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# Are Traits That Experience Reinforcement Also Under Sexual Selection?

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abstract: Where closely related species occur in sympatry, reinforcement may result in the evolution of traits involved in species recognition that are at the same time used for within-species mate choice. *Drosophila serrata* lives in forested habitat on the east coast of Australia, and over the northern half of its distribution it coexists with a closely related species, *Drosophila birchii*. Here we show that the strength of reinforcing selection in natural populations is sufficient to generate reproductive character displacement along a 36-km transect across the contact between sympatric and allopatric populations of *D. serrata*. The sympatric and allopatric populations display genetically based differences in male cuticular hydrocarbons (CHCs), while female CHCs changed with latitude across the contact. The directional changes observed in male CHCs between sympatric and allopatric regions were the same changes that were generated by experimental sympatry in the laboratory, providing direct evidence that the changes across the contact zone are due to the presence of *D. birchii*. We show that sympatric and allopatric females differ in preference for male CHCs and that females from allopatric populations prefer allopatric-like male CHCs over sympatric-like CHCs. Male attractiveness within *D. serrata* may therefore be compromised by reinforcing selection, preventing the spread of sympatric-like blends to the area of allopatry.

*Keywords:* reproductive character displacement, species recognition, mate choice, mate recognition, cuticular hydrocarbons, *Drosophila serrata*.

There are two distinct levels at which the evolution of traits involved in the selection of mates can occur. Within

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a species, the process of intraspecific mate choice (sexual selection) may drive the evolution of both mating preferences and display traits (Kirkpatrick and Ryan 1991; Andersson 1994). When closely related species are in sympatry, the need for interspecific mate choice (species recognition) may also influence mating preferences and displays (Otte and Endler 1989). In species where the same traits are used for both species recognition and sexual selection, mate choice should be examined as a continuum between these two levels (Endler 1989; Rand et al. 1992; Ryan and Rand 1993; Endler and Houde 1995; Ptacek 2000). In such species, the processes of sexual selection and species recognition may interact, with species recognition affecting how within-species mate choice occurs (Gerhardt 1982; Ryan and Rand 1993; Pfennig 1998; Hoskin et al. 2005; Phelps et al. 2006).

One way in which sexual selection and species recognition may interact is during the process of reinforcement. Reinforcement of mate recognition occurs when there is selection against individuals who make the mistake of mating with a closely related species (Dobzhansky 1951; Howard 1993). Although reinforcement has been a controversial topic in evolutionary biology, empirical evidence has been accumulating for the pattern of reproductive character displacement, generated by reinforcing selection, occurring across a wide variety of taxa (e.g., Noor 1995; Saetre et al. 1997; Nosil et al. 2003; Pfennig 2003; Hoskin et al. 2005). In addition to the finding of the pattern of reproductive character displacement in natural populations, manipulative evidence for the process of reinforcement in natural populations of *Drosophila serrata* has been established by reproduction of reproductive character displacement through the application of experimental sympatry with *Drosophila birchii* under laboratory conditions (Higgie et al. 2000). Genetic analyses of reproductive character displacement are now beginning to determine the genetic basis of the response to reinforcing selection (Blows and Higgie 2003; Ortíz-Barrientos and Noor 2005).

One of the remaining objections to the operation of the process of reinforcement in natural populations has been that gene flow would inhibit the evolution of reproductive

character displacement (Moore 1957; Bigelow 1965; Howard 1993; Hoskin et al. 2005). There are two ways in which gene flow could influence the efficacy of reinforcing selection. In systems in which hybrids are produced between divergent lineages, gene flow through hybridization may homogenize the two lineages involved (Liou and Price 1994; Servedio and Kirkpatrick 1997; Kirkpatrick 2000; reviews in Turelli et al. 2001; Servedio and Noor 2003). Alternatively, gene flow among populations of the same lineage from outside the area of sympatry may swamp those populations experiencing reinforcing selection and thus inhibit the evolution of reproductive character displacement. Although recognized as a serious problem for the theory of reinforcement (Moore 1957; Bigelow 1965; Howard 1993), this aspect of gene flow has received little attention (but see Nosil et al. 2003; Hoskin et al. 2005), and few studies have examined changes in reproductive characters over small spatial scales. However, adaptation in the face of substantial gene flow has been shown to be possible across abrupt ecotones as a consequence of abiotic environmental factors (Endler 1977; e.g., Smith et al. 1997; Schneider et al. 1999; Michalak et al. 2001), but it is not known whether reinforcing selection is sufficiently strong to maintain reproductive character displacement over similarly small spatial scales.

Examining how reproductive character displacement is maintained over a small spatial scale would enable a detailed ecological and genetic analysis of how mate recognition evolves in nature (Barton 2000), for two reasons. First, determining the geographic distribution of phenotypes over such a small spatial scale allows reproductive character displacement to be studied in an area where reinforcing selection is actively maintaining the pattern of divergent phenotypes in the presence of gene flow from areas not under reinforcing selection. In sympatric areas, both mate choice for attractiveness (sexual selection) and mate choice for correctly choosing your own lineage in the presence of a closely related lineage (reinforcing selection) are required. However, in allopatric areas, only sexual selection may be required. The relative strengths of sexual and reinforcing selection will therefore determine the distribution of reproductive character displacement across the contact zone between sympatry and allopatry. For example, an allopatric phenotype could be at a selective disadvantage in sympatry (reinforcement is occurring), while a sympatric phenotype may not be selected against in allopatry. Alternatively, sympatric phenotypes may be at a selective disadvantage in allopatry, which may occur if the allopatric phenotype is at a sexual-selection optimum. Under the assumption that gene flow is bidirectional, the spatial distribution of genetically based sympatric and allopatric phenotypes across the contact zone

can help distinguish which of these two possibilities occurs at a contact zone.

Second, patterns of reproductive character displacement are often described across large geographic regions, possibly confounding the presence/absence of the second lineage with large-scale geographic clines in other biotic and abiotic variables and therefore misidentifying phenotypic variation as reproductive character displacement (Butlin 1995). Establishing reproductive character displacement over very small spatial scales can help to minimize this potential problem to a large extent. Similarly, genetic analysis of phenotypic differences across small spatial scales is more likely to reflect the underlying genetic changes that result as a consequence of reinforcing selection than changes that have arisen from selection for abiotic or other factors that may occur across broader geographic regions.

A native Australian species of *Drosophila, D. serrata* lives in forested habitat on the east coast of Australia (Dobzhansky and Mather 1961; Ayala 1965). Over the northern half of its distribution, *D. serrata* coexists with a closely related species, *D. birchii*. In the populations that are sympatric with *D. birchii*, the cuticular hydrocarbons (CHCs) of *D. serrata*, which act as contact pheromones display the classic pattern of reproductive character displacement over a scale of 300–2,200 km (Higgie et al. 2000). In an evolutionary manipulation, the pattern of reproductive character displacement was observed to evolve in laboratory conditions when allopatric populations of *D. serrata* were exposed to experimental sympatry with *D. birchii* (Higgie et al. 2000). Hybridization is very rare between *D. serrata* and *D. birchii* because prezygotic isolation is high (Ayala 1965; Blows 1998; Higgie et al. 2000); therefore, reinforcing selection could not have operated postzygotically. Instead, the selective pressure that appeared to cause the change in CHCs in the laboratory was mating inefficiency, where allopatric *D. serrata* males inseminated almost 50% fewer *D. serrata* females when in the presence of *D. birchii* than they usually did in the presence of their own species (Higgie et al. 2000). Importantly, the same CHCs are under sexual selection in *D. serrata* (Hine et al. 2002, 2004; Blows et al. 2004; Chenoweth and Blows 2005). The use of CHCs in *D. serrata*, both for species recognition in the presence of *D. birchii* and in mate choice among individuals of its own species, allows an examination of how these evolutionary processes interact where sympatric and allopatric populations of *D. serrata* meet in the field.

Populations of *D. serrata* that are sympatric or allopatric with *D. birchii* abut at a contact zone on the Byfield Peninsula on the east coast of Australia (fig. 1A). The southernmost distribution of *D. birchii* is at the northern end of this peninsula, while allopatric populations of *D. serrata* occur in interconnected habitat for another 1,300 km south. Estimates of genetic differentiation at microsatellite
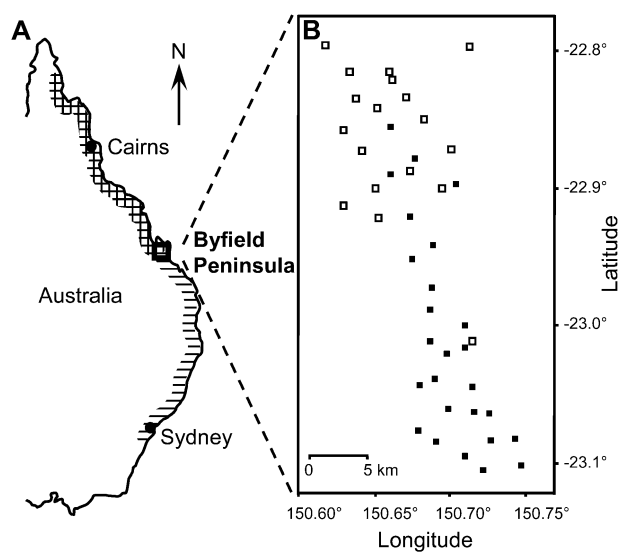
**Figure 1:** *A*, East coast of Australia, showing approximate sympatric (*cross-hatched lines*) and allopatric (*horizontal lines*) distributions of *Drosophila serrata* with *Drosophila birchii* and the Byfield Peninsula, which has the most southerly population of *D. birchii*. *B*, The Byfield Peninsula population of *D. serrata* was sampled at the border of the sympatric and allopatric populations in April 2001. *Drosophila serrata* sample sites were designated sympatric (*open squares*), if *D. birchii* was also found in the trap, or allopatric (*filled squares*), if no *D. birchii* were trapped.

loci among allopatric populations of *D. serrata* up to 900 km apart have been shown to be very low, with little or no significant population structure, consistent with high levels of gene flow among populations (Magiafoglou et al. 2002). Consequently, there are likely to be high levels of gene flow among *D. serrata* populations only a few kilometers apart on the Byfield Peninsula.

To begin, we characterize the distribution of CHCs of *D. serrata* across the Byfield Peninsula contact zone. We sampled 257 *D. serrata* females from the Byfield Peninsula along a transect of approximately 36 km and then established isofemale lines in the laboratory from each female caught in the wild (fig. 1*B*). Where environmental factors can have a large effect on phenotypes, measuring phenotypes from a common-garden experiment is essential to determine whether the phenotypic differences across the contact zone have an underlying genetic basis. First, we measured CHCs from 1,000 individuals from these isofemale lines to determine whether reproductive character displacement existed across the Byfield Peninsula contact zone. Second, we established how the reproductive character displacement in CHCs found on the Byfield Peninsula related to the evolution of reproductive character displacement seen in a manipulative selection experiment that demonstrated which CHC combinations were under

reinforcing selection in *D. serrata* (Higgie et al. 2000). Third, we examined the genetic basis of the CHC changes between sympatry and allopatry across the contact zone through a quantitative genetic analysis and compared the genetic basis of reproductive character displacement at this spatial scale with that established at a larger geographic level (Blows and Higgie 2003). On the basis of manipulative evidence from our previous selection experiment for the effect of *D. birchii* on the CHCs of *D. serrata* (Higgie et al. 2000), we were able to demonstrate that the changes in CHCs across the 36 km of the contact zone were a consequence of the presence/absence of *D. birchii*.

The pattern of reproductive character displacement established at the Byfield contact zone was consistent with the second possibility outlined above, where sympatric phenotypes are at a selective disadvantage in allopatry. We found sympatric phenotypes stopping abruptly at the contact zone but some evidence for a transition from allopatric- to sympatric-like phenotypes into the area of sympatry. To determine whether sympatric-like phenotypes may be at a disadvantage in allopatry, we evaluated female preference for male CHCs using females from sympatric and allopatric populations. We assessed female preference by presenting females with males from a population created from the hybridization of two sympatric and two allopatric populations. We show that the preferences of allopatric females discriminate against sympatric-like phenotypes, suggesting that reinforcing selection interacts with the process of sexual selection.

## Methods

### Field Collections

Flies were sampled from the Byfield Peninsula over 9 days in May 2001 (fig. 1). In April 1999, *Drosophila birchii* was found at Byfield but not at Yeppoon, approximately 30 km farther south (M. Higgie, unpublished data), indicating that the boundary between sympatric and allopatric *Drosophila serrata* was between these two locations. A bucket containing decomposing bananas sprinkled with dry yeast granules was placed approximately every 2 km in the area between Byfield and Yeppoon wherever appropriate vegetation was found. Every site was sampled on two or three different days. *Drosophila serrata* were collected from 36 sites, while *D. birchii* were collected from 16 of those sites, resulting in 20 allopatric *D. serrata* sites and 16 sympatric *D. serrata* sites. Flies were netted from buckets and anesthetized using carbon dioxide, and then *D. serrata* and *D. birchii* were identified by microscope. Female *D. serrata* were placed singly in food vials to establish 257 isofemale lines: 151 isofemale lines from allopatric sites (females per site: median = 4, range = 1–31) and 106 isofemale lines
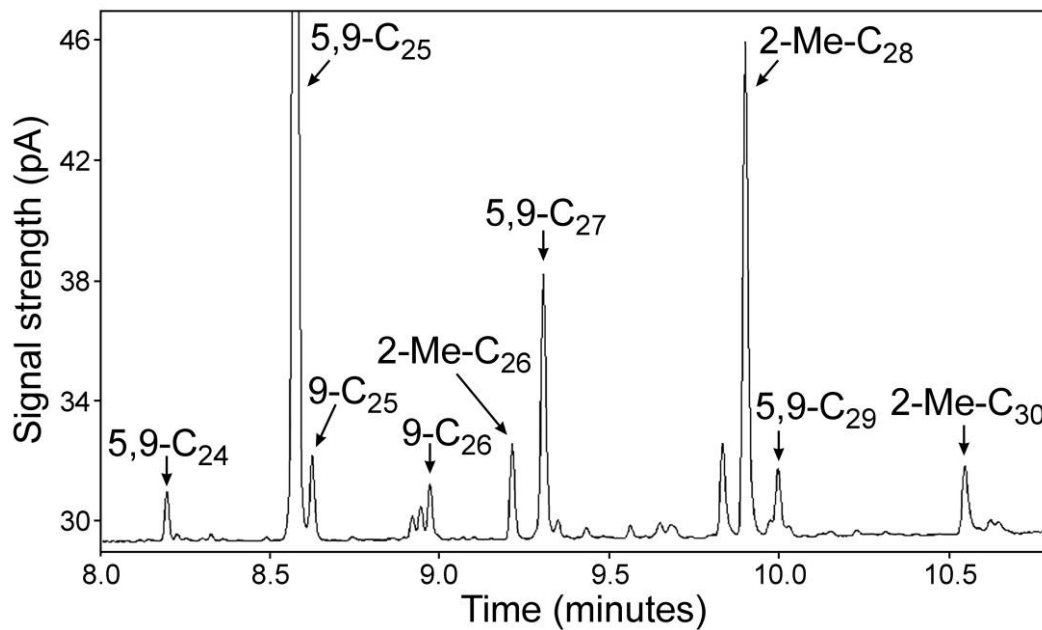
**Figure 2:** Gas chromatograph of a single *Drosophila serrata* female taken from an isofemale line established from the Byfield Peninsula. The nine cuticular hydrocarbons used were identified using gas chromatography–mass spectrometry (Howard et al. 2003; M. Higgie, unpublished data). The peak of $5,9$-$C_{25}$ has been truncated for display purposes. pA = picoamperes.

from sympatric sites (females per site: median = 3, range = 1–20). Isofemale lines were maintained at 25°C with a 12L : 12D photoperiod for seven generations before being assayed for their CHCs.

### Cuticular Hydrocarbons across the Contact

Two males and two females from each isofemale line were sexed as virgins and aged in single-sex vials for 6 days. Cuticular hydrocarbons were extracted from single flies by placing each fly in 100 $\mu$L of hexane in a microvial insert, which sat for 4 min and was vortexed for 1 min; then the fly was removed. Samples were run on an Agilent 6890N gas chromatograph using an HP-5 column 50 m long and 320 $\mu$m in diameter with a 0.17-$\mu$m film thickness. An Agilent 7683 autosampler was used to inject 1 $\mu$L of sample into a 200°C pulsed-pressure splitless inlet. The oven temperature program started at 57°C and was held for 0.9 min; it was then ramped at 50°C/min to 200°C and at 20°C/min to 340°C and held for 6 min, for a total run time of 16.76 min. The flame ionization detector was set at 250°C.

Nine CHC compounds were quantified from each fly—5,9-tetracosadiene ($5,9$-$C_{24}$), 5,9-pentacosadiene ($5,9$-$C_{25}$), 9-pentacosene ($9$-$C_{25}$), 9-hexacosene ($9$-$C_{26}$), 2-methyl-hexacosane ($2$-Me-$C_{26}$), 5,9-heptacosadiene ($5,9$-$C_{27}$), 2-methyl-octacosane ($2$-Me-$C_{28}$), 5,9-nonacosadiene ($5,9$-

$C_{29}$), and 2-methyl-triacontane ($2$-Me-$C_{30}$)—using Agilent ChemStation vB.01.01 SR1, where the area under each peak represents the relative amount of CHC present (see fig. 2). The peak areas were transformed into proportions by dividing the area under the peak by the total area of all nine peaks from an individual's CHC profile. Logcontrasts were then taken, using the proportion of $9$-$C_{26}$ as the denominator, to remove the unit-sum constraint (Aitchison 1986; Blows et al. 2004), resulting in eight log-contrast variables:

$$\text{logcontrast CHC}_n = \log_{10} \frac{\text{prop(CHC}_n)}{\text{prop}(9\text{-}C_{26})}.$$

### Reanalysis of Reinforcement Selection Experiment

Previously, we observed the evolution of reproductive character displacement in a selection experiment using *D. serrata* and *D. birchii* (Higgie et al. 2000). Briefly, three field allopatric and three field sympatric *D. serrata* populations were exposed to nine generations of experimental sympatry with *D. birchii*. Control populations of all six field populations were maintained for nine generations without *D. birchii*. Allopatric populations exposed to experimental sympatry with *D. birchii* showed significant evolution of their CHCs within nine generations to re-

semble sympatric populations of *D. serrata*, while sympatric populations remained unchanged relative to their controls (Higgie et al. 2000). Because we are interested in the evolution of reproductive character displacement, we reanalyzed the three allopatric experimental sympatry populations and their controls. Only males from the allopatric populations were reanalyzed, because they represent a manipulation of the natural situation at Byfield, where allopatric *D. serrata* individuals are being exposed to sympatry in the wild with *D. birchii*. Females were excluded because the effect of latitude at Byfield was not comparable to that in the selection experiment. The same nine CHCs used in the Byfield Peninsula data set (above) were reintegrated from this previous experiment using Agilent ChemStation vB.01.01 SR1. To examine whether experimental sympatry with *D. birchii* had an effect on the CHCs of the three allopatric populations, compared with their control populations that were maintained without *D. birchii*, a nested ANOVA was carried out using restricted maximum likelihood in the MIXED procedure of SAS v8.2 with population nested within treatment and with the Satterthwaite approximation of denominator degrees of freedom (SAS Institute 1999; table A2 in the online edition of the *American Naturalist*).

### Genetic Variance across the Contact

Blows and Higgie (2003) have shown that the phenotypic changes in *D. serrata* CHCs that result from reinforcing selection imposed by *D. birchii* also result in detectable changes in the quantitative genetic basis of these CHCs. It was therefore of interest to determine whether the phenotypic CHCs changes in the wild over the 36-km transect were strong enough to result in similarly detectable changes in additive genetic variance.

The genetic variance-covariance (**G**) matrices of males from sympatric and allopatric populations on the Byfield Peninsula were obtained from a quantitative genetic analysis of isofemale lines (app. B in the online edition of the *American Naturalist*). Genetic variances estimated from isofemale lines include variation due to both dominance and epistasis, but they have been shown to approximate estimates containing only additive genetic variance over a range of dominance values (Hoffmann and Parsons 1988). Importantly, they contain no variance due to different environments experienced by individuals in the wild, such as differing larval substrates. Estimates of all CHC variance components were obtained using restricted maximum likelihood in the MIXED procedure of SAS v8.2 with a model that nested isofemale lines within site and the Satterthwaite approximation of denominator degrees of freedom (SAS Institute 1999).

The **G** matrices from sympatric and allopatric regions

were compared using the Flury matrix comparison approach (Flury 1988; Phillips and Arnold 1999). This method was particularly useful here because it enabled the difference between the two matrices to be ascribed to changes in matrix size (proportional or nonproportional changes in eigenvalues) or changes in covariance structure (changes in eigenvectors). Since the Flury approach requires product-moment-based covariance matrices, we first calculated two new covariance matrices based on isofemale line means. We then used the CPC program written by Phillips (1998) to conduct the comparison of these two new matrices, using the jump-up approach to determine the level of matrix similarity between the two **G** matrices.

### Mating Preferences of Sympatric and Allopatric Females

Mate choice trials were carried out to examine whether *D. serrata* females from field sympatric and allopatric populations preferred different blends of *D. serrata* male CHCs. Females from two sympatric populations (Cooktown, $n = 129$; Townsville, $n = 129$) and two allopatric populations (Coffs Harbour, $n = 129$; Forster, $n = 124$) were each placed singly in a glass vial containing fly media and were allowed to choose between two randomly selected males. Males in the mate choice trials were sourced from the F3 generation of a hybrid sympatric-allopatric population. This hybrid population was created for this experiment by reciprocally hybridizing the two sympatric populations of Cooktown and Townsville with the two allopatric populations of Coffs Harbour and Forster. This created a wide range of male CHC phenotypes that represented the variation present in both sympatric and allopatric populations and additionally allowed females from the four populations to choose between males from the same population. Therefore, any difference in female preference for CHCs between sympatric and allopatric populations was unlikely to be a consequence of other male attributes specific to particular geographic populations, which might have been the case had females been presented with males from different populations. However, linkage disequilibrium between CHCs and some other (unknown) trait that is consistent among populations, in either sympatry or allopatry, may still persist in this hybrid population if the two traits are physically linked.

An overall indication of female preference for male CHCs was tested using the approach of Endler (1986). Separately for each choosing population, a canonical variate that distinguished between the CHCs of chosen and rejected males was calculated from a one-way MANOVA model using the GLM procedure in SAS v8.2. The binomial fitness measure (chosen or rejected) was then regressed onto each standardized canonical variate to estimate a standardized sexual selection gradient separately for each

population. This was carried out using restricted maximum likelihood in the MIXED procedure of SAS v8.2, with the Satterthwaite approximation of denominator degrees of freedom (SAS Institute 1999).

The difference in CHCs between sympatric and allopatric *D. serrata* populations is a quantitative, not qualitative, one. That is, they all have the same CHCs compounds, but they differ in the relative amount of each individual CHC. Because our data set consists of nine CHC compounds that are transformed to eight logcontrast variables, the differences between sympatric and allopatric CHCs are multivariate in nature. We therefore calculated a canonical variate to create a continuous, univariate variable that described the difference between sympatric and allopatric CHCs and hence represented the reproductive character displacement in *D. serrata* CHCs due to reinforcement by *D. birchii*. We call this the axis of reproductive character displacement (RCD), with low values representing allopatric-like CHCs and high values representing sympatric-like CHCs. The canonical variate was calculated using a one-way MANOVA model discriminating between the CHCs of the males from the two sympatric (Cooktown, $n = 22$; Townsville $n = 25$) and two allopatric (Coffs Harbour, $n = 25$; Forster, $n = 25$) field populations with the GLM procedure in SAS v8.2 (MANOVA: SYMALLO [sympatric vs. allopatric region] Wilks's $\Lambda = 0.1702$, $F = 53.62$, df $= 8, 88$, $P < .001$). The RCD canonical variate was then applied to the hybrid males to give them a score on the RCD axis.

Female preference for RCD was assessed by regressing whether the male was chosen or rejected onto the RCD axis for each of the four populations. To test for a difference in slope between the populations the choosing females came from, an ANCOVA and a planned contrast between sympatry and allopatry were carried out using restricted maximum likelihood in the MIXED procedure of SAS v8.2 with the Satterthwaite approximation of denominator degrees of freedom. Cubic splines were used to visualize the form of female preference on male RCD for each population, generated by the SAS v8.2 TPSPLINE procedure with a λ-smoothing value of $-0.25$.

## Results

### *Cuticular Hydrocarbons across the Contact*

Male and female CHCs were analyzed separately because the largest amount of variation in CHCs was due to the difference in sex. The common-garden experimental design allowed us to describe phenotypic change across the contact that is genetically based rather than reflecting phenotypic plasticity due to environmental variation. Latitude was used as a covariate to examine changes in phenotype occurring along the length of the contact zone because the contact

zone has a north-south orientation. The model for this experimental design is given by a nested multivariate ANCOVA (MANCOVA), where sympatric or allopatric regions (SYMALLO) was a fixed effect, isofemale lines nested within site nested within sympatry/allopatry regions were random effects, latitude (LAT) was a continuous covariate, and the interaction SYMALLO × LAT was used in the initial model to test for homogeneity of slope. All models parameters were estimated using restricted maximum likelihood in SAS v8.2 with the MIXED procedure because of an unbalanced sample size across sites and sympatry/allopatry regions. The Satterthwaite approximation of denominator degrees of freedom was specified.

Females on the Byfield Peninsula had CHCs that varied with latitude, and the association with latitude depended on whether females were from isofemale lines that were founded from sites with or without *Drosophila birchii* (MANCOVA homogeneity-of-slopes test: SYMALLO × LAT $F = 6.31$, df $= 1, 304$, $P = .013$). The CHCs of females founded from the sympatric region had a significant multivariate relationship with latitude ($\beta = 0.276$, $t = 2.69$, df $= 374$, $P = .007$), while those from the allopatric region did not ($\beta = -0.029$, $t = -0.45$, df $= 195$, $P = .655$). Of the univariate associations between female CHCs and latitude, seven of the eight CHCs had greater slopes with latitude in the sympatric region (e.g., 5,9-tetracosadiene [5,9-$C_{24}$], displayed in fig. 3), although none of the univariate interaction terms reached significance (table A1 in the online edition of the *American Naturalist*).

The association between male CHCs and latitude did not change significantly as a consequence of whether isofemale lines originated from sympatric or allopatric regions (MANCOVA: SYMALLO × LAT $F = 0.01$, df $= 1, 173$, $P = .923$). The SYMALLO × LAT interaction term was therefore removed, and the next model tested whether there was a significant effect of latitude or SYMALLO on male CHCs. Latitude did not have a significant effect on male CHCs (MANCOVA common-slope model: LAT $F = 1.22$, df $= 1, 144$, $P = .271$). Males did have a significantly different overall blend of CHCs depending on whether they were from isofemale lines that were founded from sites that were sympatric or allopatric to *D. birchii* (MANCOVA common-slope model: SYMALLO $F = 5.63$, df $= 1, 74.2$, $P = .020$), although no individual male CHC reached statistical significance (table A2). Note that the presence of a significant multivariate test with a lack of univariate tests reaching significance simply reflects the fact that a combination of traits has changed significantly but each individual trait has not changed enough to be detected with our current sample size.
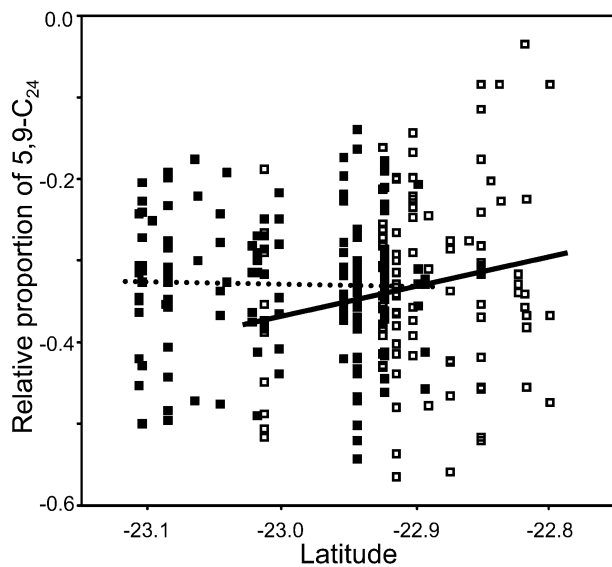
**Figure 3:** Relationship between the relative proportion of cuticular hydrocarbon 5,9-$C_{24}$ and latitude from a 36-km transect on the Byfield Peninsula, showing *Drosophila serrata* females that are allopatric (*filled squares, dotted line*) and sympatric (*open squares, solid line*) in distribution with *Drosophila birchii*. Data points represent isofemale line means after seven generations in the lab, while lines represent regression slopes.

## *Comparison with Reinforcement Selection Experiment*

Higgie et al. (2000) demonstrated the evolution of reproductive character displacement (RCD) in a selection experiment using *Drosophila serrata* and *D. birchii*, where the CHCs of allopatric populations exposed to experimental sympatry with *D. birchii* evolved to resemble the sympatric CHC phenotype within nine generations. In comparing the selection experiment result with the field pattern on the Byfield Peninsula, we found that all but one of the CHCs from allopatric populations exposed to experimental sympatry with *D. birchii* in the selection experiment changed in the same direction (fig. 4B) as in the presence of *D. birchii* at the Byfield contact zone (the exception being 5,9-pentacosadiene [5,9-$C_{25}$], which remained unchanged; fig. 4A). The only male CHC from allopatric field populations to show a significant univariate change after nine generations of experimental sympatry with *D. birchii* was 2-methyl-hexacosane (2-Me-$C_{26}$), while 2-methyl-octacosane (2-Me-$C_{28}$) showed the next-greatest amount of change, although this was not significant (table A2). In a similar fashion, 2-Me-$C_{26}$ showed the most change at Byfield, with 2-Me-$C_{28}$ showing the next-greatest change (table A2). The experimental manipulation of sympatry therefore indicates that the change in male CHCs across the sympatry/allopatry contact at Byfield was a consequence of the presence of *D. birchii*.

## *Genetic Variance across the Contact*

As a consequence of the common-garden sampling design, the phenotypic changes observed in male and female CHCs between sympatric and allopatric regions on the Byfield Peninsula have an underlying genetic basis. This, in turn, indicates that allele frequencies differ between the two regions at loci that control CHC expression. Such allele frequency changes may be manifested in changes in genetic variance and covariances, as we have previously shown for these traits (Blows and Higgie 2003). To determine whether a significant difference between the two regions existed in their genetic variance-covariance matrix (**G**), a likelihood ratio test was performed by comparing the $-2$ residual log likelihood from a restricted maximum likelihood model that allowed separate estimates of the elements of **G** in the two regions with that from a model that constrained both regions to be the same. Estimating the genetic variances and covariances independently for both males and females from sympatric and allopatric sites resulted in a significantly better model fit than using a pooled estimate (likelihood ratio test; males: $\chi^2 = 61.4$,
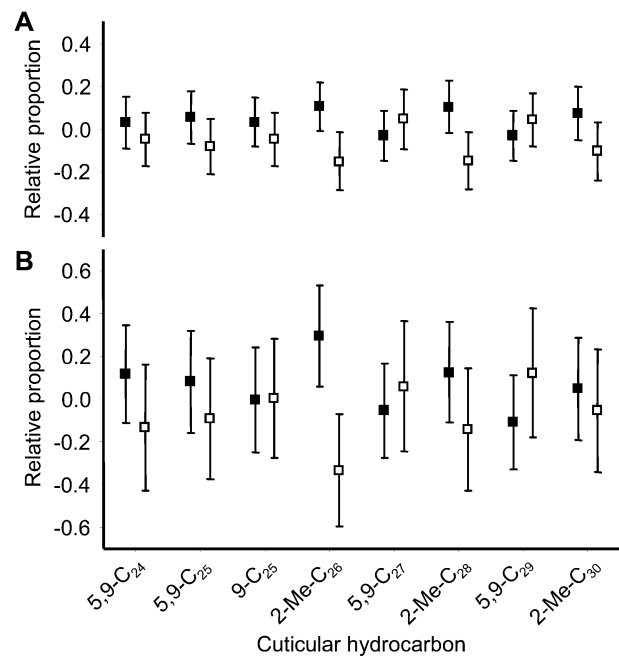


**Figure 4:** *A*, Relative proportions of *Drosophila serrata* male cuticular hydrocarbons (CHCs) of allopatric (*filled squares*) and sympatric (*open squares*) populations from a 36-km transect on the Byfield Peninsula; *B*, corresponding CHCs from Higgie et al. (2000), where allopatric populations of *D. serrata* from the field were exposed to nine generations of experimental sympatry with *Drosophila birchii* (*open squares*) or nine generations without *D. birchii* (*filled squares*). Data points are standardized logcontrasts of each CHC, using 9-$C_{26}$ as the divisor, with 95% confidence intervals based on individuals.

df $= 36$, $P = .005$; females: $\chi^2 = 57.8$, df $= 36$, $P = .012$). Consequently, the **G** matrices for both males and females are different between the sympatric and allopatric regions.

To explore the nature of the differences in **G** matrices between sympatry and allopatry, we employed the Flury approach (Flury 1988; Phillips and Arnold 1999) for comparisons of product-moment-based covariance matrices. The two product-moment-based **G** matrices were found to be different between the regions of sympatry and allopatry for both sexes using this method (equality; males: $\chi^2 = 56.7$, df $= 36$, $P = .016$; females: $\chi^2 = 63.6$, df $= 36$, $P = .003$; see table 1), in a fashion very similar to the restricted maximum likelihood modeling of the variance-component matrices in the preceding paragraph. The model of best fit for males indicated that the genetic variances had changed in magnitude in a nonproportional way, with proportionality between matrices being rejected (proportional; males: $\chi^2 = 5.1$, df $= 35$, $P = .017$; see table 1), while the female **G** matrices changed in a proportional way between the sympatric and allopatric regions, with equality between matrices being rejected (equality; females: $\chi^2 = 63.6$, df $= 36$, $P = .003$; see table 1). These results suggest that the **G** matrices changed in size but not in covariance structure between the two regions. Female genetic variances tended to get larger in sympatry, while no clear pattern was discernible in male genetic variances.

### Mating Preferences of Sympatric and Allopatric Females

That genetically based CHC changes are detectable on the Byfield Peninsula, despite high levels of gene flow recorded in this species (Magiafoglou et al. 2002), implies that there is strong selection applied to *D. serrata* making mating decisions in the presence of *D. birchii*. In addition, there may also be opposing selection in the allopatric region; otherwise, the *D. serrata* CHCs favored under sympatric conditions with *D. birchii* would spread into the region of allopatry. One possible reason for the allopatric CHC phenotype being maintained is that it may be selected for through sexual selection. The greatest CHC change in males under sympatric conditions with *D. birchii* was a relative decrease in 2-Me-$C_{26}$, while 2-Me-$C_{28}$ decreased by the second-greatest amount. Both these CHCs have been shown to be under sexual selection in *D. serrata* in the laboratory, with females choosing males with larger relative amounts of 2-Me-$C_{26}$ and 2-Me-$C_{28}$ (Blows et al. 2004; Chenoweth and Blows 2005). For the males at least, this lends support to the idea that the allopatric blend of CHC is favored under sexual selection, with the sympatric blend being selected against, thus preventing the sympatric blend from spreading into the area of allopatry.

To determine whether sympatric or allopatric females

**Table 1:** Comparison of **G** matrices from sympatric and allopatric regions for male and female *Drosophila serrata* using the Flury approach

| Hierarchy | df | Males | | Females | |
|---|---|---|---|---|---|
| | | $\chi^2$ | $P$ | $\chi^2$ | $P$ |
| Equality | 36 | 56.674 | .016 | 63.598 | .003 |
| Proportional | 35 | 55.081 | .017 | 45.440 | **.111** |
| CPC | 28 | 37.708 | **.104** | 31.220 | .307 |
| CPC(6) | 27 | 35.898 | .117 | 29.625 | .331 |
| CPC(5) | 25 | 30.998 | .189 | 29.023 | .263 |
| CPC(4) | 22 | 30.574 | .105 | 22.479 | .432 |
| CPC(3) | 18 | 24.315 | .145 | 18.013 | .455 |
| CPC(2) | 13 | 12.627 | .477 | 15.206 | .295 |
| CPC(1) | 7 | 7.855 | .346 | 4.558 | .714 |

Note: The $P$ values in boldface indicate the best-fit model for each sex, using the jump-up approach; the models listed above them are rejected at a significance level of .05. df = degrees of freedom; CPC = common principal components.

prefer different male CHCs, we conducted a formal analysis of sexual selection on male CHCs as a consequence of female mating preferences. Females from two sympatric and two allopatric populations were assayed for their preference by being allowed to choose between males from a mass-bred, sympatric-allopatric hybrid population. All four populations showed significant female preference for male CHCs (standardized sexual selection gradients for the canonical variate of female choice [Endler 1986]; Cooktown: $\beta = 0.183$, $F = 39.41$, df $= 1, 256$, $P < .001$; Townsville: $\beta = 0.073$, $F = 5.58$, df $= 1, 256$, $P = .019$; Coffs Harbour: $\beta = 0.125$, $F = 16.99$, df $= 1, 256$, $P < .001$; and Forster: $\beta = 0.140$, $F = 20.70$, df $= 1, 246$, $P < .001$), demonstrating that males CHCs are under sexual selection by female choice in both sympatric and allopatric populations.

To determine whether sympatric and allopatric CHC blends have a differential effect on sexual selection, we investigated female preference on the combination of CHCs that displayed reproductive character displacement (RCD), that is, the difference in CHCs due to being sympatric or allopatric to *D. birchii*. Females from sympatric populations showed no linear preference for the RCD axis (Cooktown: $\beta = 0.004$, $t = 0.14$, df $= 256$, $P = .887$; Townsville: $\beta = 0.027$, $t = 0.86$, df $= 256$, $P = .389$). Alternatively, females from allopatric populations showed a strong, linear preference for the RCD axis (Coffs Harbour: $\beta = 0.076$, $t = 2.46$, df $= 256$, $P = .015$; Forster: $\beta = 0.121$, $t = 3.89$, df $= 246$, $P < .001$). In particular, females from allopatric populations preferred to mate with a male the more allopatric-like his CHCs were, while the more sympatric-like his CHCs were, the less they preferred to mate with him (fig. 5).

There was a significant difference in the sexual selec-

tion gradients among the four populations (ANCOVA): RCD × POP (population) $F = 3.02$, df $= 3, 1,014$, $P = .029$). To test specifically whether this difference was due to choosing females being sourced from sympatric or allopatric populations, a planned contrast between sympatric and allopatric slopes was carried out within the ANCOVA (planned contrast "sympatric female vs. allopatric females": $F = 7.79$, df $= 1, 1,014$, $P = .005$). This result indicated that sympatric and allopatric females have different preferences for the combination of CHCs that are under reinforcing selection.

## Discussion

We have shown that reinforcing selection is capable of maintaining genetically based reproductive character displacement across a contact zone of 36 km between sympatric and allopatric regions. The reproductive character displacement in CHCs on the Byfield Peninsula persists despite sympatric and allopatric sites being separated by only a few kilometers and *Drosophila serrata* being a potentially highly vagile species (Magiafoglou et al. 2002). The evolution of reproductive character displacement on the Byfield Peninsula displayed a number of close similarities with the evolution of reproductive character displacement under experimental sympatry in laboratory conditions over nine generations (Higgie et al. 2000). Seven out of eight male trait means changed in the same direction, with the exception of a single hydrocarbon that showed no change, and two methyl alkanes (2-Me-$C_{26}$ and 2-Me-$C_{28}$) displayed the greatest changes both on the Byfield Peninsula and in the reinforcement selection experiment.

The reproductive character displacement in male CHCs between sympatric and allopatric regions on the Byfield Peninsula could be considered a step change because the
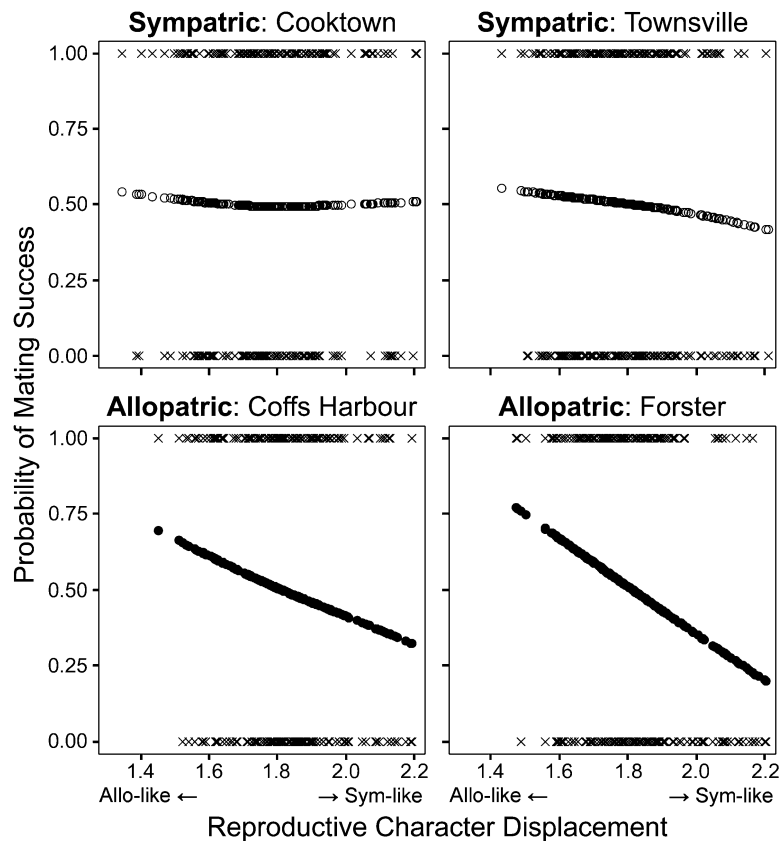


**Figure 5:** Sympatric and allopatric *Drosophila serrata* female preferences for the cuticular hydrocarbons (CHCs) of *Drosophila serrata* males along the axis of reproductive character displacement (RCD). Crosses represent whether a *D. serrata* male from a sympatry-allopatry hybrid population was chosen (success = 1) or rejected (success = 0) and the RCD value of his CHCs. Circles represent the predicted probability of mating success for these males relative to their RCD value, depending on whether they were assessed by females from field sympatric populations (*open circles*) or field allopatric populations (*filled circles*). Allopatric-like CHCs are represented by lower values of RCD (to the left on the *X*-axis), while sympatric-like CHCs are represented by higher values of RCD (to the right on the *X*-axis).

phenotypic change had no relationship with latitude. Female CHCs did change with latitude, and this depended on whether they originated from the sympatric or the allopatric region. There are at least two factors that could influence the difference in how male and female CHCs differ between sympatric and allopatric populations. First, male CHCs could be under stronger reinforcing selection than female CHCs because the change in male CHCs is more abrupt at the contact zone. This difference is possible because the CHCs of *D. serrata* have relatively low intersex genetic correlations (Chenoweth and Blows 2003), which have been shown to allow the independent evolution of male and female CHCs to some extent (Rundle et al. 2005). Second, if reinforcing selection exerted in sympatry with *Drosophila birchii* is the same for both sexes but gene flow is higher for females (e.g., females could be more vagile than males), then the balance between gene flow and selection would be diluted the closer the sympatric populations were to allopatric populations. The experiments described here do not allow us to distinguish between these two competing hypotheses.

Irrespective of the reasons why male and females differ in their association with latitude in the sympatric region of the contact zone, a prominent feature of the contact zone was the lack of any evidence for the movement of the sympatric phenotype into the allopatric region for either sex. Males from sympatric and allopatric regions and females from the allopatric region showed no change in CHC phenotype with latitude, while females from the sympatric region did. It seems unlikely that there is asymmetric female gene flow between these two regions, and therefore selection against the sympatric phenotype in allopatry appears to be stronger than selection against the allopatric phenotype in sympatry.

Allopatric female mating preferences from independent populations were biased against the sympatric phenotype, suggesting that sexual selection may play an important role in the maintenance of the contact zone, restricting the spread of the sympatric phenotype into allopatric areas. Sympatric females from this experiment appeared to show no preference for either sympatric or allopatric male CHCs, although one may expect them to show a preference for sympatric males in order for reproductive character displacement to evolve. However, from this experiment we can see that there is a difference between female mating preferences depending on whether the female is sympatric or allopatric: the sympatric females choose allopatric-like males less often than allopatric females do, and they choose sympatric males more often than allopatric females do. This difference in preference is in the correct direction for the evolution of reproductive character displacement and is all that would be required for it to evolve. What remains to be determined is whether sympatric female preference for CHCs is dependent on the physical presence of *D. birchii* individuals during mate choice.

One interpretation of the way in which reproductive character displacement has evolved at Byfield is that the CHC phenotype found in allopatric populations may be a sexual selection optimum that has been compromised in sympatry to accommodate the evolution of reproductive character displacement via reinforcing selection, creating a conflict between *D. serrata*'s species recognition and sexual selection, as has been proposed by a number of authors (Gerhardt 1982; Ryan and Rand 1993; Pfennig 1998; Hoskin et al. 2005; Phelps et al. 2006). Sympatric populations of *D. serrata* have evolved both different female preferences and different male traits in response to reinforcement of mate recognition by coexisting with *D. birchii*. As a result of this change in their mate recognition, females from allopatric populations of *D. serrata* now find the sympatric CHC blend to be less attractive. This may be due to sympatric males having relatively low amounts of two CHCs, 2-Me-$C_{26}$ and 2-Me-$C_{28}$, whereas allopatric *D. serrata* females prefer males with relatively high amounts (Blows et al. 2004; Chenoweth and Blows 2005). So not only has reinforcement changed the CHCs of *D. serrata* in sympatry with *D. birchii*, but this has also had the consequence of making sympatric males less attractive to allopatric females, potentially resulting in assortative mating between sympatric and allopatric populations of *D. serrata*. Indeed, reproductive character displacement has recently been shown to alter mate recognition to the extent of resulting in a speciation event via this mechanism (Hoskin et al. 2005).

Differences in levels of genetic variance are also discernible in the sympatric and allopatric regions, reflecting likely underlying changes in allele frequency in response to reinforcing selection. Experimental sympatry in the laboratory produced large increases in levels of genetic variance in both male and female traits (Blows and Higgie 2003), similar to that found here for sympatric female CHCs at Byfield, but no such pattern was discernible in male genetic variances at Byfield. The differing genetic variances between sympatric and allopatric regions suggest that there are allele frequency differences between the two regions. Because there is little or no natural hybridization between *D. serrata* and *D. birchii* (Ayala 1965; Blows 1998; Higgie et al. 2000), introgression due to hybridization is unlikely to be the cause of higher genetic variances in the sympatric region. Ultimately, it will be necessary to follow specific alleles in laboratory selection experiments and across the contact zone in the field to be able to fully characterize how *D. serrata* responds to the reinforcing selection generated by the presence of *D. birchii*.

Further study is required to elucidate the details of how sexual selection in allopatry and sympatry operate in this

system. Currently, we are experimentally testing whether the allopatric phenotype represents a sexual selection optimum while the sympatric phenotype is a sexual selection state compromised by reinforcement of mate recognition. Sympatric and allopatric populations will be hybridized, and then the opportunity for sexual selection will be manipulated to determine whether sexual selection drives these hybrid populations to the original allopatric phenotype hypothesized to be a sexual selection optimum.

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