TITLE: Reinforcement of conspecific sperm precedence weakens sexual selection in sympatric populations of *Drosophila*

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ABSTRACT: Sexual selection is well recognized as a driver of reproductive isolation between lineages, however selection for increased reproductive isolation could also reciprocally feed back to alter sexual selection, when these processes share a genetic basis. Direct selection for isolation is most likely to occur in the context of 'reinforcement', where selection acts to increase prezygotic barriers to reduce the cost of heterospecific matings. Many studies of reinforcement focus on premating reproductive barriers, however postmating traits--such as conspecific sperm precedence (CSP)--can also respond to reinforcing selection. When CSP and intrapopulation sperm competition (ISC) share a genetic basis, selection for increased CSP in sympatric populations could alter ISC and the strength of sexual selection. We tested this prediction with the sister species *Drosophila pseudoobscura* and *D. persimilis*, using two sympatric and two allopatric populations of *D. pseudoobscura*. We used a factorial sperm competition experiment to evaluate differences in CSP and ISC between sympatric and allopatric populations. Using multiple tester males across this factorial design also allowed us to estimate the opportunity for sexual selection within each population. Consistent with a pattern of reinforcement, the sympatric populations had higher mean CSP. Reinforcement, in turn, decreased the average offensive sperm competitive ability specifically within these sympatric populations, allowing less opportunity for sexual selection to operate among conspecific males. These data demonstrate that strong reinforcing selection for reproductive isolation can have consequences for sexual selection and sexual interactions within species, in these important postmating sperm competition traits.

SIGNIFICANCE STATEMENT

Sexual selection can accelerate speciation by driving the evolution of reproductive isolation, but forces driving speciation could also reciprocally feedback on sexual selection. Using assays of sperm competition within and between two sister species, we show that populations of *Drosophila pseudoobscura* that co-occur with sister species *D. persimilis* have an elevated ability to outcompete heterospecific sperm, a signature of direct selection to increase reproductive isolation where these species interact. We also find these *D. pseudoobscura* populations have decreased sperm competitive ability against conspecific males. Our findings demonstrate that direct selection to increase reproductive isolation against other species can reduce the opportunity for sexual selection within species, a collateral effect of reproductive traits responding to heterospecific interactions.

Introduction

The presence of heterospecifics can influence sexual interactions and therefore alter patterns of selection on reproductive traits, when closely related species come into contact. In cases where these species have the potential to interbreed, selection can favor divergence in sexual traits to avoid costs of heterospecific mating, a type of reproductive character displacement commonly called reinforcement (1-3). The frequency at which reinforcement contributes to speciation is still under debate (3-4) although several recent examples provide strong evidence for reinforcement acting on mating traits (5-10). Regardless, because reinforcement produces evolutionary change in these mating traits, it could have collateral effects on intraspecific sexual dynamics, thereby altering the magnitude and efficacy of sexual selection specifically within populations exposed to heterospecifics. These potential reciprocal interactions between sexual selection and reproductive isolation remain relatively untested (6-7), but can have important consequences for how we interpret evolution of sexual traits and interactions. For example, patterns of reproductive trait evolution in rapid radiations, where sexual selection is thought to be the primary driver, may be misinterpreted if they do not take into account species interactions.

For reinforcement and sexual selection to reciprocally affect the evolution of sexual traits, these traits must be involved in both processes and share a genetic basis. Currently the best example of a shared genetic basis for sexual selection and reproductive isolation comes from *Drosophila* sperm competition genes, several of which have been shown to mediate both sexual selection through intraspecies sperm competition (ISC) and reproductive isolation via conspecific sperm precedence (CSP) (8). Conspecific sperm

precedence occurs when a female mates with both heterospecific and conspecific males yet most of the progeny are sired by the conspecific male; this precedence can occur either through competitive mechanisms (including male sperm competition and cryptic female choice) or non-competitive mechanisms (resulting mainly from gametic incompatibilities). CSP has proven to be a strong reproductive isolation barrier among species in *Drosophila* (4,12-13) and in many other plant and animal species (4 and references therein). Although ubiquitous, CSP can be overlooked as a reproductive isolating barrier because it involves inconspicuous phenotypes that are not readily observed in the field (14). Moreover, although reinforcement studies have focused on pre-mating traits, reinforcement can act on any pre-zygotic trait (15-17), including CSP. Previous empirical studies have been equivocal about whether heterospecific interactions and reinforcement select for increased CSP specifically in sympatry, with no single study simultaneously estimating and comparing levels of CSP in allopatric and sympatric populations (13, 18-25)

While reinforcing selection (acting on CSP) and sexual selection (acting on ISC) could interact to influence evolutionary change in post-copulatory traits, the outcomes of this interaction clearly will depend upon whether these forces act in concert or in opposition. When sexual selection and reinforcing selection act in concert, trait evolution can proceed faster than otherwise expected, but the direction of trait evolution remains unchanged. In contrast, the potential feedback between sexual selection and reproductive isolation can generate complex evolutionary outcomes when these forces act at cross-purposes. For example, sperm competition is shaped by sexual conflict between males and females (i.e. antagonistic pleiotropy; 26-28) and genotype-genotype interactions

(male-male: 29-30 and male-female: 31-33). Both are expected to maintain high variance in the affected traits and, indeed, sperm competition genes are often highly variable both in terms of molecular and phenotypic variation (30, 33-34). In contrast, under models of speciation by sexual selection, genetic variance of sexual traits that act as barriers to reproduction is expected to be reduced and the overall trait mean shifted; this is because reproductive isolation is generated by strong disruptive selection between populations and directional selection within a population (35-36), including directional selection imposed by reinforcement (1-3). The net effect of selection imposed by intrapopulation sexual interactions and by reinforcement will together determine the phenotypic and genetic variation in sperm competition traits/genes. Therefore, scenarios of sexual versus reinforcing selection could produce very different optimal phenotypic and genetic variation in sperm competition traits and genes.

One way these potentially antagonistic optima could play out is when reinforcement-mediated changes in the mean and variance of sperm competition traits change the opportunity for sexual selection among conspecifics (7). Sperm competition contributes to variance in reproductive success because male genotypes that can disproportionately sire offspring increase their fitness compared to the fitness of rival male conspecifics (37-38). Strong sperm competition leads to greater opportunity for sexual selection because there is greater variance in reproductive success compared to scenarios where males have equal probability of siring offspring (sperm competition offensive/defense ability=0.5). This generates two alternative predictions of the possible effects of reinforcement on sexual selection. First, the response to strong directional selection from reinforcement on sperm competition traits could lead to greater siring

ability in intrapopulation sperm competition, increasing variance in reproductive success and opportunity for sexual selection. Alternatively, strong directional selection could reduce phenotypic variation so that competitive ability is equalized among males, thus reducing the opportunity for sexual selection.

One effective strategy to evaluate the interaction between selection for increased reproductive isolation (i.e. reinforcement) and sexual selection acting on sperm competition genes is to estimate variation between genotypes in CSP and ISC in parallel. *A priori*, these two postcopulatory interactions might be expected to be differently shaped by males and females. Sexual dynamics within species indicate that ISC should be determined by both male and female genetic effects, and their interaction (31,33). In contrast, because females experience most of the cost of heterospecific matings (39-41), variation in CSP might be predominantly controlled by females via cryptic female choice (42) and thus we might expect female genetic effects to strongly influence observed variation in CSP. Unlike ISC however, the phenotypic and genetic variance for CSP has not been empirically explored and their similarity to ISC is currently unknown.

In this study, we examine evidence for reinforcement of CSP among populations of *Drosophila pseudoobscura* that are allopatric or sympatric with their closely related sister species *D. persimilis*, and evaluate the potential consequences of these heterospecific interactions for ISC and sexual selection within *D. pseudoobscura* populations. One of the first clear empirical demonstrations of reinforcement on pre-mating isolation was described in this species pair (43). The detection of a pattern consistent with reinforcement in this system suggests that heterospecific interactions and matings are frequent and sustained over evolutionary time and can act as a substantial

selective agent on reproductive traits. Here we first determine whether there is evidence that heterospecific interactions have selected for increased CSP, by comparing CSP among populations of *D. pseudoobscura* that are allopatric or sympatric with *D. persimilis*. A pattern of stronger CSP specifically in sympatry is consistent with reinforcement. Second, we evaluate whether selection for strong CSP in sympatry has affected ISC, and thereby sexual selection, as might occur when CSP and ISC have shared genetic architecture. Throughout, we test for differences in trait variation across a set of distinct genotypes which allows us to specifically evaluate which sex is playing a more critical role in determining variation in heterospecific and conspecific postcopulatory interactions.

MATERIALS AND METHODS

Wild type fly stocks

All stocks were reared on standard media prepared by the Bloomington Drosophila Stock Center, and were kept at room temperature (~22C). We used a set of isofemale lines collected from four natural populations in the summers of 2013 and 2014 (Fig 1). Allopatric *D. pseudoobscura* were collected at Zion National Park, UT (kindly provided by N. Phadnis) and Lamoille Canyon, NV (collected by D. Castillo). Sympatric *D. pseudoobscura* and *D. persimilis* were collected at two sites: Mt. St. Helena, CA (*D. pseudoobscura* collected by A. Hish/M. Noor and D. Castillo, and *D. persimilis* collected by D. Castillo); and, near Meadow Vista and Forest Hill, CA (called here 'Sierra'; *D. pseudoobscura* and *D. persimilis* collected by D. Castillo). For both sympatric

populations, both species were present in field collections and can be considered truly co-occurring/sympatric.

Conspecific sperm competition assay

Sperm competition assays generally involve mating an individual female sequentially with two distinct male genotypes. In all experimental crosses between species, females were paired first with a *D. persimilis* male and second with a *D. pseudoobscura* male; that is, the assays are evaluating the "offensive" sperm competitive ability of conspecific males to displace heterospecific sperm (equivalent to 'P2', or second male siring ability; 44). We focused on "offensive" sperm competition because *D. pseudoobscura* females do not remate with *D. persimilis* males if they have first mated with a conspecific, therefore we cannot evaluate "defensive" sperm competition in this cross. In this experiment we partitioned the variance in CSP due to male genotype, female genotype, and the male x female genotype interaction using a "diallel-like" crossing design, which is commonly used for this purpose (31,33; Supplemental Fig 1). (Diallel designs are used to estimate additive genetic variance or heritability directly (as in 45); our design is "diallel-like" because we did not use progeny from the diallel to estimate heritability.) From each of the four populations, we used 4 isofemale lines as both the female genotype and second male genotype (resulting in 16 diallel cells). If CSP is important for reproductive isolation in sympatry it should be consistently strong across multiple heterospecific genotypes. Accordingly, rather than rely on a single tester male genotype, we aimed to use multiple wild-collected *D. persimilis* tester male lines. To do so, for each female x second male combination, we completed four crosses using each of four *D. persimilis*

tester males once as the first (heterospecific) male, and then pairing the mated female with a male from the second (conspecific) male genotype. This design produced 64 CSP replicates per population (256 replicates across all populations). Given the large scale of the experiment we completed replicates in several blocks. To reduce block effects, we included all male x female genotype combinations in each block.

Virgin individuals were collected and aged 7 days prior to the initiation of an experimental block. One day before mating, *D. persimilis* tester males were isolated individually (46). The following day, females were individually added (without anesthesia) to a vial containing a tester male and were co-housed for 24 hours, after which time the tester male was removed. We kept females housed individually in these vials for 7 days before second mating (similar to 46). After 7 days we inspected all vials for the presence of larvae to determine if females had mated with the first *D. persimilis* tester males. This was used to evaluate evidence for differences in successful first matings (pre-mating isolation) among allopatric and sympatric populations, rather than observing matings directly, as there is high variance in time to copulation in this heterospecific pairing (47). Only females that had mated (i.e. had produced larvae within 7 days) were retained for the remainder of the experiment.

For the second mating, each individual female was randomly assigned one of the four *D. pseudoobscura* male genotypes from her own population to determine the strength of CSP. These second males were also isolated one day before the introduction of the female. Seven days after mating with the first male, females were transferred, without anesthesia, to the vial containing the second male. Individual pairs were co-housed for 24 hours and the male was removed on the second morning. The female was

kept for five days (transferring after 2 days to avoid overcrowding of larvae). All progeny produced in the five-day window after the second mating were collected; from these progeny a maximum of 10 males and 10 females, randomly chosen from the total group of progeny, were used to score CSP (P2) as described below.

Intrapopulation sperm competition assay

The design for intrapopulation sperm competition (ISC) assay mirrored the experimental design for CSP except that, rather than a *D. persimilis* tester male, the first male was a *D. pseudoobscura* tester male derived from the same population as the diallel block. The same female-second male diallel genotype blocks were used in ISC and CSP experiments. For each female x second male combination we completed two replicates using two unique intraspecific *D. pseudoobscura* tester males (drawn from additional isofemale lines not used in the diallel genotype block; described further below). This allowed us to have a total sample size per population that matched the CSP experiment (64 replicates per population, 256 replicates across all populations). As with CSP assays, to reduce block effects we included all male x female genotype combinations in each block.

The details of the mating scheme (virgin collection, aging of individuals, isolation of individuals, etc.) are identical to the CSP experiment. We did not observe matings directly, but the average refractory period for *D. pseudoobscura* is 4 days (48), so we are confident that on average only a single mating occurred in the 24 hour co-housing timeframe. Each individual female was randomly assigned one of the two *D. pseudoobscura* male genotypes to determine the strength of P2 (second male siring

ability). The female was kept for five days after the second mating (transferring after 2 days to avoid overcrowding of larvae). All progeny produced in the five-day window after the second mating were collected and scored.

Generating visibly-marked tester males for quantifying CSP and ISC

To allow efficient progeny scoring, paternity was scored with the aid of visible markers in both CSP and ISC experiments. This required us to generate marked male tester lines with wild-caught *D. persimilis* (for CSP tester males) and *D. pseudoobscura* (for ISC tester males) lines from each study population. For CSP, to introduce a visible marker into wild-type wild-collected *D. persimilis* males from our sympatric sites, we introgressed an X-linked marker ("short" or *sh*) from a *D. pseudoobscura* line, into four of our collected *D. persimilis* genotypes (Supplemental Methods; Supplemental Fig 2). These four *D. persimilis* tester males were used to evaluate the mean strength and variation in CSP for all four *D. pseudoobscura* populations in the CSP experiment. For ISC experiments, the marked tester males were created by introgressing a green fluorescent protein marker (GFP) into 2 wild type *D. pseudoobscura* strains per population (therefore 8 strains in total, using wild-collected isofemale lines that were not a part of the diallel blocks for each population). The original GFP strain was obtained from the UCSD stock center (14011-0121.166) the creation of which is described in Holtzman et al. (49). We chose this marker because it is dominant (11) in this particular strain we mapped its location to the second chromosome (Supplemental Methods), which allowed us to score inheritance of the marker in both sexes.

Scoring conspecific sperm precedence

Hybrid male progeny from *D. pseudoobscura* x *D. persimilis* crosses are sterile (there are no motile sperm, observable by dissecting the testes). We used this sterility phenotype to differentiate the male progeny of heterospecific versus conspecific males and therefore to score CSP. For a given replicate we collected and dissected 10 male progeny that were produced after the second mating. Each male was dissected individually in PBS buffer, and its testes moved to a slide that had 1ul of PBS buffer. A cover slip was placed over the slide and the testes were squashed, releasing sperm into the buffer. The slides were examined under an EVOS FL microscope for the presence of motile sperm. If no motile sperm were present, the male was scored as hybrid.

Because female hybrids are fertile in these crosses, the *sh* allele was used to differentiate the female progeny of heterospecific versus conspecific males and therefore to score CSP from female offspring. Since the *sh* allele is recessive we could not score F1 females directly, but instead scored their offspring for the presence of the *sh* allele. If an F1 female was hybrid (and carrying the *sh* allele from the *D. persimilis* male) we would expect ½ of her sons and ½ of her daughters to have the *sh* phenotype. We previously confirmed that the ½ segregation held for known hybrid progeny. For each cross, ten F1 females (that could be hybrid or purebred) were housed individually with a *D. pseudoobscura* male that also carried the *sh* allele (UCSD stock center *Dpse co*;*sh* 14011-0121.13). We chose a *D. pseudoobscura* male for these crosses to increase the number of progeny to score since *D. pseudoobscura* females (and therefore any purebred female progeny in our experiment) exhibit premating isolation with *D. persimilis* males; hybrid females do not demonstrate a mating preference. After a week the parental individuals

were cleared from the vials and the vials were retained to score progeny. As progeny eclosed they were scored for the presence of *sh* allele. Any F1 female that produced *sh* progeny was considered hybrid. We required each F1 female to produce at least 10 progeny to be used in scoring CSP.

Our measure of CSP was then the number of purebred progeny out of the total number of F1 individuals scored for a particular cross. If all progeny produced in a cross were scored as hybrid, we did not use this replicate in our analyses because we could not ensure that a second mating had taken place. Note that the frequency of this failure to remate following a first mating does not differ between populations (47). Every CSP estimate was based on at least 10 scored progeny and, for the majority of the crosses, we scored close to 20 individuals. In addition, to ensure that CSP estimated here does not simply reflect stronger fecundity stimulation by conspecific males, in a pilot experiment we determined that there was no difference in progeny production in heterospecific vs. conspecific matings, consistent with previous work (47,50). There was also no correlation between the total number of progeny scored for CSP and the magnitude of CSP, and the number of progeny scored did not differ between populations.

Scoring intrapopulation sperm competition

We scored all progeny that eclosed in the five days after the second mating for the presence/absence of the GFP phenotype. Our measure of sperm competition (P2) for ISC was then the number of wild-type (non-GFP) progeny out of the total number of progeny scored for a particular cross. If all progeny produced in a cross were GFP, we did not use this replicate because we could not ensure that a second mating had taken place. (As with

CSP, the proportion of females that did not remate was not significantly different between populations). Individuals were scored as they eclosed, using a Leica M205FA Stereo Microscope that has an Hg fluorescent lamp attached and GFP filter. Individuals were anesthetized and the ocelli were examined for GFP signal as described in Castillo and Moyle (11).

Statistical analyses

All analyses were completed in R v 3.01.

Differences in the probability of first mating with heterospecifics

We evaluated evidence for a pattern consistent with reinforcement acting on first mating (simple prezygotic isolation) in two ways. First, we used a chi-square test of independence to test the null hypothesis that the mating rate with heterospecifics was the same for alternative geographic scenarios (allopatric vs. sympatric), after combining both allopatric and both sympatric populations for this single comparison (pairwise tests among individual populations gave the same result; Supplemental Table 1). Second, because χ^2 tests might lack power, and since mating events can be coded as a binary variable (0 for did not mate, 1 for successful mating), we used a logistic regression model with all four populations represented by a categorical variable using the glmer function. We then tested whether there were any differences in heterospecific mating between populations by conducting a Wald's test (using the wald.test function from the aod package; 51)..

To evaluate whether there was significant variation within each population (i.e., among isofemale line genotypes) in the probability of mating with a heterospecific, we

used logistic regression. We first fit a full model where the probability of mating with a heterospecific depended on the isofemale line, the *D. persimilis* tester line, and the male x female genotype interaction, and tested significance of these effects using a Wald's test. Because there was no significant interaction for any population, we fit a reduced model that only contained the effects of isofemale line and *D. persimilis* tester line without the interaction, and report these models in the results.

Differences in mean and variance of CSP and ISC between populations

We evaluated evidence for a pattern in CSP consistent with reinforcement, by evaluating whether the allopatric and sympatric populations had a mean difference in CSP or whether they differed in variance. For analyses of mean differences, we pooled the two allopatric populations because there was no significant difference in mean CSP between them (Allopatry t = -0.45064, df = 123.62, P = 0.653) and pooled the two sympatric populations for the same reason (Sympatry t = -0.86678, df = 125.87, P = 0.3877). We tested the hypothesis that the mean CSP differed between geographic scenarios using a Welch's t-test that accounts for unequal variances between samples, and (given that the data are not normally distributed) we also confirmed these results with a Wilcoxon ranked sum test. To evaluate differences in variance, we again pooled the allopatric and sympatric populations because the variance was equivalent between allopatric populations (χ^2 = 0.031899, P = 0.8585), and between sympatric populations (χ^2= 0.80562, P = 0.3711). We compared the total phenotypic variation between geographical classes of population with a Levene-type test implemented in the lawstat package in R (52; Supplemental Methods).

Using the same statistical approach as for CSP, we tested for differences in the mean and variance between sympatric and allopatric populations for ISC, again pooling the individual allopatric and sympatric populations as they were not significantly different from one another for either measure (Allopatric mean t=-1.136, df=118,66, P=0.2593; Sympatric mean t=0.191, df=125.72,P=0.8488; Allopatric variance χ^2=0.949, P=0.3316; Sympatric variance χ^2=0.0796, P=0.7782). Note that, although we report results from tests with these pooled data in the main text, we also observed significant differences in pairwise tests between individual allopatric and sympatric populations, for both average and variance measures of CSP and ISC (Supplemental Tables 2 and 3).

Genetic variation and genotype effects on CSP and ISC

Within each population we assessed whether female, male, or female x male genotype predicted variation in the strength of CSP and ISC. While this can be tested using a two-way ANOVA with interaction, we used binomial regression as this more naturally models our count/binomial data (Supplemental Methods). The model is of the form

$$logit(p_{ijk}) = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$$

The variable α is a categorical variable with four levels that represents male genotype. The variable β is also a categorical variable with four levels that represents female genotype. The variable $(\alpha\beta)$ represents the male x female genotype interactions. Since we were interested in partitioning the variance and estimating the variance components $(\sigma_\alpha^2, \sigma_\beta^2, \sigma_{\alpha\beta}^2)$ we assumed that each variable was a random variable. To test the significance of each variance component, we used a binomial regression in a mixed modeling framework with parametric bootstrap (53). In this bootstrap procedure, data are

simulated from the null model which lacks the random effect of interest. Then the full and reduced models are fit to the simulated data to determine the bootstrap distribution of the Likelihood Ratio test statistic. To the model above we also included a random effect of tester male (*D. persimilis* for CSP and GFP *D. pseudoobscura* strain for ISC). To provide an assessment of the relative importance of each variable we calculated the intraclass correlation for each coefficient; a high correlation indicates that the variable explains much of the variance in the data (Supplemental Methods).

Quantifying sexual selection and variance in male reproductive success

To evaluate whether the intensity/opportunity for sexual selection differs among populations we require an estimate of variance in male reproductive success (54). In a natural scenario most males can gain fitness through offensive (P1) and defensive (P2) sperm competition, so the best estimate for variance in reproductive success would be total progeny produced. In our experiment we did not score lifetime progeny production, and specific male genotypes were either used as offensive or defensive males. As such we estimated male fitness as the proportion of progeny sired, taking into consideration that we had two distinct classes of males--tester (defensive) and diallel block (offensive) males--that may differ in their frequency and variance in fitness in the experiment. Following Shuster et al. (55) we define total variance in male reproductive success as the sum of within and between male class variance

$$V_{total} = (f_{P1})(V_{P1}) + (f_{P2})(V_{P2}) + (\bar{X}_{P2} - \bar{X}_{P1})^2(f_{P2})(f_{P1})$$

The two terms on the left hand of the equation represent the within class variance (for example, V_{P1} is the variance in sperm competitive success between tester males and f_{P1} is

the frequency of tester males used in the experiment). The last term represents the between class variance.

We were interested in reproductive variance at the level of male genotype so we averaged biological replicates to generate mean fitness values for each individual genotype. We determined significant differences in variance and calculated confidence intervals for each variance parameter using empirical bootstrapping methods (Supplemental Methods).

RESULTS

No difference between allopatric and sympatric populations in first mating rates with heterospecifics

We did not find evidence for a pattern consistent with reinforcement of first mating. The average probability of heterospecific matings ranged from 46-52% between populations, and did not differ between allopatric and sympatric populations (χ^2 test of independence: χ^2=1.185, df=1, P=0.2763; Wald's Test: χ^2=1.9, df=4, P=0.75; Table 1). In pairwise tests we also failed to reject the null hypothesis (Supplemental Table 1). There was, however, substantial genetic variation for heterospecific mating rate between females within each population (Fig. 1; Supplemental Table 2). Only in one of the populations (Lamoille, which is allopatric) did the identity of the *D. persimilis* tester line affect variation in this trait (Supplemental Table 2).

Reinforcement acts on conspecific sperm precedence

Unlike first mating, we observed a pattern consistent with reinforcement for conspecific sperm precedence. Specifically, in sympatry we find both greater average CSP and less phenotypic variation in this trait (Table 1; Fig. 2A). The mean CSP for the allopatric populations was significantly different (t=-6.5898, df=210.92, P<0.001; Wilcox W=4427.5, P<0.001), as was the phenotypic variance in CSP (Levene-type test χ^2=22.82, P<0.0001; data pooled by geographic region). These differences in both the average and variance of CSP were also observed in pairwise tests between individual allopatric and sympatric populations (Supplemental Table 2).

Reinforcement has collateral effects on intrapopulation sperm competition

ISC also differed between allopatric and sympatric populations, in both mean and variance (Table 1; Fig 2B). First, mean offensive ability for ISC was significantly lower in sympatric populations (t=3.738, df=246.55,P=0.0002; Wilcox's W=10280, P=0.0004). This contrasts with the observed increase in offensive CSP in sympatric populations. Second, there was more variation in ISC in the sympatric populations compared to the allopatric populations (Leven-type test χ^2=5.74, P=0.0172). Given the differences in ISC and CSP across populations, we used the mean CSP and ISC phenotype for each male x female genotype combination (i.e., cells of the diallel crossing design) to examine the pattern of relationship between the two phenotypes across the four populations. We observed a significant negative relationship between CSP and ISC (Pearson's r=-0.31, P=0.01; Fig. 5).

Female genotype effects contribute to CSP and male x female genotype effects explain both CSP and ISC

Of male, female, and male x female genotype effects that could contribute to explaining the variance in CSP, we found that three out of the four populations had a significant female genotype effect on CSP (Table 2; Fig 3), and all populations had a significant male-female genotype interaction effect. The *D. persimilis* tester male line was also significant in three out of four populations. There was no consistent pattern among populations in which effect had the largest intraclass correlation (i.e. which explained the largest proportion of variance); in some populations the female genotype effect had the largest intraclass correlation, while in others the male x female genotype interaction had the largest intraclass correlation (Table 2). In contrast, for ISC in all four populations we only observed significant male-female genotype interaction and a significant effect of the GFP tester male (Table 3; Fig. 4). In every case, the male-female genotype effect had a larger intraclass correlation (usually two to three times greater) than the identity of the GFP tester male.

The opportunity for sexual selection is decreased in sympatry

The sympatric populations had significantly lower variance for reproductive success compared to the allopatric populations (Figure 6; Supplemental Table 3). The variance in reproductive success across all male genotypes (both offensive and defensive) in the allopatric Lamoille population was significantly greater than both sympatric populations (Mt. St Helena F=1.96, Bootstrap P=0.003; Sierra F=2.08, Bootstrap P=0.008), as was the variance in reproductive success in the allopatric Zion population compared to the

sympatric populations (Mt. St Helena F=2.65, Bootstrap P=0.003; Sierra F=2.83, Bootstrap P=0.004).This reduced variance in reproductive success in sympatry is a product of lower P2 values in sympatry, that result in equalized differences in the siring success between offensive and defensive males.

DISCUSSION

Interactions with heterospecifics have the potential to drive divergent sexual selection and the evolution of reproductive isolation, via reproductive character displacement and reinforcement (6-7,56). Using *D. pseudoobscura* and *D. persimilis*, here we assessed whether there was evidence for reinforcement of species barriers in sympatry via elevated conspecific sperm precedence, a trait that is known to contribute to reproductive isolation across numerous taxa (2). We saw a clear signal of increased CSP in sympatric populations, consistent with a pattern of reinforcement. Specifically, the average CSP was higher, and the overall level of phenotypic variation was lower, in sympatric populations, a pattern consistent with recent or recurrent directional selection acting on CSP in these populations. We further asked whether reinforcement could have collateral effects on intraspecific sperm competition and sexual selection, given that these two traits are mechanistically and genetically linked (11,57). We found that sympatric populations also had lower ISC ability (lower offensive ability – P2) than allopatric populations, consistent with weakened sexual selection in sympatry.

Our results indicate that CSP can strongly contribute to reproductive isolation in response to reinforcing selection. While conspecific sperm precedence is known to be a barrier to gene flow in *Drosophila* (12-13,46) and other taxa (2), its overall importance in

nature has been difficult to ascertain (14,16). In our study we observed not only an increase in mean CSP in sympatric populations but also a decrease in the phenotypic variation. Previous studies of reinforcement sometimes qualitatively describe variation in the target premating traits, but trait variance is typically not quantified (5,9,17). Nonetheless, models of speciation by sexual selection show that strong divergent selection will erode phenotypic variation in selected traits (58-59). Our observations of both increased mean CSP and reduced variation specifically in sympatry provide compelling support for the inference that CSP has responded to strong selection imposed by heterospecific interactions, and underscores the important role that CSP can play in maintaining species boundaries.

These findings, in turn, suggest that other premating and postmating barriers that precede CSP are not strong enough to limit the efficacy of selection on CSP in our sympatric populations (14,16). Indeed, our analysis of premating isolation (propensity to mate with a heterospecific in the first mating) indicated that this potential barrier was no stronger in sympatry than in allopatry. This is interesting because one of the first studies demonstrating reinforcement on premating barriers used the *Drosophila pseudoobscura* and *D. persimilis* sister pair (43), although subsequent studies have found more variable patterns (47,60-61; but see 62). Our observation of a strong response in CSP also suggests the populations of *D. pseudoobscura* and *D. persimilis* we examined are not strongly isolated by non-competitive (gametic) isolation, in agreement with inferences from other studies of this specific species pair (47,50). In contrast, other species pairs with incomplete premating isolation, such as *D. yakuba-santomea* (63), have detected evidence for reinforcing selection on non-competitive gametic incompatibility (17).

Our second major inference is that the response to reinforcing selection observed in CSP has had a collateral effect on the magnitude of offensive ISC and the opportunity for sexual selection in sympatric populations. The decrease in the opportunity for sexual selection in sympatry appears to be the result of a negative genetic correlation between CSP and ISC, an inference that differs from both of our *a priori* expectations. One *a priori* hypothesis was that selection for increased CSP in sympatry would select for increased P2 among conspecifics, if offensive sperm competitive ability were a general trait that acted regardless of whether the competitor was a conspecific or heterospecific male. In contrast, we observed that ISC, as measured by offensive sperm competition, was lower for sympatric populations compared to allopatric populations; that is, average P2 was closer to 0.5, indicating a greater equalization in sperm competitive ability among competing males. Our additional *a priori* expectation was that strong directional selection would alter sexual selection by reducing phenotypic variation. However, the reduced phenotypic variation seen for CSP in sympatry was not mirrored by reduced phenotypic variation for ISC. Instead, we infer that selection for stronger CSP in sympatry has reduced mean ISC in sympatric populations via a negative genetic correlation between these two sperm competitive phenotypes.

For reinforcing selection to influence and interfere with sexual selection in this way, the selection favoring increased CSP must outweigh selection acting to maximize ISC. This means that heterospecific mating must occur at an appreciable rate in nature to allow opportunities for reinforcing selection to act. Two lines of evidence suggest that this is the case. First, from our data we observe a large range in the frequency with which *D. pseudoobscura* females accept *D. persimilis* males in no-choice experiments, with

some genotypes on average accepting *D. persimils* 90% of the time (i.e., line averages for premating isolation ranged from 0.1-0.6). While no estimates for heterospecific mating rate exist from natural observations, rare F1 progeny have been identified from wild collections (64). Second, genetic evidence suggests there has been post-speciation gene flow (i.e., evidence of movement of alleles between species) between *D. pseudoobscura* and *D. persimils* (65-66). Notably, these estimates of realized gene flow will systematically underestimate the rate of heterospecific matings, because they will only capture events that result in F1 progeny that themselves then successfully reproduced. However, given the presence of strong CSP, for example, many heterospecific matings may never produce hybrid progeny.

In addition to requiring sufficient hybridization events to exert reinforcing selection, CSP must have a larger effect on fitness than ISC to systematically influence ISC. One way this could occur is via a higher selective premium specifically for females. While weaker CSP results in substantial fitness deficits for females (via reproductive investment in low or no fitness hybrids), weaker ISC likely has a comparatively marginal, or even beneficial, effect on female fitness outcomes. For example, if weaker ISC is correlated with weaker manipulation of female reproduction--as predicted under antagonistic pleiotropy models of ISC--weaker ISC may directly benefit female reproduction (26-28). Although studies do not usually partition variance in CSP in this way, our design allowed us to partition the variance of both CSP and ISC into female genotype, male genotype, and genotype x genotype effects, to better understand which sex is playing a more critical role in each post-mating prezygotic phenotype. We observed significant male x female genotype interactions for all populations for both CSP

and ISC but, interestingly, only saw significant female genotype effects for CSP. Female genotypes can contribute to CSP via cryptic female choice (42) and the effect of female genotype on CSP, but not ISC, is consistent with the hypothesis that females face more costs of hybridization (39-41) and that choice manifests as female control of sperm use patterns (67-69). These observations suggest that, for CSP, cryptic female choice may be operating similarly to premating isolation mechanisms where females are observed to be the more "choosy" sex and female effects control the level of reproductive isolation more so than male effects (70).

Overall, our data suggest that strong reinforcing selection for reproductive isolation can have consequences for sexual selection and sexual interactions, in these important postmating sperm competition traits. The direction of this interaction provides an interesting inversion to standard expectations about the connection between sexual selection and speciation. Sexual selection is often thought of as a driver of sexual characteristics whose evolutionary divergence then contributes to reproductive isolation. But a direct genetic connection between these processes implies reproductive isolation also has the reciprocal potential to shape sexual selection (71). Based on our observations of higher mean but lower variance in CSP in sympatry, a negative correlation between CSP and ISC, and reduced variance in reproductive success via ISC among sympatric conspecific males, we infer that strong selection for reproductive isolation within populations exposed to heterospecific species has reduced the efficacy of sexual selection in these populations, a collateral effect of reinforcing selection that has not previously been demonstrated.

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Table 1. The average levels of reproductive isolation for each *D. pseudoobscura* population measured from two barriers to reproduction: female preference (proportion of females that did not mate with heterospecifics) and conspecific sperm precedence (CSP). Higher values indicate stronger reproductive isolation. Interpopulation sperm precedence (ISC) is included for comparison. The mean and variance estimates for CSP and ISC are based on 64 replicates per populations A = allopatric; S = sympatric

	Fem. Pref.	CSP		ISC	
Population	Proportion (n)	Mean	Variance	Mean	Variance
Lamoille (A)	0.481 (179)	0.75	0.054	0.76	0.028
Zion (A)	0.476 (145)	0.77	0.041	0.80	0.047
Mt. St. Helena (S)	0.540 (200)	0.90	0.017	0.79	0.057
Sierra (S)	0.505 (222)	0.92	0.018	0.68	0.052

Table 2. The genotype effects that predict CSP. The maximum likelihood estimate (ML est.) and intraclass correlation (ICC) are reported as point estimates from the full model. The *P*-value for each term was calculated by comparing the observed Likelihood ratio test statistic (LR) to the distribution generated by parametric bootstrap. Data were bootstrap sampled according to the null hypothesis where the random effect of interest is not included. The full and reduced models are then fit to each bootstrap sample to determine the distribution for the LR test statistic. A = allopatric; S = sympatric. Bold indicates significance at $P<0.05$. Italics indicates marginal significance $P<0.06$.

Lamoille (A)				
Effect	ML est.	LR	*P*-Value	Intraclass Corr.
Female	**0.4024**	**8.10**	**0.0067**	0.096
Male	0.0000	0.00	0.7509	0.00
M x F	**0.1154**	**3.52**	**0.0383**	0.027
D. persimilis	**0.3413**	**37.49**	**0.0013**	0.082
Zion (A)				
Effect	ML est.	LR	*P*-Value	Intraclass Corr.
Female	*0.2683*	*2.72*	*0.05632*	0.067
Male	0.0000	0.00	0.4190	0.00
M x F	**0.3315**	**16.30**	**0.00238**	0.0833
D. persimilis	**0.0865**	**6.44**	**0.0068**	0.0217
Mt St. Helena(S)				
Effect	ML est.	LR	*P*-Value	Intraclass Corr.
Female	**0.8408**	**5.77**	**0.0068**	0.188
Male	0.0000	0.00	0.9891	0.000
M x F	**0.3266**	**8.76**	**0.0026**	0.0737
D. persimilis	0.0000	0.00	0.9851	0.000
Sierra (S)				
Effect	ML est.	LR	*P*-Value	Intraclass Corr.
Female	0.3287	0.72	0.1760	0.071
Male	0.1529	0.27	0.2673	0.033
M x F	**0.5975**	**7.28**	**0.0046**	0.129
D. persimilis	**0.2487**	**8.16**	**0.0012**	0.053

Table 3. The genotype effects that predict ISC. The maximum likelihood estimate (ML est.) and intraclass correlation (ICC) are reported as point estimates from the full model. The *P*-value for each term was calculated by comparing the observed Likelihood ratio test statistic (LR) to the distribution generated by parametric bootstrap. Data were bootstrap sampled according to the null hypothesis where the random effect of interest is not included. The full and reduced models are then fit to each bootstrap sample to determine the distribution for the LR test statistic. A = allopatric; S = sympatric. Bold indicates significance at $P<0.05$.

Lamoille (A)				
Effect	ML est.	LR	*P*-Value	Intraclass Corr.
Female	0.0668	0.825	0.2131	0.018
Male	0.0000	0.000	0.5037	0.000
M x F	**0.2098**	**29.93**	**0.0023**	0.057
GFP male	**0.0879**	**23.88**	**0.0010**	0.024
Zion (A)				
Effect	ML est.	LR	*P*-Value	Intraclass Corr.
Female	0.3003	3.202	0.0647	0.074
Male	0.0405	0.170	0.3721	0.010
M x F	**0.3056**	**22.47**	**0.0022**	0.076
GFP male	**0.0835**	**12.21**	**0.0011**	0.020
Mt. St. Helena (S)				
Effect	ML est.	LR	*P*-Value	Intraclass Corr.
Female	0.0000	0.000	1.0000	0.00
Male	0.0184	0.096	0.4120	0.005
M x F	**0.2195**	**52.44**	**0.0019**	0.060
GFP male	**0.0825**	**35.24**	**0.0010**	0.022
Sierra (S)				
Effect	ML est.	LR	*P*-Value	Intraclass Corr.
Female	0.0000	0.000	0.3744	0.00
Male	0.0000	0.000	1.0000	0.00
M x F	**0.4139**	**70.85**	**0.0021**	0.111
GFP male	0.0077	0.902	0.0886	0.002

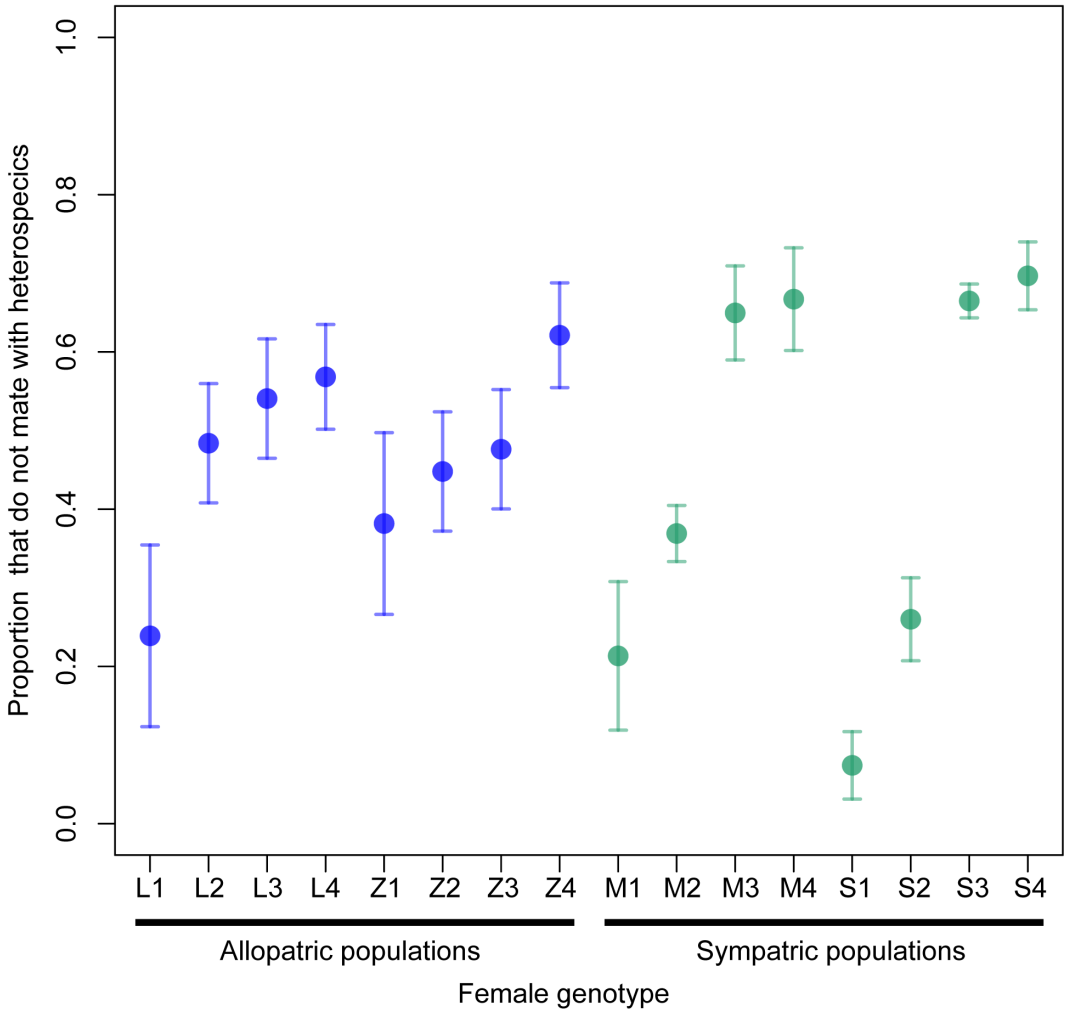

Figure 1. Reproductive isolation via female preference does not show a pattern consistent with reinforcement. Reproductive isolation is measured by the proportion of females that did not mate with heterospecifics in individual no-choice trials. Significant variation among *D. pseudoobscura* female genotypes in female preference occurs in each population (Supplemental Table 2). . Each point is the mean reproductive isolation for each isofemale line tested against each of four *D. persimilis* tester males. Error bars represent ± one standard error.

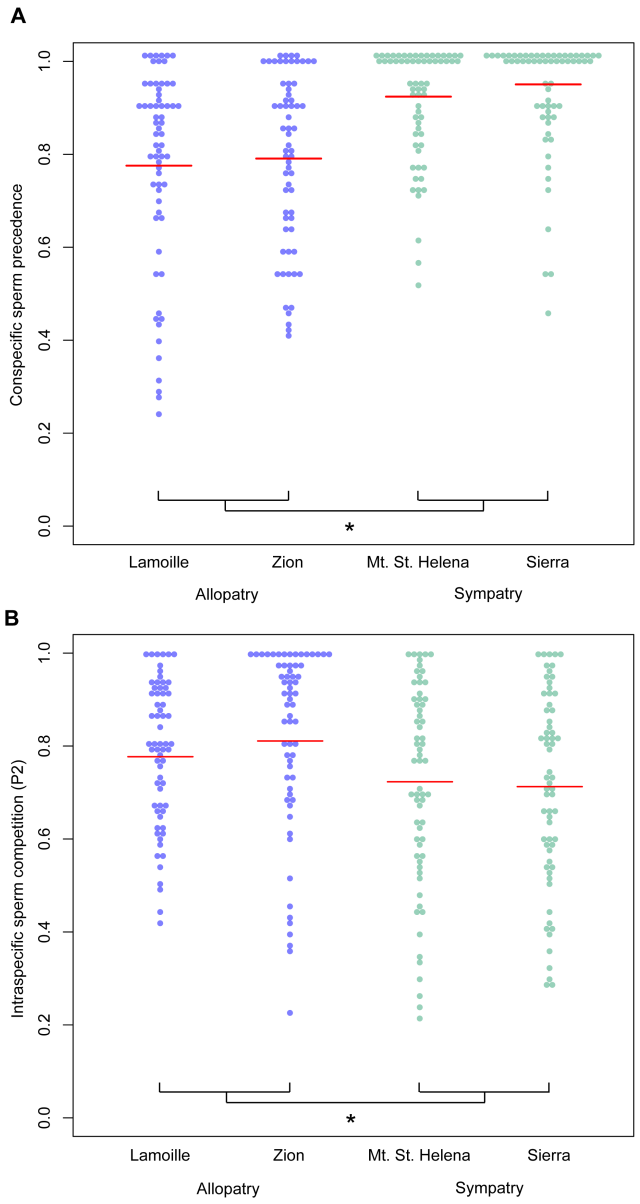

Figure 2. The phenotypic distributions of CSP (panel A) is consistent with a pattern of reinforcement. The distribution of ISC (panel B) shows a shift in ISC in the opposite direction compared to CSP for sympatric populations. The red line in each distribution represents the mean value. Significant differences determined by Welch's t-test and Wilcox tests between the allopatric and sympatric populations is denoted by *.

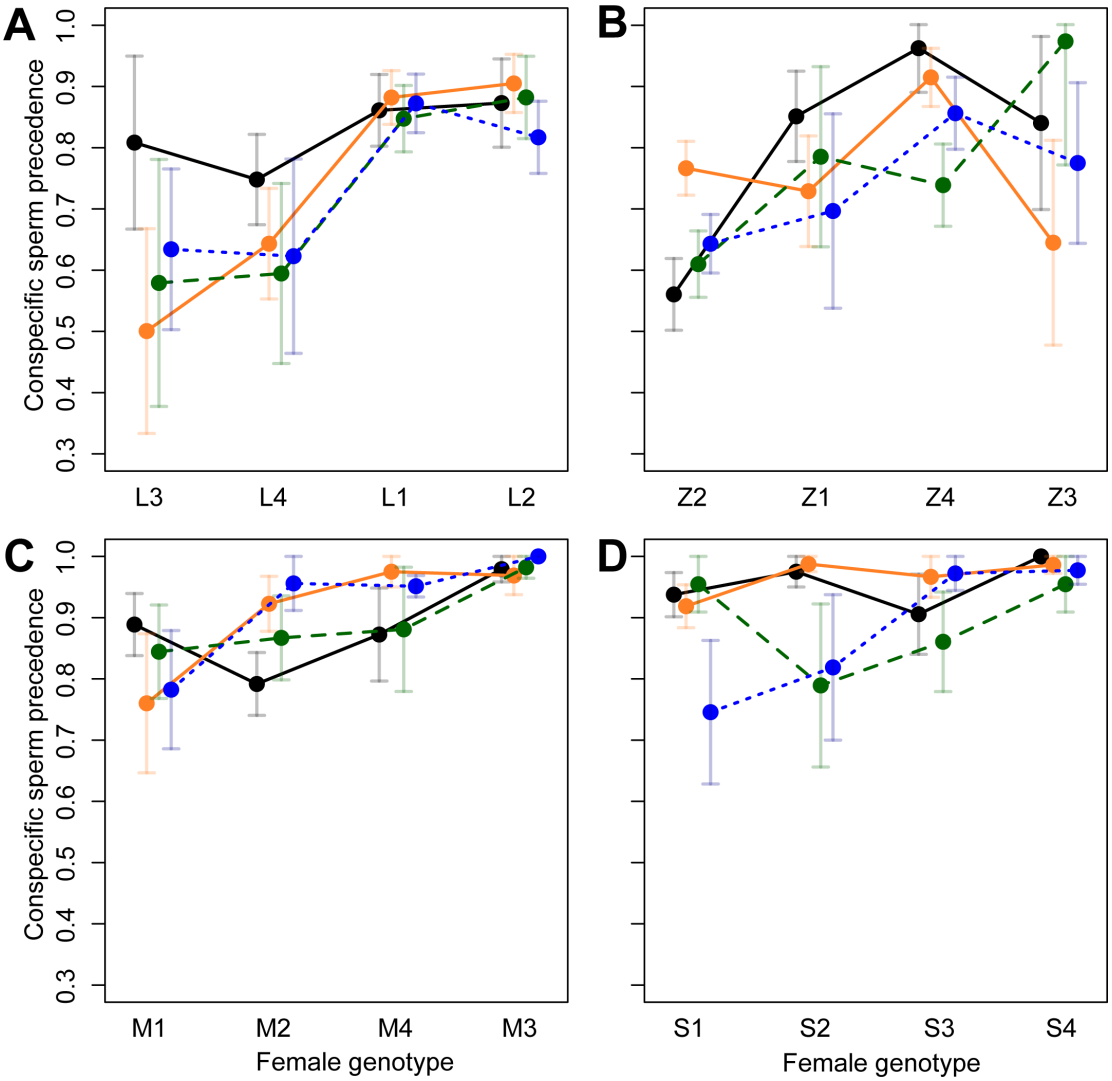

Figure 3. Conspecific sperm precedence (CSP) for all male-female genotype combinations in each population demonstrating a significant effect of female genotype and male-female genotype interaction on the outcome of CSP. A) Lamoille-Allopatry, B) Zion-Allopatry, C) Mt. Dt. Helena-Sympatry, and D) Sierra-Sympatry. Each point represents a specific male-female genotype combination. Error bars are ± one standard error. Female genotypes are ordered by mean CSP. Each color represents a single male genotype for each population. Colors were re-used between each population panel, but actual second male genotypes were unique to each population.

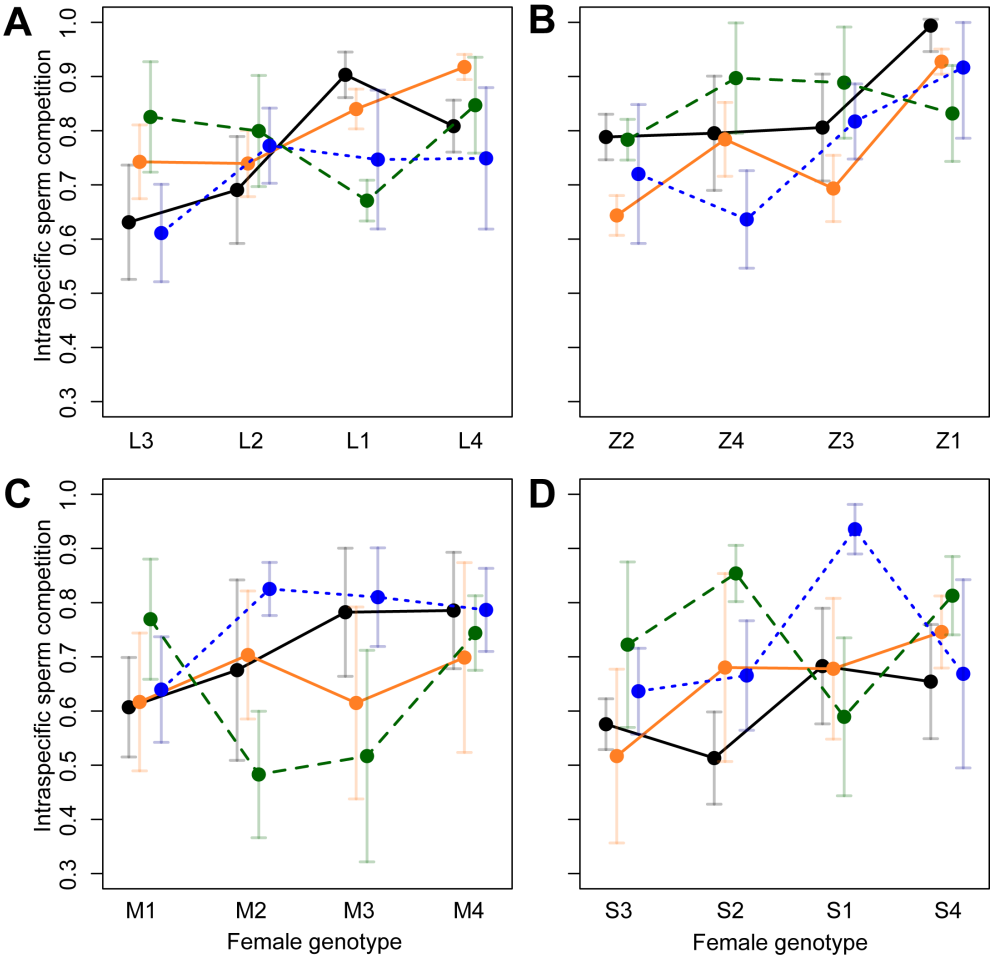

Figure 4. Intrapopulation sperm competition (ISC) for all male-female genotype combination in each population demonstrating a significant male-female genotype interaction on the outcome of ISC. A) Lamoille-Allopatry, B) Zion-Allopatry, C) Mt. Dt. Helena-Sympatry, and D) Sierra-Sympatry. Each point represents a specific male-female genotype combination. Error bars are ± one standard error. Female genotypes are ordered by mean ISC. Each color represents a single male genotype for each population. Colors were re-used between each population panel, but actual second male genotypes were unique to each population.

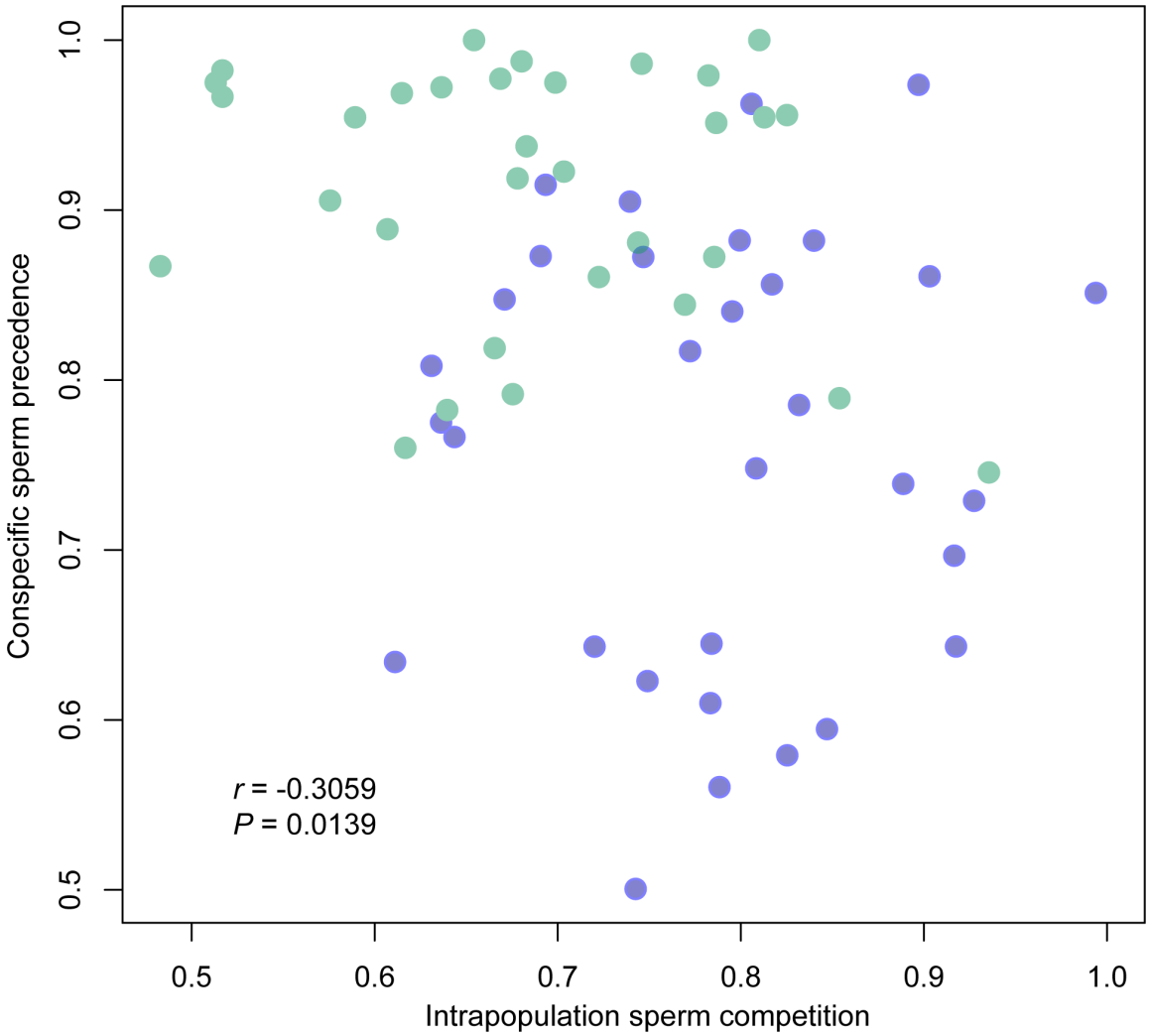

Figure 5. The negative correlation between intrapopulation sperm competition (ISC) and conspecific sperm precedence (CSP) across all four populations with each point representing a male-female genotype combination. Blue points are from allopatric populations and green points are from sympatric populations.

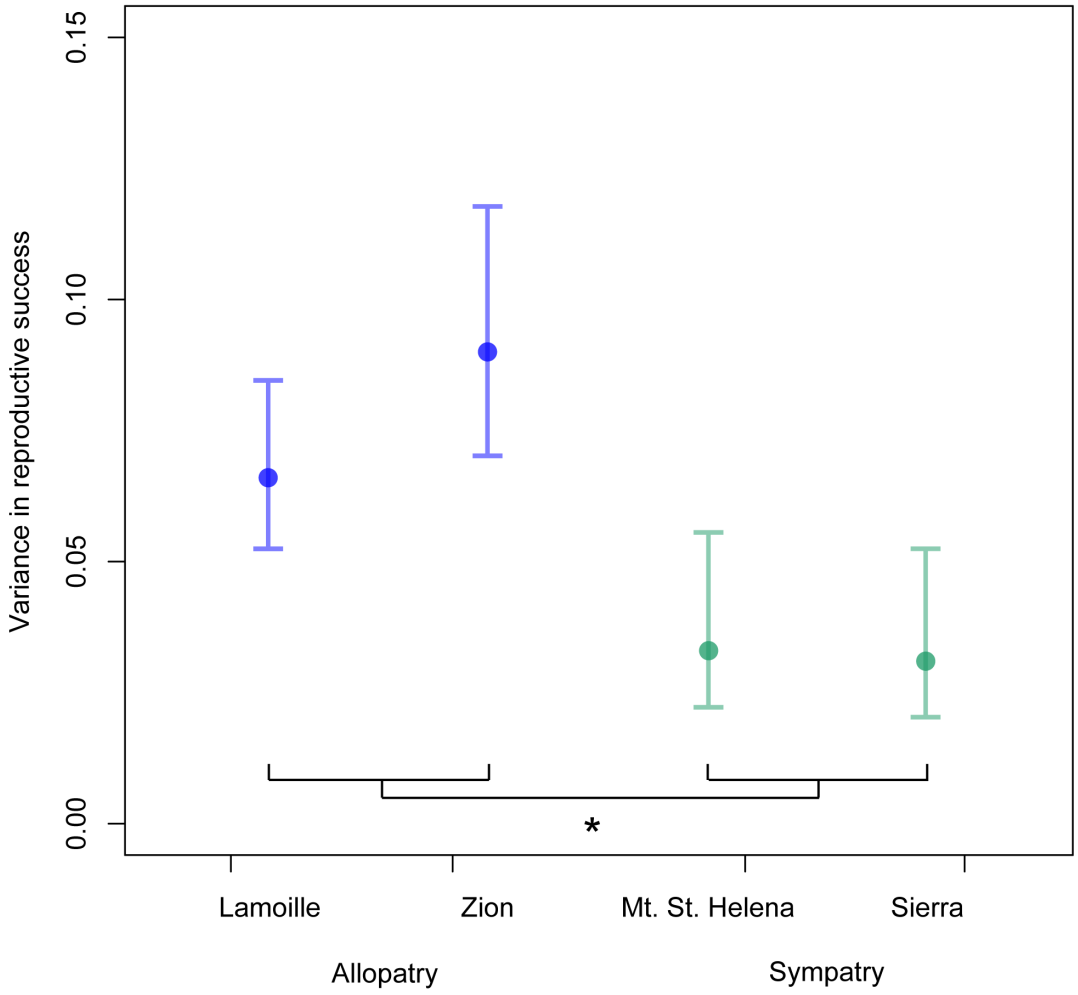

Figure 6. The variance in reproductive success across populations calculated in the framework that combines offensive and defensive males. Each point represents the estimate for the variance in fitness for each population. The error bars are confidence intervals generated from the empirical bootstrap distribution. Significance, denoted by *, was assessed in pairwise comparisons between allopatric and sympatric populations using empirical bootstrap hypothesis testing.