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Reproductive character displacement of female mate preferences for male cuticular hydrocarbons in *Drosophila subquinaria*

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Several lines of evidence implicate sexual isolation in both initiating and completing the speciation process. Although its existence is straightforward to demonstrate, understanding the evolution of sexual isolation requires identifying the underlying phenotypes responsible so that we can determine how these have diverged. Here, we study geographic variation in female mate preferences for male sexual displays in the fly *Drosophila subquinaria*. Female *D. subquinaria* that are sympatric with its sister species *D. recens* discriminate strongly against both *D. recens* and allopatric conspecific males, whereas females from allopatric populations do not. Furthermore, female mate preferences target at least in part a suite of cuticular hydrocarbons (CHCs) in males and geographic variation in CHCs mirrors the pattern of mate discrimination. In this study, we quantify female mate preferences for male CHCs from populations that span the geographic range of *D. subquinaria*. We find that the direction of linear sexual selection varies significantly between populations that are sympatric versus allopatric with *D. recens* in a pattern of reproductive character displacement. Differences in preference partially align with existing differences in CHCs and patterns of sexual isolation, although discrepancies remain that suggest the involvement of additional traits and/or more complex, nonlinear preference functions.

KEY WORDS: **Behavioral isolation, *Drosophila recens*, reinforcement, sexual isolation, sexual selection, speciation.**

Sexual (i.e., behavioral) isolation is often considered to be one of the most important reproductive barriers between existing species (Mayr 1963) and a number of lines of evidence suggest that its evolution may be key in initiating speciation (Coyne and Orr 2004). Consistent with this, partial sexual isolation can sometimes be detected among populations within a species (e.g., Nosil et al. 2002; Jiggins et al. 2004; Yukilevich and True 2008) and has been observed to evolve over relatively short time-frames in some laboratory evolution experiments (Rice and Hostert 1993; Coyne and Orr 2004). There is also mounting evidence that sexual isolation can be reinforced following secondary contact between incompletely isolated species (Noor 1995; Saetre et al. 1997; Rundle and Schluter 1998; Hoskin et al. 2005; Silvertown et al. 2005; Jaenike et al. 2006; Kronforst et al. 2007; Nosil et al. 2007; Urbanelli and Porretta 2008), suggesting that its evolution may also be key in the late stages of the speciation process.

Data archival location: All CHC data will be deposited to Dryad upon acceptance.

Sexual isolation is straightforward to demonstrate and is usually inferred from the outcome of mating trials in which opposite-sex individuals are confined together ("no-choice" trials), or in which males and/or females are given a choice between conspecific and heterospecific mates ("choice" trials). Sexual isolation is commonly assumed to arise from differences in mate preferences and the sexual displays they target, but in the absence of phenotypic measurements of candidate traits (i.e., sexual displays) from individuals used in these trials, the underlying traits cannot be identified. If we want to understand how this isolation has evolved, the phenotypes responsible need to be identified so that we can determine how these have diverged (Kirkpatrick and Ryan 1991; Coyne and Orr 2004; Maan and Seehausen 2011;

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Shaw and Mullen 2011). Sometimes the male signal is obvious (e.g., plumage color as in *Ficedula* flycatchers; Sætre and Sæther 2010), but much of the time it is not, in part because females may assess multiple traits during mate choice (Candolin 2003; Chenoweth and Blows 2006). Even for a well-studied group such as *Drosophila* in which patterns of sexual isolation are well characterized (Coyne and Orr 1989, 1997, 2004; Yukilevich 2012), in only a few cases have the signal traits been identified (Coyne et al. 1994; Tomaru and Oguma 1994; Ritchie et al. 1999; Howard et al. 2003).

Once the male signals are known, only then can female preferences for them can be quantified. A focus on mate preferences is useful not only to ascertain their involvement in sexual isolation (and ultimately whether and how they diverged), but when measured at the population-level the average mate preferences among a group of females is equivalent to the sexual selection gradient on the male traits they target (Wagner 1998; Chenoweth and Blows 2006). Therefore, assaying mate preferences within populations, and their divergence among populations, may not only provide insight into the causes of sexual isolation, but also into how sexual selection acts on male phenotypes and how this differs among populations. Although much attention has been given to assaying mate preferences and/or sexual selection within populations, comparatively little has been given to quantifying differences among populations (but see Arnqvist 1992; Gosden and Svensson 2008; Rundle et al. 2008), most notably with respect to the evolution of sexual isolation during speciation (but see Boughman 2001; Mendelson and Shaw 2002; Hobel and Gerhardt 2003; Rundle et al. 2005, 2009; Higgie and Blows 2007).

Here, we quantify and compare female mate preferences for male sexual displays among populations of *Drosophila subquinaria*. *Drosophila subquinaria* occurs in western N. America but in central Canada its range overlaps that of its eastern sister species *D. recens*. This area of sympatry is thought to represent a zone of secondary contact that has occurred within the last 12,000 years since the end of the Wisconsin glaciation (Jaenike et al. 2006). Both *D. subquinaria* and *D. recens* are generalists on basidiomycete mushrooms and in sympatry the adults of both species can be found on the same mushrooms at the same time of the year. The two species are morphologically indistinguishable except for the internal male genitalia (Wheeler 1960).

A pattern of reproductive character displacement exists in *D. subquinaria* such that females from sympatry discriminate against *D. recens* males more strongly than do females from allopatry, consistent with the reinforcement of sexual isolation (Jaenike et al. 2006; Bewick and Dyer 2014). *Drosophila recens* females also discriminate against *D. subquinaria* males, although this does not differ on average between sympatry and allopatry and is weaker than that observed by sympatric *D. subquinaria* females (Jaenike et al. 2006). Strong postzygotic isolation between the species exists in part due to the sterility of F1 hybrid males in both directions of the cross, but a *D. recens*-specific *Wolbachia* infection further strengthens this in one direction by greatly reducing the survival of hybrid offspring of both sexes produced by *D. subquinaria*, but not *D. recens*, females (Werren and Jaenike 1995; Shoemaker et al. 1999). This asymmetrical sexual isolation is consistent with a higher cost of hybridization for *D. subquinaria* compared to *D. recens* females. Such concordant asymmetries between pre- and postzygotic isolation are common between sympatric but not allopatric species-pairs of *Drosophila* and suggest that reinforcement may be common in this genus (Yukilevich 2012).

In addition to increased discrimination against *D. recens* males, sympatric *D. subquinaria* females also discriminate against their own allopatric males, although less strongly than they do against *D. recens* males (Jaenike et al. 2006; Bewick and Dyer 2014). There is no known postzygotic isolation between sympatric and allopatric *D. subquinaria* populations in the laboratory (Jaenike et al. 2006; K. A. Dyer, unpubl. data) and moderate genetic structuring of nuclear genome variation between sympatric and nearby allopatric populations implies some gene flow across the sympatry-allopatry barrier (Jaenike et al. 2006; Bewick and Dyer 2014). This suggests that increased discrimination by sympatric *D. subquinaria* females may have evolved through a process of reinforcement in response to low hybrid fitness, and as a by-product these females have evolved to find their own allopatric males less attractive. In support of this by-product scenario, there is some evidence of a shared genetic basis of discrimination against *D. recens* and allopatric *D. subquinaria* males (Bewick and Dyer 2014), although these behaviors have not been mapped genetically. The initiation of a secondary speciation process as a side effect of reinforced premating isolation implicates the evolution of sexual isolation in both the early and latter stages of separate speciation events, and has been termed "cascade" reinforcement (Ortiz-Barrientos et al. 2009; Hoskin and Higgie 2010).

Although morphological differentiation is limited to the internal male genitalia, *D. subquinaria* and *D. recens* share a suite of long-chain hydrocarbons and hydrocarbon-based derivatives (e.g., fatty acids) on their cuticles that differ in relative concentration such that the species are easily differentiated by their multivariate cuticular hydrocarbon (CHC) phenotypes (Curtis et al. 2013). All pure hydrocarbons (i.e., those containing only carbon and hydrogen) are also shared between the sexes, although there is extensive sexual dimorphism in their relative concentrations (Curtis et al. 2013). In both species there are also a few male-specific oxygen-containing compounds including 11-*cis*-Vaccenyl acetate (cVA) and several tri-acylglycerides that are common in other *Drosophila* (Yew et al. 2011; Curtis et al. 2013; Chin et al. 2014).

CHCs are used as sexual display pheromones in several *Drosophila* (Chenoweth and Blows 2005; Van Homrigh et al. 2007; Etges et al. 2009; Veltsos et al. 2012) and evidence strongly implicates a similar role in *D. subquinaria*. In particular, a detailed study of courtship and mating, including manipulations of various sensory modalities in both sexes, revealed that both allopatric and sympatric females are unusual in their near-exclusive reliance on olfaction for successful copulation. Rendering females deaf or blind had little effect on mating rates, but removal of their antennae, which prevents olfaction, decreased mating to near-zero (Giglio and Dyer 2013). Perfuming assays further implicate CHCs in the discrimination of *D. subquinaria* females against both *D. recens* and their own allopatric males (Dyer et al. 2014), although perfuming did not fully recover mating rates and therefore does not exclude the possibility of other traits contributing to sexual isolation as well. Finally, consistent with mate preferences for these traits, in binomial mate choice trials using *D. subquinaria* females from a single geographic population, variation in male mating success correlated with their CHC profile (Curtis et al. 2013).

Further suggestive of a central role for CHCs in sexual isolation, these traits exhibit a pattern of reproductive character displacement that closely mirrors that found for mate discrimination, with strong differentiation between sympatric and allopatric *D. subquinaria* populations but not among *D. recens* populations (Dyer et al. 2014). CHCs are also differentiated between allopatric *D. subquinaria* populations located on either side of the Coast Mountains in British Columbia (termed allopatric-coastal and allopatric-inland populations; Dyer et al. 2014). This coincides with a major phylogenetic break in mitochondrial but not nuclear DNA (Jaenike et al. 2006; Bewick and Dyer 2014). Despite differences in CHCs between these allopatric regions, there is little evidence of any mate discrimination between them, although sympatric females discriminate more strongly against coastal allopatric than inland allopatric males (Jaenike et al. 2006; Bewick and Dyer 2014).

The reproductive character displacement of mate discrimination in *D. subquinaria* suggests the underlying divergence of sexual displays and mate preferences as the cause. CHCs have been implicated in mate choice and sexual isolation and exhibit a matching pattern of character displacement consistent with their involvement in mate discrimination (Dyer et al. 2014). Here, we test for a similar pattern of reproductive character displacement in female mate preferences for male CHCs. To do this, we estimate preferences functions in eight separate *D. subquinaria* populations, including five allopatric and three sympatric with *D. recens*. In each population, we conduct replicate binomial-choice mating trials in which individual females are given the choice between two males that subsequently have their CHCs quantified by gas chromatography. In all cases males come from a common "mixed" (i.e., pooled allopatric–sympatric) stock, meaning that differences among populations can be attributed to the females and not the males among which they are choosing. After phenotyping more than 2700 males, we estimate population-average mate preferences using standard statistical techniques for quantifying selection and then compare these between sympatry and allopatry, treating populations as replicates.

Materials and Methods

POPULATIONS

We used flies from eight natural populations that span the geographic range of *D. subquinaria* and are from regions that are sympatric ($n = 3$) and allopatric ($n = 5$) with *D. recens*. The populations are listed in Table 1 along with the number of isofemale lines from each. Flies were reared at 20°C on a 12-h light: 12-h dark schedule on Instant *Drosophila* food (Carolina Biological, Burlington, NC) supplemented with fresh commercial *Agaricus bisporus* mushroom. All flies used in experiments were reared at a controlled density. Virgins were collected using light CO_2 anesthesia within 24 h of eclosion and stored on standard media, separately by sex, at a density of 10–15 flies per vial.

These populations were used to create a mixed stock that included lines from both sympatric and allopatric regions, thereby increasing variation in CHCs. As there is strong discrimination by sympatric females against mating with allopatric males, we used a crossing design that minimized the opportunity for mate preference expression. The stock was initiated with 384 vials, where each vial consisted of a cross between two lines. These crosses were set up such that each population was crossed to every other population in equal proportions (i.e., each population contributed females to 48 vials, of which six were crossed to males from each of the eight populations). Lines were chosen randomly from each population, with each line sampled more than once. From the second generation onward, we collected virgins from each cross, and then randomized the vials to cross. We did this for eight generations, and from the second generation onward we crossed a single virgin female and male per vial to minimize mate choice. When we collected virgin males from this stock to use in the mating trials, we first combined flies across vials and then randomly selected males to use.

MATING TRIALS

We conducted binomial mate choice trials in which we paired a single female with two males from the mixed stock. Mating trials took place in 4 mL vials that contained a blended mushroom-agar medium, and commenced within 2 h of the incubator lights turning on. All flies were virgin and seven to 10 days post adult eclosion at the time of testing, and were transferred into the mating vials

Table 1. **Populations used in this study. The location, abbreviation (Abbr.), and region with respect to overlap with *D. recens* are indicated for each. Allopatric (allo) populations are further designated as being west (coastal) or east (inland) of the Coast Mountains. Also shown is the abundance of *D. recens* relative to *D. subquinaria* at the time of collection (data from Bewick and Dyer 2014), and the number of isofemale lines included in the mating trials and in the creation of the mixed stock.**

Population	Abbr.	Region	Year collected	No. of *D. subquinaria* lines	Percentage of *D. subquinaria* among wild flies
Portland, OR	Po	allo-coastal	2010	9	100
Seattle, WA	Se	allo-coastal	2010	14	100
Deary, ID	De	allo-inland	2009	3	100
Missoula, MT	Mi	allo-inland	2010	5	100
Shuswap, BC	Sh	allo-inland	2010	2	100
Canmore, AB	Ca	Sympatric	2010	2	47
Hinton, AB	Hi	Sympatric	2010	14	27
Kawtikh, AB	Ka	Sympatric	2010	5	8

by air aspiration without the use of CO_2. Females were placed in the mating vials first; two males were then chosen randomly from different virgin vials and simultaneously added to the mating vial. Vials were observed until the female began mating with one of the two males, at which point the flies were anesthetized and the mating pair was gently separated using a paintbrush. Following Dyer et al. (2014), CHCs were extracted from the chosen and rejected males by placing an individual fly in 100 μl of hexane for 3 min and then vortexing the sample for 1 min, after which the fly was removed and discarded. CHCs were stored at −20°C until they were shipped from Athens, GA, to Ottawa, ON, for analysis. A total of 100–250 mating trials were conducted for each of the eight populations, with females chosen evenly from across the isofemale lines. Mating trials were conducted in a randomized block design to minimize the effects of day, time of day, and order of extraction.

QUANTIFYING CHCs

Samples were analyzed on an Agilent 6890N dual-channel "fast" (220 V oven) gas chromatograph (Agilent Technologies, Wilmington, DE) employing flame ionization detection and using previously published method parameters (Curtis et al. 2013). Individual profiles were determined by integration of the area under 17 peaks corresponding to those CHCs identified and quantified in past studies with the exception of hentria-*n*-*n*-contadiene ($C_{31:2}$), a very low concentration CHC that was not detectable in many individuals and was therefore excluded (Curtis et al. 2013; Dyer et al. 2014). These compounds consist of only odd carbon numbers (C_{29}, C_{31}, C_{33}, and C_{35}) and include methyl-branched alkanes, alkenes, and alkadienes, all of which are present in both sexes although many are sexually dimorphic in relative concentration (Curtis et al. 2013). The male-specific cVa and six triacylglycerides were not integrated as these compounds, which are known to be secreted exclusively from the ejaculatory bulb in other *Drosophila*, are transferred to females during mating and function to inhibit courtship by subsequent males (Yew et al. 2011; Curtis et al. 2013; Chin et al. 2014).

To correct for technical error associated with quantifying absolute abundances via gas chromatography, after integration the relative concentration of each CHC was calculated by dividing the area under each peak by the total area of all peaks for that individual. Proportions such as this are a form of compositional data to which standard statistical methods should not be applied (Aitchison 1986; Egozcue and Pawlowsky-Glahn 2011). To address this, we calculated centered log-ratio (CLR) coefficients as (Aitchison 1986):

$$CLR_n = \ln\left(\frac{p_n}{\left(\prod_{n=1}^{k} p_n\right)^{1/k}}\right), \quad (1)$$

where p_n is the relative concentration (i.e., proportional area) of CHC_n and the divisor is the geometric mean of the proportions of all $k = 17$ CHCs within an individual. CLR coefficients provide isometric properties with respect to the Aitchison geometry and are standard for use in principal components analyses (PCAs; Aitchison 1983). PCA was necessary because multicolinearity among the traits was high (maximum variance inflation factor > 25). CLR transformation results in homogeneously scaled trait values such that covariances among these traits play the role of correlations between nonscaled real variables (Aitchison 1983) and PCA was therefore performed on the covariance as opposed to correlation matrix of CLR-transformed traits. Rather than performing separate PCAs for each population, a single analysis was performed across all males used in the trials with females from all eight populations so that the resulting principal components (PCs) were comparable across populations (e.g., PC1 is the same trait in every population; Table S1). Prior to this, 32 outliers were removed using the Mahalanobis distance-based technique in the multivariate platform of JMP version 11.2 (SAS Institute Inc.,

Cary, NC), possibly representing contaminated samples or errors during integration. The resulting dataset consisted of 2769 males with an average of 346 males/population (range: 230–421). Because CLR-transformed trait values have a zero-sum constraint, the first 16 PCs account for all of the variation in relative CHC concentration, whereas the 17th has an eigenvalue of zero and was discarded.

QUANTIFYING MATE PREFERENCES WITHIN POPULATIONS

When the mating responses of a group of females from a population are individually tested against a range of male phenotypes, the resulting sexual selection gradient is equivalent to the population-average mate preference (Wagner 1998; Chenoweth and Blows 2006). Standardized sexual selection gradients were calculated separately for each population using first-order polynomial regression (Lande and Arnold 1983), fit using ordinary least squares, in which relative mating success was modelled as a function of the 16 PCs of the CLR-transformed CHCs (standardized separately by population: mean = 0, standard deviation = 1). Fixed effects of day of the mating trial and isofemale line designation for the females were never significant and were therefore excluded from all models. The overall importance of CHCs in explaining variation in male mating success was given by the adjusted coefficient of determination (R^2_{adj}).

In males, the relative concentrations of several of the CHCs, and hence at least some of their resulting PCs, were bimodally distributed suggestive of a locus or larger region of major effect on CHC expression segregating within the mixed stock (Fig. 1). Although this bimodality was unrelated to male mating success (Fig. S1), it violates the assumption of multivariate normality underlying polynomial regression, making standard statistical inference inappropriate. We therefore used a randomization procedure to determine overall model significance, as well as the significance of the individual gradients, separately in each population. In each of 10,000 iterations, mating success scores were randomly shuffled among males, the polynomial regression was rerun, and the resulting test statistics (F-value for overall model fit, t-values for the individual gradients) were recorded. P-values were calculated as the proportion of cases in which the value of the test statistic from a randomization was equal to, or greater than, the observed test statistic. Absolute t-values were used in all cases to provide a two-tailed test.

Our analysis of sexual selection within populations, and its comparison among populations (see below), was restricted to linear selection because we lacked sufficient replication to fully quantify nonlinear selection given the large number of CHCs in *D. subquinaria*. This problem is particularly acute when one wishes to compare selection among populations because doing so requires that it should be estimated on the same traits within each population, preventing the use of a canonical analysis to condense nonlinear selection onto the major axes of the response surface and hence reduce dimensionality (Blows and Brooks 2003; Chenoweth et al. 2012). Moreover, there was evidence of significant nonlinear selection in only two populations, one allopatric and one sympatric, when analyzing the entire suite of traits (i.e., 16 PCs; Table S2), suggesting that a focus on linear selection was reasonable.

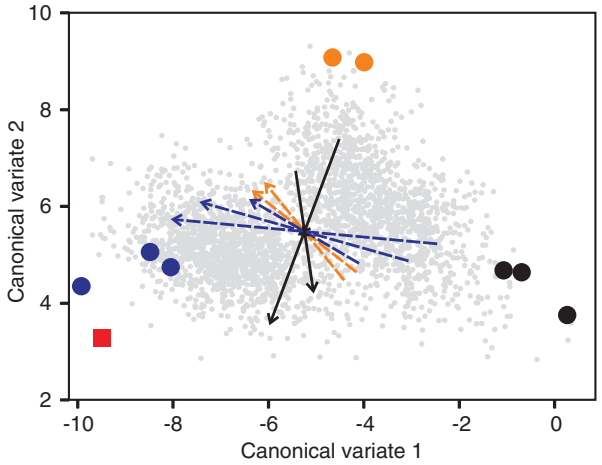

Figure 1. Standardized sexual selection vectors on cuticular hydrocarbons in male *D. subquinaria* when estimated from mating trials using males from a mixed stock (gray dots) and females from each of eight populations that are either sympatric (black points, solid lines) or allopatric (dashed lines) to *D. recens*. Allopatric populations are subdivided into inland (blue) and coastal (orange). Vectors are depicted for the first two combinations (i.e., canonical variates) of male CHCs that differ most among the eight *D. subquinaria* populations (Table S3). Circles indicate the average phenotype of virgin males from each of the eight populations in this phenotypic space (colors correspond to those of the selection vectors), and the red square denotes the average phenotype of *D. recens* males. Selection vectors have been magnified 20 times to make them visible on the scale of the among-population variation.

COMPARING MATE PREFERENCES AMONG POPULATIONS

Our primary interest is to compare average mate preferences of females that are sympatric versus allopatric to *D. recens*, treating populations as replicates. Differences in average female mate preference would be manifested as variation in the multivariate direction of sexual selection on male CHCs. The statistical comparison of selection among populations or other groups can be challenging and a number of different techniques have been used in past studies (Rundle et al. 2008, 2009; Chenoweth et al. 2012). One approach uses a multivariate mixed linear model that allows the regression coefficients for the continuous covariates

(i.e., the selection gradients on the male CHCs) to vary between the fixed effects of region (e.g., sympatry vs. allopatry) and that accommodates the random effect variation of these coefficients among populations nested within each regions via a random coefficient component (Meyer and Kirkpatrick 2005; Chenoweth et al. 2012). Such models are normally fit using restricted maximum likelihood, but preliminary analyses indicated likelihood convergence problems that rendered this approach unfeasible in our case.

As an alternative approach to testing for variation in preference, we compared linear selection gradients using a randomization technique based on their vector correlation. The correlation of $\boldsymbol{\beta}$ vectors provides a measure of the overall similarity of the multivariate direction of linear sexual selection and ranges from $+1$ (indicating the same direction, equivalent to an angle of 0°) to -1 (indicating opposite directions, or 180°), with 0 indicating orthogonal vectors (i.e., oriented at 90°). Correlations are calculated as the dot product of two vectors standardized to unit length. To test for differences in mate preference between sympatry and allopatry, we summed the standardized selection gradients (i.e., $\boldsymbol{\beta}$ vectors) separately for all allopatric and all sympatric populations, yielding a single vector that provides an overall measure of how sexual selection acts within each region. The orientation of these vectors was quantified via their correlation/angle. This observed vector correlation was compared to a distribution of 1000 correlations generated from a randomization procedure in which status (i.e., sympatry vs. allopatry) was shuffled among populations, the sympatric and allopatric vectors were re-summed, and the correlation of the resulting vectors was recomputed. This procedure does not alter the vectors of selection within each population, but generates a distribution of sympatric–allopatric vector correlations under the null hypothesis of no average difference between these regions. We performed this test when simply summing the $\boldsymbol{\beta}$ vectors for each region as described above, but also after standardizing the vector within each population to a unit length prior to summing. The former weights the contribution of each population to the total vector by the overall strength of selection in the population (i.e., $|\boldsymbol{\beta}|$), whereas in the latter all populations contribute equally because all vectors have the same magnitude. Finally, we also used an analogous randomization procedure to test for differences in preference when allopatric populations were subdivided into inland versus coastal, separately testing all three pairwise combinations of sympatric, allopatric inland, and allopatric coastal. However, with only five to six populations in total involved in these comparisons, power was limited.

Our tests of sexual selection within populations used all the PCs of CHC variation to fully describe the association between trait variation and mating success. However, not all CHCs are necessarily (or equally) divergent among populations and differences in preference are most likely to exist for combinations of traits that differ. Our comparison of mate preferences above therefore utilized the combinations of male CHCs that differed the most among populations, thereby reducing dimensionality, removing multicolinearity, and focusing inference on a smaller number of traits of biological interest. In a previous paper (Dyer et al. 2014), we quantified the CHCs of virgin males from these same populations. Using these data (males only), here we performed a discriminate analysis on the CLR-transformed CHCs to identify the trait combinations that differed among these eight *D. subquinaria* populations. The first five canonical variates were significant (see Results) and the resulting coefficients were used to score males from the mating trials to calculate their values of these five traits. Standardized selection gradients were estimated by regressing relative mating success against the standardized values of these traits separately in each population, as for the analysis of the PCs above, and it was these selection vectors that were used in the randomization procedure above to compare sexual selection between sympatry and allopatry. Significance of selection on these trait combinations within each population was evaluated via a randomization procedure that shuffled mating success scores among males, as previously described for the PCs.

Results

Linear sexual selection on CHCs in males was significant in each of the eight populations ($P \leq 0.028$ in all cases) and explained 9.7% of the variance in mating success on average (Table 2), consistent with female mate preferences for these traits. In general, selection gradients appeared variable across populations with a few exceptions. Sexual selection consistently favored lower values of PC7 in all populations, for example, although this was nonsignificant in one case. Differences in mate preference between sympatry and allopatry were also apparent for some trait combinations, with selection on PC3 being negative and significant in all three sympatric populations but weaker and nonsignificant in the five allopatric populations, and selection on PC4 was positive and significant (or nearly so) in all allopatric populations but weaker and nonsignificant in the sympatric populations.

Among virgin males, five CHC trait combinations (i.e., canonical variates) differed significantly among these eight populations and accounted for 99.2% of the among-population variation in CHCs (Table S3). When males from the mating trials were scored for these trait combinations, sexual selection on the five resulting canonical variates was significant overall in seven of the eight populations, with Canmore (Ca) being the single exception (Table 3). In sympatry, the overall vector of selection (a measure of the average population-average mate preference) was oriented 62.9° from that in allopatry, representing a vector correlation of 0.455. Consistent with reproductive character

Table 2. Quantifying female mate preferences for male CHCs separately in eight populations[1] of *D. subquinaria* that are allopatric to, or sympatric with, *D. recens*. Vectors of standardized directional sexual selection gradients (β) and adjusted coefficient of determination (R^2_{adj}) were estimated using first-order polynomial regression of relative mating success on the 16 principal components (PCs) of variation in CHC relative concentration (Table S1). Significance of the individual gradients[2], and overall model significance (*P*), were determined by a randomization procedure (see Methods).

	β (allopatric coastal)		β (allopatric inland)			β (sympatric)		
	Se	Po	De	Sh	Mi	Ca	Hi	Ka
P	<0.0001	0.0025	<0.0001	<0.0001	<0.0001	0.0275	<0.0001	<0.0001
R^2_{adj}	0.071	0.053	0.125	0.099	0.088	0.055	0.159	0.129
PC1	0.019	0.004	**−0.219**[5]	0.060	**−0.139**[4]	−0.007	**0.113**[3]	**0.177**[4]
PC2	−0.006	−0.052	0.007	−0.004	0.038	−0.002	**0.114**[3]	0.031
PC3	0.019	**0.083**	−0.045	0.048	−0.052	**−0.161**[3]	**−0.102**[3]	**−0.106**[3]
PC4	**0.199**[5]	**0.185**[5]	**0.077**	**0.231**[5]	**0.148**[4]	0.035	0.039	0.028
PC5	−0.021	**−0.066**	**−0.075**	**−0.089**[3]	−0.048	**−0.195**[4]	**−0.178**[4]	−0.032
PC6	−0.037	−0.008	**−0.206**[5]	−0.001	−0.044	0.025	−0.042	0.040
PC7	**−0.070**	**−0.095**[3]	**−0.123**[4]	**−0.175**[5]	**−0.080**[3]	−0.065	**−0.154**[4]	**−0.285**[5]
PC8	**−0.165**[5]	**−0.093**[3]	−0.014	−0.012	**−0.081**[3]	**−0.127**[3]	−0.041	0.023
PC9	**0.069**	−0.020	**−0.069**	−0.032	0.039	−0.054	−0.073	−0.003
PC10	0.027	0.008	**0.087**[3]	0.036	0.025	**0.087**	−0.001	0.003
PC11	**0.082**[3]	0.063	**−0.094**[3]	−0.029	0.017	**0.123**[3]	**0.193**[5]	**0.127**[3]
PC12	−0.023	0.029	0.019	−0.033	0.041	0.012	−0.050	−0.028
PC13	−0.057	**−0.096**[3]	**−0.184**[5]	**−0.135**[4]	**−0.165**[5]	−0.016	**−0.210**[5]	**−0.186**[5]
PC14	−0.055	−0.026	0.007	0.047	**−0.159**[4]	−0.056	**−0.096**	−0.067
PC15	**0.081**	**0.094**[3]	−0.045	−0.034	0.006	−0.002	0.054	0.068
PC16	**0.071**	0.024	0.058	**0.124**[4]	**0.082**[3]	**0.177**[4]	0.056	0.066

[1]Population abbreviations are given in Table 1.
[2]Bold denotes selection gradients for which $P < 0.1$.
[3]$P < 0.05$.
[4]$P < 0.01$.
[5]$P < 0.001$.

displacement of mate preferences, this difference in orientation was significantly greater (i.e., the vector correlation was significantly smaller) than that expected under the null hypothesis of no effect of sympatry versus allopatry (randomization procedure, $P = 0.013$). This does not change qualitatively if populations are weighted equally (instead of by the strength of selection) when summing the selection vectors in sympatry and allopatry (vector correlation = 0.508, angle = 59.5°, $P = 0.015$), nor if Canmore is excluded and the comparison is performed using only the seven populations in which selection on these traits is significant overall (vector correlation = 0.396, angle = 66.6°, $P = 0.04995$).

The strongest differences in selection were observed for canonical variates 1 and 2 (CV1 and CV2), the two trait combinations that are the most divergent among populations in males (accounting for 72.6% and 21.1% of the among-population variation, respectively; Table S3). CV1 primarily distinguished sympatric from nearby allopatric-inland populations, with allopatric-coastal populations being intermediate between these whereas CV2 distinguished coastal populations from the others (Fig. 1). For CV1, allopatric females tended to prefer lower values more strongly in did sympatric females, whereas for CV2, sympatric females tended to prefer lower values while coastal allopatric females tended to prefer higher values (Fig. 1). If allopatric populations are further subdivided into coastal versus inland, none of the pairwise comparisons of sexual selection among these three regions were significant, although the separate contrasts of sympatric with allopatric inland and allopatric coastal both approached significance ($P < 0.1$; Table 4).

Discussion

Phenotypic analyses revealed significant sexual selection on CHCs in male *D. subquinaria* when using females from each of the eight populations, consistent with results from an earlier study that used females and males from a single allopatric population (Deary, ID; Curtis et al. 2013). Variation in male CHCs explained 9.7% (range 5.3–15.9%) of the variance in male mating success on average, similar to that observed for sexual

Table 3. Standardized sexual selection gradients (β) in eight different populations[1] for the first five canonical variates defining the combinations of male CHCs that differ most among these populations (Table S3). Significance of the individual gradients[2], and overall model significance (*P*), were determined by a randomization procedure (see Methods).

	β (allopatric coastal)		β (allopatric inland)			β (sympatric)		
	Se	Po	De	Sh	Mi	Ca	Hi	Ka
P	0.014	<0.001	<0.001	<0.001	0.003	0.589	<0.001	0.005
CV1	−0.091	**−0.118**[3]	**−0.304**[5]	**−0.124**[3]	**−0.238**[5]	−0.014	−0.079	0.021
CV2	**0.114**	0.094	0.029	0.074	0.069	−0.007	**−0.217**[4]	**−0.141**[3]
CV3	−0.029	−0.064	**−0.213**[5]	**−0.120**[3]	−0.045	−0.020	−0.062	−0.057
CV4	**−0.121**[3]	**−0.120**[3]	**−0.160**[4]	**−0.191**[5]	**−0.158**[4]	−0.098	**−0.233**[5]	−0.057
CV5	0.065	**0.129**[3]	0.049	**0.115**	0.006	0.076	**0.251**[4]	**0.232**[4]

[1]Population abbreviations are given in Table 1.
[2]Bold denotes selection gradients for which $P < 0.1$.
[3]$P < 0.05$.
[4]$P < 0.01$.
[5]$P < 0.001$.

Table 4. Pairwise comparison of average sexual selection vectors among the three geographic regions. Vector correlations (and equivalent angles) are shown below the diagonal and significance (randomization *P*-values) above.

Region	Allopatric coastal	Allopatric inland	Sympatric
Allopatric coastal	NA	0.180	0.081
Allopatric inland	0.879 (28.4°)	NA	0.099
Sympatric	0.446 (63.5°)	0.441 (63.8°)	NA

selection on male CHCs in two other *Drosophila* species (Van Homrigh et al. 2007; Sztepanacz and Rundle 2012; Gershman et al. 2014). Standardized directional selection gradients on some trait combinations were also large, with one or more gradients in every population approaching or exceeding 0.18 (the median absolute strength in studies measuring variation in mating success in nature; Kingsolver et al. 2001). When estimated from a random sample of females the resulting sexual selection gradients are equivalent to the population-average female mate preference for these traits (Wagner 1998; Chenoweth and Blows 2006). The existence of female mate preferences for male CHCs in *D. subquinaria* is consistent with the results of two previous manipulative experiments showing that sympatric *D. subquinaria* females were more likely to mate with allopatric *D. subquinaria* males, and with *D. recens* males, when each was perfumed with sympatric *D. subquinaria* CHCs (Dyer et al. 2014). Perfuming, however, only partially recovered mating compatibility and neither these results nor the current rule out a contribution of other traits to mate discrimination. Mate choice has been shown to be multimodal in other *Drosophila*, for example, involving both wing song and pheromones (Etges et al. 2009; Veltsos et al. 2012), and although sensory manipulations in *D. subquinaria* suggest a predominant role for olfaction, signal modalities may interact during mate choice (Rybak et al. 2002) and this could help explain some of the variance in male mating success that is not accounted for by CHCs on their own.

Sexual selection on CHCs in males also varied among the eight populations and differed significantly between those that are sympatric versus allopatric with *D. recens*. Because females from all populations were presented with males from a common mixed stock, this variation in sexual selection can be attributed to differences in the females themselves, implying the divergence of female mate preferences in a pattern of reproductive character displacement. Reproductive character displacement in this species has been previously shown for sexual isolation (Jaenike et al. 2006) and CHCs (Dyer et al. 2014), and a difference in mate preferences between sympatry and allopatry suggests that the stronger sexual isolation observed in sympatry is the result at least in part of the combined divergence of both male CHC displays and female preferences for them. Although consistent with the reinforcement of sexual isolation in sympatry in response to maladaptive hybridization, there are other processes that can create such a pattern including "differential fusion" and ecological character displacement (Noor 1999). Definitive support for reinforcement will therefore require manipulative evidence that the existing patterns of reproductive character displacement in male sexual displays, female preferences, and sexual isolation itself, will evolve under experimental sympatry and that this occurs in response to reduced hybrid fitness in particular (see Higgie et al. 2000; Matute 2010).

A pattern of reproductive character displacement consistent with reinforcement has been shown in multiple studies

involving specific species pairs, and broader-scale comparative studies also support a role for reinforcement (Coyne and Orr 1989, 1997, 2004; Howard 1993; Noor 1997; Servedio and Noor 2003; Yukilevich 2012). The vast majority of these data quantify divergence in terms of some measure of sexual isolation, or sometimes sexual signals or displays involved in mate discrimination, whereas demonstrations of character displacement of mate preferences are much rarer. Female preference functions for male red nuptial coloration in sympatric Limnetic threespine sticklebacks differ significantly from that of the Benthic and solitary ecomorphs (Boughman 2001). Mate preferences of female *D. serrata* for male CHCs also differ significantly between populations that are sympatric versus allopatric with the related *D. birchii* (Higgie and Blows 2007). Female preference for conspecific over heterospecific signals in green tree frogs (*Hyla cinerea*) were also shown to be significantly stronger in sympatry than in allopatry, although preference function for male call frequency did not differ in shape as expected (Hobel and Gerhardt 2003). Finally, in Hawaiian cricket in the genus *Laupala*, male pulse rate and female preferences for this male song characteristic have diverged among populations and species in correlation with one another and sympatric species can always be distinguished by their pulse rate, although a clear pattern of reproductive character displacement is not apparent (Mendelson and Shaw 2002; Grace and Shaw 2011, 2012). Beyond these few case studies, the limited attention that has been given to quantifying differences in preferences is surprising, as whether and how they diverge is a distinguishing feature of many models for the evolution of sexual isolation (Kirkpatrick and Ravigné 2002; Maan and Seehausen 2011). Reproductive character displacement, for example, can arise from direct or indirect selection on signal traits or preferences, yet little is known about the relative importance of these (Servedio 2001; Kirkpatrick and Ravigné 2002; Servedio and Noor 2003).

Differences in sexual selection among the three regions (i.e., sympatric, allopatric inland, allopatric coastal) partially correspond with existing differences in male CHCs (i.e., selection vectors are rotated in the direction of the current phenotypes in these regions; Fig. 1), suggesting that divergent sexual selection has contributed to CHC divergence. However, in no case is this alignment perfect, suggesting that male CHCs are not at their sexual selection optimum in any of these populations. In addition to their role as sexual displays, CHCs form a waxy layer on the cuticle of the fly that reduces water loss and thus protects against desiccation (Gibbs 1998; Foley and Telonis-Scott 2011), making these traits likely targets of natural as well as sexual selection (e.g., see Gibbs et al. 1997; Kwan and Rundle 2010). Current trait values may therefore represent a compromise between those favored by natural versus sexual selection. In addition to the demonstrated differences in sexual selection, natural selection on CHCs may also vary among regions due to differences in climate or other environmental variables (Frentiu and Chenoweth 2010).

The discrepancy between the direction of sexual selection and current trait values appears greatest in sympatry (Fig. 1), suggesting that males in these populations are furthest from their sexual selection optimum. In an evolution experiment using mixed sympatric–allopatric populations of *D. serrata*, Higgie and Blows (2008) similarly found that under conditions of experimental allopatry, male CHCs and female preferences for them evolved in the presence of sexual selection to resemble those seen in allopatric populations in nature, suggesting that allopatric populations were closer to, or at, their sexual selection optimum, whereas changes in selection caused by the presence of *D. birchii* caused sympatric populations to deviate from this. We estimated sexual selection in the absence of *D. recens*, but its presence in sympatric populations may cause reproductive interference that alters mate choice and the resulting selection gradients. Indeed, selection arising from costly interspecific interactions during mate acquisition may itself drive the evolution of increased premating isolation in sympatry, thereby producing a pattern of reproductive character displacement without selection via reduced hybrid fitness (Gröning and Hochkirch 2008). In *D. serrata*, for example, changes in selection on CHCs in sympatry appear not to arise from reduced hybrid fitness but rather to be caused by some form of reproductive interference (Higgie et al. 2000).

Selection vectors represent the average female mate preference and differences in preferences among regions may also help explain existing patterns of sexual isolation. For example, for these trait combinations, inland allopatric males are quite similar to *D. recens* males whereas the geographically adjacent sympatric males have diverged from this along CV1, the primary axes of among-population differentiation (Fig. 1). Inland allopatric females, and to a lesser extent coastal allopatric females, prefer more allopatric-like males for this trait combination (i.e., those with lower CV1 values), whereas mate preferences of sympatric females are on average relatively insensitive to this trait combination, although there is no evidence that females have reversed their preference to favor higher values (Fig. 1). Sympatric females also discriminate against *D. recens* males more strongly than do nearby inland allopatric females, and mate preferences of sympatric females are more poorly aligned with *D. recens* phenotypes than are those of inland allopatric females (Fig. 1). Sympatric females also discriminate against their own allopatric males, and they do this more strongly against coastal as compared to inland males. The preference of sympatric females for low values of CV2 is consistent with this as coastal males will be less attractive than inland males. Finally, coastal allopatric populations differ from the other *D. subquinaria* populations along CV2 and females from these populations show a stronger preference for higher CV2 values than do females from any of the other populations.

Nevertheless, patterns of mate preferences for these trait combinations do not fully explain those of sexual isolation. For example, although both coastal and inland allopatric females show some discrimination against sympatric males in mating trials, the effect is weak relative to the discrimination by sympatric females against allopatric males (Jaenike et al. 2006; Bewick and Dyer 2014). However, our preference estimates suggest that allopatric females should find sympatric males quite unattractive due to their high values of CV1 (Fig. 1). Some caveats to the interpretation of our results may be important here. First, our analyses focused on linear preferences because we lacked sufficient replication to fully quantify nonlinear selection given the large number of traits involved (see Methods). Although nonlinear selection was generally nonsignificant, this may in part be due to insufficient power to detect it as some of the estimated quadratic gradients were not small (Table S2). It is therefore possible that preferences include nonlinear components that affect mating compatibilities and that have not been accounted for in our current analyses. Second, as noted earlier, other traits may also contribute to sexual isolation. Mate choice is clearly based on multiple signals in some *Drosophila* (Etges et al. 2009; Veltsos et al. 2012) and male *D. subquinaria* do perform wing extensions and vibrations during courtship. Interestingly, sympatric males do this more frequently than allopatric males and removal of male wings significantly reduces mating rates (Giglio and Dyer 2013). However, neither blinding females nor removing their aristae (which renders them deaf) had an effect on mating rates, suggesting that these wing actions do not function as auditory courtship songs or visual displays, but rather may serve to fan pheromones during courtship. Finally, our analysis also focused on the combinations of CHCs that differ the most among populations, but preferences may also differ for trait combinations that are less divergent.

In addition to female mate preferences, male–male interactions may also contribute to the outcome of binomial choice mating trials and the resulting sexual selection gradients may therefore represent the combined influence of both processes. In *D. subquinaria*, however, there is little evidence of male–male competition, at least during mating trials like those we used, and even if present, this is unlikely to have produced the differences in sexual selection we detected. With respect to the prevalence of male–male competition, compared to *D. melanogaster* in which males are visibly aggressive with one another, *D. subquinaria* males are much more subdued, suggesting that direct male–male interactions are less important. Forced copulations also do not occur in laboratory mating trials, as is evidenced by the low mating rate of sympatric *D. subquinaria* with allopatric conspecific males and the complete lack of mating of these females with *D. recens* males (Bewick and Dyer 2014). Although males of several other *Drosophila* have been shown to compete over territories consisting of food (live yeast) and egg-laying substrate needed by females (e.g., Hoffmann 1987; White and Rundle 2014), our mating trials were designed to minimize the opportunity for this to occur. In particular, the trials combined two males from separate vials (i.e., males that had no previous social interactions with one another) into a new vial in which the female was already present. Females prefer to oviposit onto fresh mushrooms whereas these vials contained only blended mushroom within the medium and also lacked live yeast over which males may compete for territories. Mating generally occurred quickly under these circumstances (i.e., < 15 min.) and males were rarely observed interacting with each other (K. Dyer, pers. obs.). Most importantly, even if intrasexual selection did contribute to our estimates of selection, the males in all trials came from the same mixed stock so variation in this is unlikely to have caused the observed differences in selection gradients among populations in females.

Our analysis of among-population variation in selection focused on the combination of CHCs that were most divergent among populations, with the idea that differences in preference will be more readily detected for trait combinations that also differ. Although sexual selection on CHCs was significant in all eight populations when considering the entire suite of CHCs, it was not significant in one—Canmore, AB (Ca)—when tested on these particular trait combinations. Why sexual selection on these traits was nonsignificant in this population is unknown, although it had the lowest replication of all populations (230 vs. an average of 346 males phenotyped) so power was reduced. Sexual selection was correspondingly weak in this population overall, with a low R^2 and the highest P-value of any of the populations (Table 2). Binomial mate choice trials tend to be noisy and it is therefore possible that female mate preferences do target these traits combinations in this population but we failed to detect them due to sampling error. In *D. serrata*, for example, sexual selection on male CHCs have been estimated via binomial choice trials in at least a half-dozen separate studies, yet despite remarkably strong consistency in the resulting gradients (Gershman et al. 2014), assays on this scale fail to detect it on occasion (H. Rundle, unpubl. results). Alternatively, female mate preferences in Ca may differ from that in the other sympatric populations for other reasons, targeting different traits or combinations of CHCs that those captured by the among-population canonical variates. Independent assays will be needed to distinguish sampling error from a true preference difference for a single population like this, although the pattern of character displacement is significant whether Ca is included in the analysis.

In conclusion, we demonstrate differences in sexual selection among *D. subquinaria* populations that imply the divergence of female mate preferences for male sexual display pheromones in a pattern of reproductive character displacement. Reproductive character displacement was previously demonstrated for mate

discrimination and CHCs (Jaenike et al. 2006; Dyer et al. 2014) and its existence for mate preferences implicates their divergence in the latter stages of the *D. subquinaria–D. recens* speciation. Variation in mate preferences partially, but not fully, aligns with existing differences in CHCs, suggesting that differences in sexual selection have contributed to CHC divergence but that other unidentified factors may also be involved (e.g., spatial variation in natural selection). Variation in mate preferences also help to explain some of the existing patterns of sexual isolation including the stronger discrimination of sympatric as compared to allopatric females against *D. recens* males, and the stronger discrimination of sympatric females against coastal as compared to inland allopatric males. However, other patterns, like the weak sexual isolation of allopatric females from sympatric males, remain unaccounted for, suggesting the potential involvement of other traits, combinations of CHCs, and/or more complex (nonlinear) preferences. A comprehensive understanding of the evolutionary origins of enhanced isolation in sympatry will require further study to determine the source and targets of selection that are ultimately responsible.

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DATA ARCHIVING

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Supporting Information

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Table S1. Eigenvectors from a principal component analysis of the covariance matrix of 17 CLR-transformed CHCs across all male *D. subquinaria* used in the mating trials.

Table S2. Standardized quadratic selection gradients on the major axes of γ (the matrix of quadratic and cross-product terms quantifying quadratic and correlational selection on the original traits).

Table S3. Scoring coefficient for the first five canonical variates from a discriminate analysis among populations of CLR-transformed CHCs in virgin males.

Figure S1. Biplot of the first two principal components of variation in relative concentrations of cuticular hydrocarbons from all the males from mixed stock used in the mating trials.

Table S1. Eigenvectors from a principal component analysis of the covariance matrix of 17 CLR-transformed CHCs across all male *D. subquinaria* used in the mating trials, and percent variance explained by each principal component (PC). The 17th PC has an eigenvalue of zero (i.e. accounts for no variance) due to the unit-sum constraint inherent in compositional data and is therefore not shown.

Trait	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12	PC13	PC14	PC15	PC16
% var	60.68	17.86	5.47	3.24	2.43	2.12	2.09	1.61	1.26	1.02	0.62	0.49	0.39	0.35	0.21	0.16
CHC2	0.0648	-0.0182	0.0895	-0.1388	0.2793	-0.3222	0.3173	0.3119	0.1074	0.4433	0.3624	0.1073	-0.1205	0.3915	-0.0621	0.0842
CHC3	0.2506	0.3718	0.0162	-0.0528	-0.1503	0.3645	0.2544	0.0615	0.4513	-0.0095	0.1021	-0.2642	-0.1154	-0.2577	-0.3776	-0.0825
CHC4	0.2863	0.4722	-0.2623	0.0663	-0.2193	0.0010	0.2387	-0.1396	-0.2909	0.0322	-0.1067	-0.0911	0.2840	0.2734	0.4016	0.1283
CHC5	0.1071	-0.0052	-0.1418	-0.2583	0.6107	-0.0728	0.2030	0.1632	-0.2549	-0.2593	-0.2189	0.0495	0.2224	-0.3920	-0.0742	-0.0751
CHC6	0.4675	0.1463	0.1325	-0.2547	-0.0300	-0.1717	-0.6554	-0.0507	-0.1969	-0.0842	0.2381	0.0291	-0.2211	-0.0366	-0.0418	-0.0318
CHC7	0.0248	0.0634	-0.1678	0.3275	0.0413	0.3733	-0.1643	0.3156	0.0941	-0.0991	-0.1173	0.6863	-0.1043	0.1384	0.0068	-0.0003
CHC9	-0.1230	-0.0340	0.5416	0.3646	-0.1986	-0.0297	0.0865	0.3396	-0.3562	-0.3008	0.1473	-0.1699	0.1934	0.0225	-0.1666	0.0440
CHC10	0.2223	-0.2655	0.4013	0.0422	-0.1715	-0.0723	0.1864	-0.2473	0.1608	0.2353	-0.0654	0.3155	0.0670	-0.4521	0.3483	0.1127
CHC11	-0.0912	-0.1420	0.1292	-0.0168	0.3290	0.2424	-0.0285	-0.1180	0.2499	-0.3762	0.0453	-0.3047	-0.2794	0.2152	0.4284	0.3274
CHC12	0.0569	-0.1171	0.1051	0.2424	0.1650	-0.0043	0.0932	-0.4183	-0.0466	0.0063	-0.1177	-0.0458	-0.1272	0.2678	-0.0664	-0.7301
CHC13	0.2340	-0.3981	-0.1106	0.1080	0.0050	0.0085	-0.2352	-0.0437	0.1461	0.2006	-0.3855	-0.2223	0.3445	0.2199	-0.3602	0.3005
CHC14	-0.0953	-0.2229	-0.3748	0.2254	-0.0991	0.0372	-0.1317	0.3530	-0.1442	0.3253	0.0563	-0.3667	-0.2314	-0.3175	0.2895	-0.1885
CHC17	-0.4035	0.3380	0.2267	-0.2034	-0.1020	-0.2190	-0.0958	0.0800	0.0206	0.1509	-0.6207	-0.0187	-0.2934	0.0147	-0.0265	0.0404
CHC18	-0.1700	-0.3505	-0.0935	-0.5755	-0.3543	0.3698	0.1754	-0.0723	-0.3059	-0.0369	0.0786	0.1119	-0.0733	0.1393	-0.1100	-0.0260
CHC19	-0.4182	0.1567	0.0504	-0.1680	0.0680	0.0973	-0.3281	-0.0340	0.2690	0.1211	0.2586	0.0053	0.6021	-0.0154	0.1568	-0.2162
CHC20	-0.3260	0.1398	-0.1904	0.2784	0.1437	-0.0295	-0.0047	-0.4960	-0.2320	0.1298	0.2655	0.0858	-0.1408	-0.1982	-0.3092	0.3617
CHC21	-0.0872	-0.1347	-0.3512	0.0136	-0.3169	-0.5726	0.0890	-0.0049	0.3284	-0.4788	0.0779	0.0928	-0.0064	-0.0132	-0.0368	-0.0486

Table S2. Standardized quadratic selection gradients on the major axes of γ (the matrix of quadratic and cross-product terms quantifying quadratic and correlational selection on the original traits). Gradients are the eigenvalues from the diagonalization of γ when estimated by regressing relative mating success against the 16 standardized principal components of CLR-transformed CHCs separately in each population. *P*-values (in parentheses) are based on 1000 permutations/population using the method of Reynolds et al. (2010)[a].

	Allopatric coastal		Allopatric inland			Sympatric		
Axis of γ	Se	Po	De	Sh	Mi	Ca	Hi	Ka
1	0.594 (0.840)	0.485 (0.518)	**0.761 (0.020)**	0.361 (0.797)	0.437 (0.518)	1.091 (0.976)	0.736 (0.891)	0.828 (0.137)
2	0.366 (0.617)	0.418 (0.648)	0.434 (0.467)	0.256 (0.990)	0.371 (0.521)	0.953 (0.499)	0.486 (0.994)	0.657 (0.310)
3	0.301 (0.782)	0.359 (0.522)	0.379 (0.433)	0.248 (0.952)	0.235 (0.977)	0.685 (0.918)	0.476 (0.485)	0.477 (0.104)
14	-0.372 (0.179)	-0.390 (0.417)	-0.321 (0.412)	-0.266 (0.504)	-0.391 (0.169)	-0.547 (0.954)	-0.381 (0.323)	-0.615 (0.321)
15	-0.576 (0.092)	-0.530 (0.195)	-0.513 (0.375)	-0.294 (0.515)	-0.405 (0.608)	-0.666 (0.999)	-0.410 (0.373)	**-0.900 (0.002)**
16	-0.638 (0.115)	-0.632 (0.143)	-0.561 (0.890)	-0.369 (0.705)	-0.622 (0.933)	-1.223 (0.698)	-0.597 (0.835)	**-1.127 (0.039)**

[a]To minimize the number of comparisons, only the three most positive and the three most negative gradients, representing concave and convex selection respectively, were tested (Morrissey 2014). Bold values denote nominal $P < 0.05$.

Table S3. Scoring coefficient for the first five canonical variates from a discriminate analysis among populations of CLR-transformed CHCs in virgin males, and the percent variance and significance of each. Data from Dyer et al. (2014).

Trait	CV1	CV2	CV3	CV4	CV5
% var (*P*)	72.60 (<0.001)	21.07 (<0.001)	2.97 (<0.001)	1.79 (<0.001)	0.80 (0.040)
CHC2	0.304	0.183	0.764	-1.643	0.060
CHC3	-0.951	-0.164	2.706	-0.641	-1.711
CHC4	0.816	-1.278	-0.011	0.385	1.681
CHC5	-1.509	-1.260	0.122	2.482	-1.402
CHC6	-0.731	0.030	-0.074	-0.431	0.721
CHC7	-0.752	0.696	-0.211	0.254	0.119
CHC9	-0.967	0.555	0.192	-0.011	0.246
CHC10	1.139	-0.329	-0.338	0.069	0.227
CHC11	-0.175	-1.590	0.527	1.254	1.863
CHC12	-3.566	2.701	3.080	0.060	1.750
CHC13	0.752	1.097	-0.748	0.397	-1.600
CHC14	-1.887	-0.963	1.958	-0.443	2.521
CHC17	-1.269	-0.830	-0.520	0.222	-0.182
CHC18	-0.119	0.113	1.051	0.203	0.242
CHC19	-0.939	0.078	1.257	0.410	-0.046
CHC20	-0.979	0.726	-0.320	-0.346	-1.608
CHC21[a]	0	0	0	0	0

[a]CLR-transformed traits have a zero-sum constraint and variation in the 17 relative concentrations is therefore be fully explained as a function of the first 16 traits.

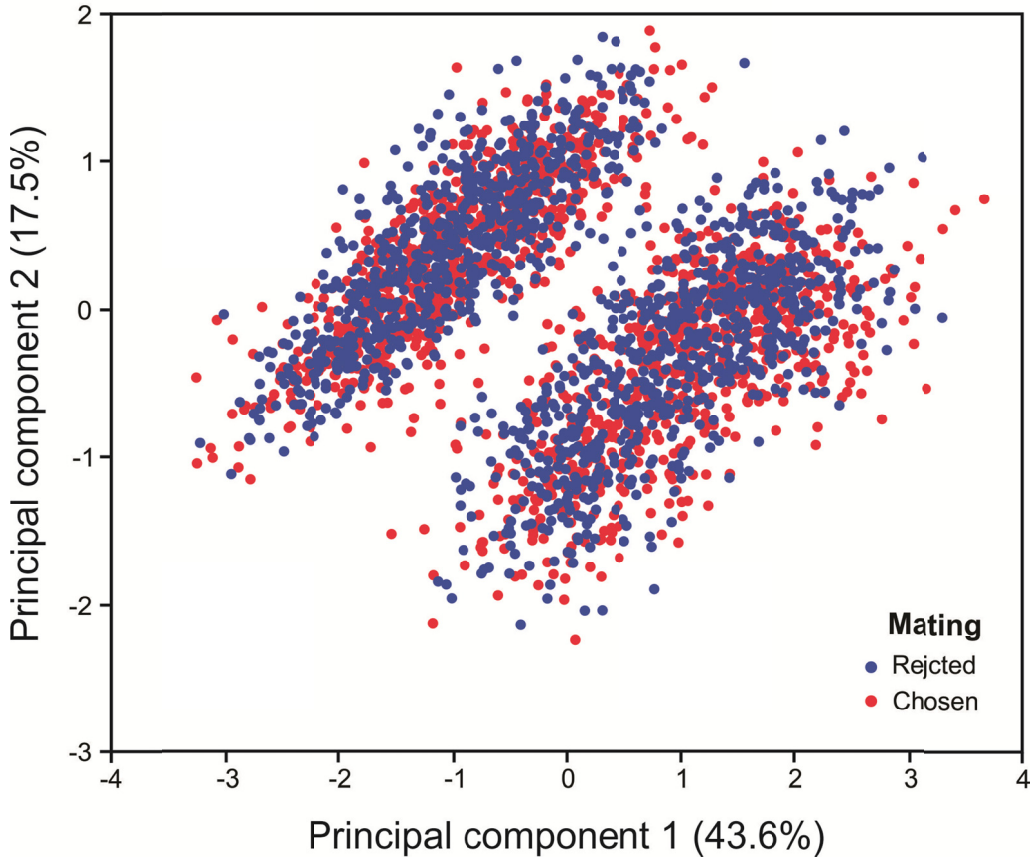

Figure S1. Biplot of the first two principal components of variation in relative concentrations of cuticular hydrocarbons from all the males from mixed stock used in the mating trials. Eigenvectors defining the trait combinations are given in Table S1.

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