowski and Møller 1995). Sexual selection's role in the exaggeration of sexual displays is also well established, the end result of which is the pervasive sexual dimorphism that constitutes a substantial component of existing phenotypic diversity (Darwin 1871; Andersson 1994). The continual exaggeration of sexual displays, however, is generally not observed in contemporary populations (e.g., Kruuk et al. 2002), and sexual selection appears insufficient to drive the divergence of sexual displays among populations on its own (Svensson and Gosden 2007).

Opposing natural selection, arising from costs to nonsexual fitness of the sexual displays themselves, has historically been recognized as a potential limit to the sustained exaggeration of sexual displays (Fisher 1930; Kirkpatrick 1987). Consistent with this, costs of sexual displays to specific components of nonsexual fitness have been demonstrated in some cases, for example, in guppies sexual selection favors brightly colored males but these males experience a greater risk of predation due to their ornamentation (Godin and McDonough 2003; see also Ryan et al. 1982; Moller 1989; Fernandez and Morris 2008). However, direct evidence that natural selection opposes further trait exaggeration in unmanipulated populations is generally lacking (Jennions et al. 2001; Kotiaho 2001).

Opposing selection may arise not only as a direct cost to nonsexual fitness of a sexual display itself, but it may also occur due to the pleiotropic effects of the underlying alleles on other traits affecting fitness. The distinction is important because opposing selection arising from pleiotropic effects will be hard to identify in phenotypic analyses because it may arise through any number of other traits. Two general observations implicate widespread pleiotropy throughout the genome, suggesting that pleiotropic costs may be an important limit to trait evolution (McGuigan et al. 2011). First, data on mutation rates indicates that the per-trait rate may be as high as one tenth of the mutation rate for an individual (Johnson and Barton 2005), suggesting that there are few genetically independent traits within an organism. Second, quantitative genetic analyses of suites of traits generally find that a small number of independent trait combinations account for the majority of genetic variance, again suggesting the presence of strong pleiotropic covariances among traits (Kirkpatrick 2009).

If traits, including sexual displays, are held at an evolutionary limit due to opposing natural selection, genetic variance underlying these traits will be subject to stabilizing selection with respect to net fitness, and the genetic basis of the traits should be characteristic of a pleiotropic model of mutation-selection balance (Keightley and Hill 1988, 1990; Johnson and Barton 2005; McGuigan et al. 2011). In the Hill–Keightley model of mutation-selection balance, alleles affecting a measured quantitative trait also have pleiotropic effects on many other traits. Although not measured, these effects are captured in the model as net pleiotropic effects on fitness. At mutation-selection balance, while a given mutation may increase or decrease the value of the measured trait, its pleiotropic effect on fitness through unmeasured traits will be deleterious. Individuals with more extreme values of the measured traits are expected to harbor more mutations, thereby generating the appearance of stabilizing selection on the focal traits. Selection is apparent in this model because it arises from the pleiotropic effects of the alleles on unmeasured traits. With a measure of net fitness, such stabilizing selection may be detectable in a phenotypic analysis. However, environmentally generated correlations between traits and fitness may obscure this selection such that it is more easily uncovered in a genetic analysis (Rausher 1992; Stinchcombe et al. 2002; McGuigan et al. 2011).

Here we apply a recently suggested empirical approach (McGuigan et al. 2011) to demonstrating the presence of a fitness optimum arising from the pleiotropic costs of a measured trait on

other fitness components. The approach tests for the signature of stabilizing selection by comparing genetic variance in measured traits among individuals of high and low fitness. Under stabilizing selection, low-fitness individuals will tend to have more extreme values of a measured trait (both high and low), meaning that genetic variance of this trait will be greater in these individuals than in high-fitness individuals (which will all tend to have intermediate trait values). This approach has been applied in a recent study of sexual display traits in *Drosophila bunnanda*, uncovering a difference in genetic variance between sexually successful and sexually unsuccessful males consistent with stabilizing selection (McGuigan and Blows 2009). The genetic basis of these traits differed significantly between the two fitness groups, consistent with a difference in genetic variance, although a direct statistical test of this difference was lacking. The results suggest that opposing selection arising through pleiotropy may be a key evolutionary limit for sexual displays.

Here we apply this approach within the context of a half-sibling breeding design in *Drosophila serrata*. *D. serrata* is an ideal system in which to study the genetic basis of evolutionary limits to sexual display trait exaggeration because sexual selection on male pheromonal displays, arising through female mate preferences, has been previously investigated using a series of quantitative genetic studies, behavioral assays, and evolution experiments. In particular, female mate choice within populations targets a particular combination of long chain cuticular hydrocarbons (CHCs) that act as contact pheromones, generating consistent and strong directional sexual selection on these traits (Higgie et al. 2000; Chenoweth and Blows 2003, 2005; Higgie and Blows 2007; Rundle et al. 2009; Delcourt et al. 2010). Results of a recent evolution experiment have implicated opposing natural selection in generating a new evolutionary limit when artificial selection on CHCs was applied in the direction of female mate preferences (Hine et al. 2011). This experiment also demonstrated that alleles conferring increased male attractiveness were segregating in the original base population at low frequency, presumably due to opposing natural selection on them. Here we undertake a test for the signature of the opposing selection in an outbred and unmanipulated *D. serrata* laboratory population.

Material and Methods

HALF-SIBLING BREEDING DESIGN

A paternal half-sibling breeding design was conducted using a previously described outbred and laboratory adapted stock population of *D. serrata* (Rundle et al. 2006; Chenoweth et al. 2008). Eighty sires were each mated individually to three virgin dams in two successive rounds (i.e., dam 1, 2, 3, 1, 2, 3). Dams were allowed to oviposit for 72 h following each mating round, and sons from these half-sibling families were collected upon emergence for use in binomial mate choice trials and subsequent extraction of their CHCs. The breeding design was conducted in two blocks, each consisting of 40 sires, spanning two generations of the laboratory stock population, resulting in 240 full and half-sibling families. Virgin males were collected from both oviposition vials for each dam using light CO_2 anesthesia within 24 h of eclosion, with a total of 10 sons collected per family. All flies used in experimental assays were held as virgins at a density of six flies per vial, and were five to seven days old at the time of the assays.

MATING SUCCESS AND CHC ASSAY

For the binomial mate choice assay, randomly chosen virgin females from an outbred *D. serrata* population fixed for a recessive mutation causing an orange-eye phenotype were presented with a random virgin male from the same population (competitor male) and a virgin son from the breeding design ("focal male," wild-type eye color). Trios were observed until a male and female pair had begun copulating, and the mating success (chosen or rejected) of the focal male was recorded. Orange-eye competitor males were chosen in 49% of the mating trials overall, indicating that this phenotype had little effect on mating success.

Female *D. serrata* actively control mating and can prevent males from mounting and achieving intromission (Hoikkala et al. 2000), implicating a central role of female mate choice in determining male fitness. Although the design of these mating trials does not preclude male–male competition nor the possibility that female choice may target other correlated traits, several lines of evidence indicate that CHCs are a direct target of sexual selection arising from female mate preferences, and that binomial choice trials are a suitable technique for quantifying the resulting sexual selection (Higgie and Blows 2008; Delcourt et al. 2010). The evidence includes results of a manipulative evolution experiment in which artificial selection in the direction of female mate preferences, as determined from binomial choice trials, was shown to increase male mating success over control populations (Hine et al. 2011). Because mating success is the primary determinant of male fitness in species where males contribute only their genes to their offspring, the outcome of binomial mate choice trials is also a straightforward way to sort males into high- and low-fitness groups.

Before copulation between the female and chosen male was complete, flies were anesthetized with light CO_2 and the focal male (chosen or rejected son from the breeding design) was isolated for immediate extraction of its CHCs. Individuals can alter their CHCs in response to various social interactions (Petfield et al. 2005) and CHCs were extracted in this timeframe to minimize the possibility of confounding effects caused by any changes in CHCs in response to mating itself. Although we could not test for such effects directly in our study, supplementary analysis of

data from a previous social manipulation experiment (Petfield et al. 2005) confirmed no effect of mating itself on CHCs when they are extracted in this way (Supporting information).

Focal males were individually washed in 100 μl of hexane for 3 min, followed by 1 min of agitation on a vortex mixer. Flies were then removed from the hexane and the resulting CHC samples were stored at −20°C. Individual CHC samples were analyzed using a dual-channel Agilent 6890N fast gas chromatograph fitted with HP-5 phenylmethyl siloxane columns of 30 m length and 250 μm internal diameter (0.1 μm film thickness), pulsed splitless inlets (at 275°C), and flame ionization detectors (at 310°C). The injection volume was 1 μl and the temperature program began by holding at 140°C for 0.55 min, ramping at 100°C/min to 190°C, then slowing to 45°C/min to 320°C and holding for 1 min.

Individual CHC profiles were analyzed by quantifying the area under nine peaks corresponding to those used in previous studies (e.g., Chenoweth and Blows 2005; Delcourt et al. 2010). These peaks have been previously identified in order of their retention times as: (Z,Z)-5,9-$C_{24:2}$; (Z,Z)-5,9-$C_{25:2}$; (Z)-9-$C_{25:1}$; (Z)-9-$C_{26:1}$; 2-Me-C_{26}; (Z,Z)-5,9-$C_{27:2}$; 2-Me-C_{28}; (Z,Z)-5,9-$C_{29:2}$; and 2-Me-C_{30} (Howard et al. 2003). The relative abundance of each hydrocarbon was calculated by dividing the area under each peak by the total area of all nine peaks for that individual. Expressing each CHC as a relative abundance corrects for technical error associated with quantifying absolute abundances and is less prone to experimental error than the use of internal standards (Blows and Allan 1998; Savarit and Ferveur 2002). These proportions were transformed into log contrasts, using (Z,Z)-5,9-$C_{24:2}$ as the common divisor, to break the unit-sum constraint inherent in compositional data and thereby permit multivariate analyses (Atchison 1986). The resulting eight log-contrast CHCs were used in subsequent analyses.

PHENOTYPIC AND GENETIC ANALYSES

A total of 10 multivariate outliers (0.5% of the total data) were identified and removed using the multivariate Mahalanobis distance technique implemented in the software package JMP (version 9.0.0; SAS Institute Inc., Cary, NC). Log-contrast CHC values were subsequently standardized globally $\{\sim N[0,1]\}$ prior to analyses.

Standardized phenotypic sexual selection gradients on the eight log-contrast CHCs were estimated using standard first and second-order polynomial regression on relative male mating success (Lande and Arnold 1983). Experimental block and gas chromatography channel were included as fixed effects in the models. The overall importance of CHCs in explaining variation in male mating success was given by the adjusted coefficient of determination (r^2_{adj}). Because mating success is binomially distributed, significance testing employed a generalized linear model with a logistic link function, fit via maximum likelihood, implemented in the GENMOD procedure of SAS (version 9.2; SAS Institute Inc.). The significance of overall linear and nonlinear selection was determined through likelihood ratio tests employing a sequential model building approach (Draper and John 1988; Rundle and Chenoweth 2011).

The sire-level covariance matrix for the eight log-contrast CHCs was estimated for successful and unsuccessful males together, using restricted maximum likelihood, and employing the following multivariate mixed model:

$$Y_{ijlcm} = \mu + \mathbf{S}_i + \mathbf{D}_{j(i)} + B_l + C_c + \varepsilon_{ijlcm}. \quad (1)$$

Fixed effects included the intercept (μ), experimental block (*B*), and gas chromatography channel (*C*). Sire (**S**) and dam nested within sire (**D**) were random effects. The additive genetic covariance matrix (**G**) for CHCs was estimated as four times the sire-level covariance matrix. Statistical support for the genetic dimensions underlying **G** was evaluated using a series of nested likelihood ratio tests employing the factor analytic modeling approach implemented in the MIXED procedure of SAS and described in Hine and Blows (2006). In these analyses, the dam effect was fixed at eight dimensions.

As a direct test of whether **G** differed when estimated using successful versus unsuccessful male offspring, a fixed effect of success (chosen or rejected) was added to equation 1, thereby removing any difference in trait means between these groups. A likelihood ratio test was then used to compare the fit of this model (which estimated a single 8 × 8 sire-level covariance matrix) to a model that allowed separate covariance matrices to be estimated for chosen and rejected sons at the sire level (as implemented using the "group" statement in the SAS MIXED procedure). In these analyses, the dam effect was again fixed at eight dimensions. A difference in **G** between high- and low-fitness sons may arise due to a difference in the genetic variances of these eight log-contrast CHCs, and/or differences in their covariance structure. We note here that the nonrandom sorting of individuals into successful and unsuccessful groups, based in part on their CHC phenotypes, may affect the interpretation of sire-level variance components as additive genetic variances and covariances. We retain the classic interpretation of these variance components for simplicity, but return to this issue in more detail in the Discussion section.

In a previous study (McGuigan and Blows, 2009), a difference in CHC genetic variance between high- and low-fitness groups of another species (*D. bunnanda*) was inferred by the presence of a significant sire × mating success interaction, demonstrating the presence of genetic variation in the effect of using successful versus unsuccessful sons in estimating **G** (i.e., that sire-level reaction norms vary). Although such an interaction is necessary for genetic variance to differ between groups, and is therefore consistent with such a difference, it is not sufficient to

demonstrate it for the same reason that the presence of a significant G × E interaction does not necessitate genetic variance to differ between environments. A more straightforward and direct test for a difference in genetic variance is possible within a univariate framework by employing a likelihood ratio test to compare the fit of a model with a single sire-level variance to one that allows separate estimates for successful and unsuccessful classes at this level. Therefore, to determine whether the genetic variance of CHCs differed between high- and low-fitness males, we applied such a test to the univariate phenotypic trait for which the absolute difference in CHC genetic variance is greatest between groups. Given only two **G** matrices, this trait combination is described by the first eigenvector of the difference matrix ($\mathbf{G}_{U-S}$), calculated by subtracting the sire-level covariance matrix estimated using successful males ($\mathbf{G}_S$) from that estimated using unsuccessful males ($\mathbf{G}_U$). This trait combination is equivalent to the leading eigenvector of the first eigentensor of the fourth-order genetic covariance tensor ($\mathbf{\Sigma}_G$) that characterizes the variation among replicate **G** matrices, as explained in Hine et al. (2009). The latter approach has the advantage, however, of being applicable to situations in which there are more than two **G** matrices.

Following McGuigan et al. (2011), the trait described by the first eigenvector of $\mathbf{G}_{U-S}$ (i.e., the trait for which genetic variance differs the most, termed CHC($\mathbf{G}_{U-S})_{max}$) was calculated by scoring each male's multivariate CHC phenotype using CHC($\mathbf{G}_{U-S})_{max} = (\mathbf{G}_{U-S})_{max}{}^{T}\mathbf{Z}$, where **Z** is a row vector of the eight observed log-contrast CHC values for an individual and $(\mathbf{G}_{U-S})_{max}$ is the leading eigenvector of $\mathbf{G}_{U-S}$. This same method was used to generate phenotypic scores for a second biologically relevant trait, CHCβ, representing the combination of male log-contrast CHCs most strongly associated with male mating success. This trait was calculated by applying to each male's CHC phenotype the vector of linear sexual selection gradients (i.e., β) from the phenotypic analysis of mating success above. Likelihood ratio tests were used to test whether genetic variance in CHC($\mathbf{G}_{U-S})_{max}$, and CHCβ, differed between high- and low-fitness males.

Results

Consistent with previous studies of this (Delcourt et al. 2010) and other populations of *D. serrata* (Hine et al. 2004, 2011; Chenoweth and Blows 2005), phenotypic analysis revealed that male log-contrast CHCs were under significant directional sexual selection overall ($\chi^2 = 203.5$; df = 8; $P < 0.001$). Variation in male CHCs explained 9.1% of the variance in male mating success ($r^2_{adjusted}$) and sexual selection was significant individually on five of the eight log-contrast CHCs (Table 1). Directional sexual selection was also strong, with three standardized gradients exceeding the median absolute value of 0.18 in Kingsolver et al.'s (2001) review of the strength of phenotypic selection in natural populations. Although the addition of nonlinear selection was significant overall ($\chi^2 = 80.02$; df = 36; $P < 0.001$), only three of the 36 nonlinear gradients were individually significant and the inclusion of all nonlinear selection explained only an additional 2.1% of the variance in mating success (i.e., an increase in r^2_{adj} from 9.1% to 11.2%). A nonparametric fitness function for CHCβ, estimated via a univariate cubic spline (Schluter 1988), also provided no indication of a fitness optimum within the range of phenotypic values (Fig. 1).

Factor-analytic modeling of the genetic covariance matrix for all males (i.e., irrespective of mating success; pooled **G**) revealed statistical support for three underlying genetic dimensions, accounting for 96.8% of the total genetic variance in the eight log-contrast CHCs (reducing from three to two dimensions

Table 1. The multivariate trait combination describing the standardized sexual selection gradient β, the first eigenvector of the difference matrix $\mathbf{G}_{U-S}$ ($(\mathbf{G}_{U-S})_{max}$), and the first eigenvector of **G** (pooled g_{max}) for the pooled dataset (i.e., successful and unsuccessful males together).

CHC	β	$(\mathbf{G}_{U-S})_{max}$	Pooled $\mathbf{g}_{max}$
(Z,Z)-5,9-$C_{25:2}$	0.068*	0.420	0.567
(Z)-9-$C_{25:1}$	−0.077*	−0.284	0.232
(Z)-9-$C_{26:1}$	0.011	0.293	0.267
2-Me-C_{26}	−0.045	−0.113	0.223
(Z,Z)-5,9-$C_{27:2}$	−0.221**	0.428	0.407
2-Me-C_{28}	0.060	0.320	0.391
(Z,Z)-5,9-$C_{29:2}$	0.539**	0.229	0.226
2-Me-C_{30}	−0.234**	0.554	0.366

*$P < 0.02$; **$P < 0.0001$.

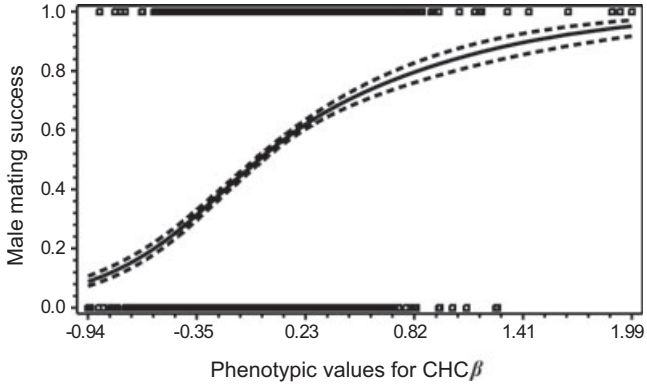

Figure 1. A nonparametric fitness function, ±1 SE (generated from 100 bootstrap replicates), depicting sexual selection on the phenotypic trait CHCβ. The fitness function was fit using a univariate cubic spline with smoothing parameter $\lambda = -1$ (chosen to minimize the general cross-validation score; Schluter 1988). Individual points are the mating success scores for separate sons from the breeding design as determined in the mate choice assays.

Table 2. Model fit statistics of the number of effective dimensions of the genetic covariance matrix (G), separately for unsuccessful and successful males. The percent of the total genetic variance in CHCs (% variance) was calculated from the full (i.e., eight-dimensional) factor analytic model.

		Unsuccessful males			Successful males		
Dimension	df	% variance	−2LL	P^1	% variance	−2LL	P^1
0	–	–	18,230.74	–	–	17,906.04	–
1	8	63.5	18,143.28	<0.001	70.1	17,854.98	<0.001
2	7	23.4	18,105.57	<0.001	16.1	17,810.88	<0.001
3	6	8.2	18,082.85	<0.001	9.5	17,799.26	0.071
4	5	3.5	18,077.93	0.425	3.7	17,796.09	0.674
5	4	1.2	18,076.27	0.798	0.7	17,794.88	0.878

[1]Results of a likelihood ratio test (−2LL), with degrees of freedom as indicated (df), of whether excluding the current factor significantly worsens the fit of the model.

Table 3. Genetic covariance matrix (G) for eight log-contrast CHCs for unsuccessful males, estimated as four times the sire-level covariance matrix. Genetic variances are along the diagonal (in bold), with covariances below and correlations above (in italics).

	(Z,Z)-5,9-$C_{25:2}$	(Z)-9-$C_{25:1}$	(Z)-9-$C_{26:1}$	2-Me-C_{26}	(Z,Z)-5,9-$C_{27:2}$	2-Me-C_{28}	(Z,Z)-5,9-$C_{29:2}$	2-Me-C_{30}
(Z,Z)-5,9-$C_{25:2}$	**1.508**	*0.587*	*0.264*	*0.622*	*0.644*	*0.870*	*0.393*	*0.757*
(Z)-9-$C_{25:1}$	0.561	**0.605**	*0.179*	*0.418*	*0.249*	*0.358*	*−0.124*	*0.244*
(Z)-9-$C_{26:1}$	0.279	0.120	**0.742**	*−0.239*	*0.884*	*0.740*	*0.788*	*0.705*
2-Me-C_{26}	0.657	0.280	−0.177	**0.741**	*0.103*	*0.705*	*−0.036*	*0.337*
(Z,Z)-5,9-$C_{27:2}$	0.755	0.185	0.727	0.085	**0.911**	*0.742*	*0.875*	*0.919*
2-Me-C_{28}	0.922	0.240	0.304	0.524	0.612	**0.746**	*0.565*	*0.902*
(Z,Z)-5,9-$C_{29:2}$	0.287	−0.057	0.404	−0.018	0.497	0.290	**0.354**	0.750
2-Me-C_{30}	0.809	0.165	0.529	0.252	0.764	0.678	0.388	**0.758**

significantly worsened the fit of the model: $\chi^2 = 24.4$; df = 6; $P < 0.001$). However, the genetic basis of these traits differed between successful and unsuccessful males, as indicated by a significantly better fit of a model that permitted separate sire-level covariance matrices to be estimated for these two groups ($\chi^2 = 154.9$; df = 33; $P < 0.001$). Analysis of unsuccessful males alone revealed statistical support for three dimensions of **G**, accounting for 95.1% of the genetic variance (Table 2). Consistent with reduced genetic variance among them (and therefore less power to detect it given similar samples sizes), only two dimensions of **G**, accounting for 86.1% of genetic variance in CHCs, were statistically supported for successful males (Table 2). Visual inspection of **G** for unsuccessful (Table 3) and successful (Table 4) males reveals that for seven of eight log-contrast CHCs, unsuccessful males have greater genetic variance than successful males, a difference that is significant overall (cumulative binomial probably of seven or more traits having greater genetic variance in unsuccessful than successful males = 0.035). This is reflected in the sum of the eigenvalues of these matrices (i.e., the trace of **G**), which reveals that total genetic variance of these traits is 1.24 times higher for unsuccessful as compared to successful males (a total difference in genetic variance of 1.22).

A direct test for a difference in CHC genetic variance between successful and unsuccessful males is possible within a univariate framework and was applied to the phenotypic trait for which genetic variance differs most between successful and unsuccessful males (termed CHC($\mathbf{G}_{U-S})_{max}$), calculated from the first eigenvector of the difference matrix $\mathbf{G}_{U-S}$ (i.e., $\mathbf{G}_U - \mathbf{G}_S$; Table 1). This single trait combination accounts for the majority of the difference in genetic variance between unsuccessful and successful males (0.80 of the total difference in genetic variance of 1.22). Although this represents 1.37 times more genetic variance among unsuccessful than successful males for this trait, this difference was not statistically supported ($\chi^2 = 1.62$; df = 1; $P = 0.20$). For CHCβ, the trait combination under the strongest directional sexual selection, there was relatively little genetic variance in the population as a whole ($V_A = 1.07 \times 10^{-3}$), although it was 3.91 times greater in unsuccessful than successful males (a difference in genetic variance of 0.0075). Again, however, this difference was not significant ($\chi^2 = 2.43$; df = 1; $P = 0.12$).

A phenotypic analysis of the trait combination CHC($\mathbf{G}_{U-S})_{max}$ revealed that it was not a target of directional sexual selection ($\beta = 0.006$, $\chi^2 = 0.155$, $P = 0.69$) through female mate choice. Consistent with the lack of directional sexual

Table 4. Genetic covariance matrix (G) for eight log-contrast CHCs for successful males, estimated as four times the sire-level covariance matrix. Genetic variances are along the diagonal (in bold), with covariances below and correlations above (in italics).

	(Z,Z)-5,9-$C_{25:2}$	(Z)-9-$C_{25:1}$	(Z)-9-$C_{26:1}$	2-Me-C_{26}	(Z,Z)-5,9-$C_{27:2}$	2-Me-C_{28}	(Z,Z)-5,9-$C_{29:2}$	2-Me-C_{30}
(Z,Z)-5,9-$C_{25:2}$	**1.234**	*0.630*	*0.413*	*0.733*	*0.679*	*0.839*	*0.522*	*0.714*
(Z)-9-$C_{25:1}$	0.597	**0.728**	*0.579*	*0.601*	*0.424*	*0.516*	*0.366*	*0.400*
(Z)-9-$C_{26:1}$	0.356	0.384	**0.602**	*0.141*	*0.866*	*0.993*	*0.872*	*0.759*
2-Me-C_{26}	0.563	0.354	0.075	**0.478**	*0.362*	*0.783*	*0.311*	*0.502*
(Z,Z)-5,9-$C_{27:2}$	0.652	0.313	0.580	0.216	**0.747**	*0.841*	*0.960*	*0.949*
2-Me-C_{28}	0.715	0.337	0.349	0.415	0.557	**0.587**	*0.763*	*0.928*
(Z,Z)-5,9-$C_{29:2}$	0.294	0.159	0.344	0.109	0.421	0.297	**0.258**	*0.867*
2-Me-C_{30}	0.565	0.243	0.420	0.247	0.584	0.507	0.314	**0.508**

selection on this trait combination, it lies almost orthogonal to β (92.0°). Although significant stabilizing sexual selection was detected on this trait ($\gamma = -0.010$, $\chi^2 = 7.25$, $P = 0.007$), it was relatively weak and accounted for little of the total variance in male mating success (r^2_{adj}, linear + nonlinear = 0.25%). Statistical support, therefore, likely reflects the substantial statistical power associated with phenotyping 1978 males.

Discussion

The often observed lack of contemporary evolution (Svensson and Gosden 2007; Kingsolver and Diamond 2011), despite directional selection on phenotypic traits (Endler 1986; Hoekstra et al. 2001; Kingsolver et al. 2001; Hereford et al. 2004), implies a limit to trait evolution. Artificial selection experiments suggest the importance of opposing selection, but empirical evidence from unmanipulated populations for the genetic basis of these evolutionary limits is generally lacking. Ascertaining these limits has proven empirically difficult and remains a central issue in evolutionary genetics. Opposing natural selection will generate net stabilizing selection around a fitness optimum, although characterizing these optima through phenotypic analyses is likely to prove challenging because, in the presence of widespread pleiotropy, this selection may arise through any number of unidentified traits, and environmental covariances between the traits and fitness may also obscure the underlying genetic associations (Rausher 1992; Stinchcombe et al. 2002). Using a genetic analysis, however, the signature of stabilizing selection can be detected through asymmetries in genetic variance of traits for high- and low-fitness individuals, allowing the existence of an evolutionary optimum to be inferred (McGuigan et al. 2011). Here, we have used this approach to demonstrate a genetic limit to the exaggeration of a suite of male sexual displays (CHCs) in *D. serrata*.

Consistent with past studies, CHCs were under strong directional sexual selection via female mate preferences. The genetic covariance structure of these sexual displays differed among high- and low-fitness individuals, characteristic of stabilizing selection, with genetic variance being greater among low- than high-fitness males for seven of the eight log-contrast CHCs, representing 1.24 times more genetic variance in the former as compared to the latter group. A difference in seven of the eight traits is significantly more than would be expected by chance (binomial probably, $P = 0.035$). Two particular trait combinations of interest also differed substantially in genetic variance, with CHC($\mathbf{G}_{U-S}$)$_{max}$ (the trait for which the difference in genetic variance is greatest) having 1.37 times more genetic variance among unsuccessful than successful males, and CHCβ (the trait combination under the strongest directional sexual selection) having 3.91 times more genetic variance among unsuccessful than successful males. Statistical support for these differences was lacking, however, in separate tests of each trait combination. Although genetic variance in CHCβ was extremely low overall, likely weakening the power of this test, more generally such tests are univariate and therefore accommodate only a subset of the total genetic variance (e.g., CHC($\mathbf{G}_{U-S}$)$_{max}$ accounts for 0.8 of the total difference in genetic variance of 1.22), thereby reducing their power. As discussed below, such tests may, therefore, require substantial sample sizes and/or a more sensitive measure of male fitness.

The interpretation of sire-level variance components when estimated separately for successful and unsuccessful males is a potential issue of concern. Because CHCs are phenotypically correlated with mating success, individuals are assigned to these two groups nonrandomly with respect to their CHC phenotypes. This nonrandom assignment may affect the interpretation of sire-level variances components as additive genetic variances and covariances. Such nonrandom sampling of individuals is not unique to the current study, and often arises in other situations including studies of natural populations that employ the animal model (e.g., the "invisible fraction"; Hadfield 2008) and in selective breeding programs where best linear unbiased predictors (i.e., BLUPs) are estimated for use in trait selection in populations where these traits have already been subject to ongoing artificial selection. This issue has only recently been recognized in the evolutionary genetic literature (e.g., Hadfield 2008) and its implications with respect to

the interpretation of our current analyses are not straightforward to determine. While our terminology has reflected the classic interpretation of sire-level variance components as additive genetic variance and covariances (e.g., **G** matrices) for simplicity of presentation, this interpretation should be treated with some caution when estimates are done separately for successful and unsuccessful males.

An alternative approach to uncovering a fitness optimum is through the genetic covariance of measured traits with fitness, allowing stabilizing selection on the genetic variance of these traits to be estimated directly (McGuigan et al. 2011). This approach, however, requires estimates of lifetime fitness for many replicate individuals within the context of a known pedigree, representing a substantial empirical challenge. An advantage of the current approach is that it requires only a broad classification of individuals into high- and low-fitness groups. Such classification can be relatively straightforward to obtain in species such as *Drosophila*, in which mating success is a substantial component of male fitness and can be assayed under laboratory conditions. Also, because mating success is likely to depend on an individual's condition, mutations that are deleterious to overall fitness are likely to be deleterious to mating success as well (Whitlock and Agrawal 2009); males of low mating success should therefore also carry alleles deleterious to nonsexual fitness. Nevertheless, the outcome of a single binomial mate choice trial is not a particularly sensitive measure of male mating success and is likely to have underestimated the true variance (Briscoe et al. 1992; Andersson 1994; McGuigan and Blows 2009), thereby reducing the estimated difference in genetic variance between groups. Repeated measures of mating success for individual males are empirically feasible in *Drosophila* (e.g., Rundle et al. 2007) and may increase the power to detect a difference.

The general inability of sexual selection to increase male mating success in unmanipulated populations (Hall et al. 2004; McGuigan et al. 2008) implies that unconditionally beneficial alleles are not segregating for male sexual displays, and emphasizes the potential importance of opposing selection in limiting the exaggeration of such traits. In *D. serrata* in particular, artificial selection on CHCs in the direction of female mate preferences has been shown to increase male mating success. However, trait responses were halted after a number of generations, despite an increase in genetic variance for this combination of CHCs as the new selective limit was approached (Hine et al. 2011). After the relaxation of artificial selection, traits rapidly decayed toward their initial values, implicating opposing selection in generating this new evolutionary limit. More importantly, the response of males to artificial selection, and the increase in genetic variance during this response, indicates that alleles conferring an increase in CHC attractiveness, and hence higher male mating success, were segregating at low frequency in the ancestral population. These alleles are presumably held at a low frequency by opposing natural selection on them. Here, we have provided evidence consistent with the signature of stabilizing selection on the genetic variance underlying CHCs in an unmanipulated *D. serrata* population.

If male CHCs are at an evolutionary optimum generated by opposing selection, in the absence of a direct benefit of mate choice, costly female preferences for these traits may depend on the indirect benefits females gain by discriminating against males carrying a greater number of deleterious mutations. The combination of CHCs in *D. serrata* preferred by females is unusually condition-dependent relative to other possible combinations (Delcourt and Rundle 2011). Consequently, males expressing higher values of this trait may carry fewer deleterious mutations and directional female mate preferences may therefore contribute to stabilizing selection on genetic variance underlying CHCs. Consistent with this, despite strong direction selection along β, significant stabilizing sexual selection is observed on CHC$\boldsymbol{g}_{max}$ ($\gamma = -0.005$; $P = 0.017$) and CHC$(\mathbf{G}_{\mathrm{U-S}})_{max}$ ($\gamma = -0.010$, $\chi^2 = 7.25$, $P = 0.007$). This selection is weak, however, consistent with stabilizing selection on CHC genetic variance arising in large part from pleiotropic effects on other unmeasured, and currently unidentified, traits.

The vector of directional sexual selection (β) is oriented 91.5° from $\boldsymbol{g}_{max}$ (Table 1). Consequently, very little standing genetic variance in CHCs lies in this direction (1.8×10^{-4}% of the total), consistent with previous results from this (Hine et al. 2004; Delcourt et al. 2010) and other species (Hall et al. 2004; Hunt et al. 2007; McGuigan et al. 2008). The lack of genetic variance in the direction of sexual selection suggests that female mate preferences are sufficiently strong and persistent to deplete standing genetic variation and that at mutation-selection balance, the maintenance of costly mate preferences may depend on a female's ability to discriminate against males carrying novel deleterious mutations every generation (Whitlock 2000; Tomkinks et al. 2004). Although it has been demonstrated that females can discriminate against males carrying large effect deleterious mutations (Sharp and Agrawal 2008; MacLellan et al. 2009), and those that have been artificially induced (Radwan 2004), there is little evidence to indicate whether females discriminate against naturally arising deleterious alleles.

In summary, our results suggest that, despite directional phenotypic selection on CHCs via one component of male fitness (mating success), the genetic variance underlying these traits is subject to stabilizing selection. Directional female mate preferences may contribute to selection against males carrying more deleterious mutations, thus aligning natural and sexual selection (Whitlock and Agrawal 2009). McGuigan and Blows (2009) provide similar results in an unmanipulated population of *D. bunnanda*, emphasizing the importance of characterizing

selection at the genetic level. The commonly observed failure of directional selection to produce a prolonged evolutionary response may therefore be explained by stabilizing selection on trait genetic variances arising from widespread pleiotropy. Additional empirical studies of this nature will be needed to assess the generality of these results and to provide insight into the nature of evolutionary limits.

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