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

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It is typically assumed that sexual selection unilaterally drives the evolution of reproductive isolation, but selection for increased reproductive isolation could feed back on 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 pre-zygotic barriers to reduce the costs of heterospecific matings. Most studies of reinforcement focus on pre-mating barriers to reproduction, however post-mating traits, such as conspecific gamete precedence, are also ubiquitous barriers to reproduction that can potentially respond to reinforcing selection. Conspecific sperm precedence (CSP) also has a known shared genetic basis with intrapopulation sperm competition, allowing for the possibility that selection for increased CSP in sympatry could alter intrapopulation sperm competition specifically in these sympatric populations. We test this prediction with the sister species *Drosophila pseudoobscura* and *D. persimilis*, using two sympatric and two allopatric populations of *D. pseudoobscura*. Consistent with a pattern of reinforcement, the sympatric populations had higher mean CSP. Reinforcement, in turn, constrained sexual selection in sympatric populations by decreasing the average offensive sperm competitive ability within populations, allowing less opportunity for sexual selection to operate. These data demonstrate that strong reinforcing selection for reproductive isolation can have consequences for sexual selection and sexual interactions, in these important postmating sperm competition traits.

Introduction

The presence of heterospecifics can influence reproductive traits, sexual interactions, and potentially sexual selection when mating with heterospecifics is costly. In cases where closely related species come into contact—with the potential to interbreed—selection can favor divergence in sexual traits to avoid costs of heterospecific mating. This type of reproductive character displacement is commonly called reinforcement (Dobzhansky 1951; Howard 1993; Servedio and Noor 2003). The frequency at which reinforcement contributes to speciation is still under debate (Marshall et al 2002; Servedio and Noor 2003) though several recent examples provide strong evidence for reinforcement acting on mating traits (Nosil et al. 2003; Jaenike et al. 2006; Higgie and Blows 2007, 2008; Porretta and Urbanelli 2012; Dyer et al. 2014; Kozak et al. 2015). Reinforcement studies have focused on pre-mating traits, but reinforcement can act on any pre-zygotic trait (Servedio 2001; Lorch and Servedio 2007; Matute 2010). Indeed some have argued that the frequency of reinforcement might be underestimated because of the focus on easily observable, often showy, traits (Marshall et al 2002).

Conspecific gamete/sperm precedence is one such trait that has proven to be a strong barrier to reproductive isolation, but can be overlooked because it involves inconspicuous phenotypes that are not readily observed in the field (Marshall et al. 2002). Conspecific gamete precedence occurs when a female mates with both heterospecific and conspecific males and most of the progeny are sired by the conspecific male. Gamete precedence can be competitive (including male sperm competition and cryptic female choice) or non-competitive (resulting mainly from gametic incompatibilities) and is common both in plants and animals (Howard 1999 and references therein). Nonetheless,

because previous conspecific sperm precedence (CSP) studies have not compared both allopatric and sympatric populations concurrently (Hewitt et al 1989; Lacquer et al 1990; Carney et al 1994; Metz et al. 1994 Hauser et al 1997; Rieseberg et al 1995; Klips 1999; Williams et al 1999; Chang 2004; Peterson et al 2011), it is unclear whether heterospecific interactions select for increased CSP specifically in sympatry, or if divergence in postmating sexual traits drives the evolution of CSP between closely related species regardless of the presence of heterospecifics.

Seminal fluid proteins in *Drosophila* contribute to both intraspecific sperm competition (ISC) and conspecific sperm precedence (CSP) (Castillo and Moyle 2014; Civetta and Finn 2014) and are among the best-characterized examples of molecules involved in sexual selection and sexual conflict within species. Their underlying genes are expressed specifically by males in the accessory gland, however they are known to influence female oviposition rate, female remating rate, and female lifespan (Sirot et al. 2009) and evidence for male x female genetic interactions at these loci is consistent with coevolution between the sexes (Clark et al. 1999, Chow et al. 2010). These seminal fluid proteins evolve rapidly between species (Begun et al. 2000; Wong et al. 2008) suggesting strong selection imposed by sexual selection and/or sexual conflict, that could easily drive divergence between species. Conversely, because CSP and ISC share components of their genetic basis (Castillo and Moyle 2014), divergence between species may also have feedback effects on sexual selection and interactions within species.

This potential feedback between sexual selection and reproductive isolation has consequences for evolutionary outcomes when these processes act at cross-purposes, including in their expected outcomes for the mean and variance in the affected traits. For

example, forces proposed to act on sperm competition genes include sexual conflict between males and females (i.e. antagonistic pleiotropy; Prout and Clark 1996; Civetta and Clark 2000; Fiumera et al 2006), male-male genotype interactions (Clark 2002; Zhang et al. 2012), and male-female genotype interactions (Clark et al 1999; Bjork et al. 2007; Chow et al 2010), all of which are expected to maintain high variance in the affected traits. Indeed, sperm competition genes are often highly variable both in terms of molecular and phenotypic variation (Wong et al. 2008; Chow et al. 2010; Zhang et al 2012). In contrast, under models of speciation by sexual selection, reproductive isolation is generated by strong disruptive selection between populations and directional selection within a population (Panhuis et al 2001; Kirkpatrick and Ravigne 2002), including directional selection imposed by reinforcement (Dobzhansky 1951; Howard 1993; Servedio and Noor 2003), thereby reducing genetic variance and shifting the overall trait mean of the affected reproductive traits. Therefore we expect a shift in the mean level and reduced genetic variation for CSP compared to ISC, specifically in sympatry.

The primary determinants of phenotypic variation might also be expected to differ between CSP and ISC. Sexual dynamics within species indicate that variation in ISC should be determined by both male and female genetic effects, and their interaction (Clark et al. 1999; Chow et al. 2010). In contrast, because females experience most of the cost of heterospecific matings (Trivers 1972; Saetre 1997; Bonduriansky 2001), variation in CSP might be predominantly controlled by females via cryptic female choice (Manier et al 2013b). Indeed, although older evidence suggests that CSP might involve male traits such as displacement or incapacitation of rival male sperm (Price et al. 2000; Rugman-Jones and Ready 2007), more recent experiments have demonstrated that females control

sperm use patterns (Manier et al 2013b), and thus we might expect female genetic effects to strongly influence observed variation in CSP. One effective strategy to evaluate these potential differences between selection for increased reproductive isolation and sexual selection acting on sperm competition genes is to estimate variation between genotypes in CSP and ISC in parallel.

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 (Noor 1995). Subsequent studies suggest variation in the presence and strength of reinforced premating isolation is larger than previously reported (Anderson and Kim 2005, 2006; Barnwell and Noor 2008; Davis et al in review; and see below), making it a potentially valuable system in which to also analyze the evolution of CSP in response to heterospecific interactions. Here we first determine whether 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 affects ISC and sexual selection, as might occur when CSP and ISC have shared genetic architecture. Throughout, we test for both changes in the mean levels of CSP and ISC, and for differences in the level of phenotypic variance; reduced phenotypic variance could reflect strong directional selection in sympatry. Moreover, because we used a

breeding design (similar to a diallel design) that evaluates trait variation across a set of distinct genotypes, we were able to determine whether male, female or male-female genotype interactions predict variation in ISC and CSP. This 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, we are evaluating the "offensive" sperm competitive ability of conspecific males to displace heterospecific sperm (equivalent to 'P2', or second male siring ability). We focused on "offensive" sperm competition because *D. pseudoobscura* females do not remate with *D. persimilis* males after mating first with conspecifics. 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 (Clark et al 1999; Chow et al 2010; Supplemental Fig 1); Diallel designs are used to estimate additive genetic variance or heritability directly (as in Lupold et al 2013). 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). 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. 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. 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 (Dixon et al 2003). 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 in these individual vials for 7 days before second mating (similar to Dixon et al 2003). 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 (Davis et al. in review). Only females that had mated (i.e. had larvae at 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 the first male was a *D. pseudoobscura* tester male derived from the same population as the diallel block, rather than a *D. persimilis* male. 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 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 (Markow and O'Grady 2005), so we are confident that only a single mating occurred in this 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.

Tester males for conspecific sperm precedence experiments

In this experiment our goal was to examine both the mean strength and the total amount of variation in CSP. Because paternity is scored via visible markers in these sperm competition experiments, we could not simply use wild-type wild-collected *D. persimilis* males from our sympatric sites. Instead, 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. These stable lines were used as testers for the CSP experiments.

Tester males for intraspecific sperm competition assays

Similar to the CSP tester lines, for ISC assays our aim was to use wild-caught tester lines that nonetheless expressed a visible marker that allowed progeny scoring. The tester males for ISC experiments were created by introgressing a green fluorescent protein marker (GFP) into wild type *D. pseudoobscura* strains (2 strains per population, using isofemale lines that were not a part of the diallel blocks, therefore 8 strains in total). The original GFP strain was obtained from the UCSD stock center (14011-0121.166) the creation of which is described in Holtzman et al. (2010). We chose this marker because it is dominant (Castillo and Moyle 2014) and 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 squished, 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.

As 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. 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 hybrid females do not demonstrate a mating preference whereas *D. pseudoobscura* females (and therefore purebred female progeny) exhibit premating isolation with *D. persimilis* males. 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. If the 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. 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 (Davis et al. *in*

review). Every CSP estimate was based 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 (Lorch and Servedio 2005; Davis et al. *in review*). There was also no correlation between the 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. (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 (2014). GFP can be seen in both the pseudopupil and ocelli, but is more apparent in the ocelli.

Statistical analyses

All analyses were completed in R v 3.01.

<u>Differences in the probability of first mating with heterospecifics</u>

We first tested for a pattern consistent with reinforcement acting on first mating (simple prezygotic isolation) using a chi-square test of independence. We combined both allopatric and both sympatric populations for a single comparison between allopatry and sympatry (pairwise tests among individual populations provided the same result; Supplemental Table 1), and we tested the null hypothesis that the mating rate with heterospecifics was the same for both geographic scenarios (allopatric vs. sympatric). 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 also 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 Lesnoff and Lancelot 2012). The Wald's test evaluates the omnibus test, specifically the null hypothesis assumes $B_1=B_2=B_3=B_4$. (A one-way ANOVA test is similar to the Wald's Test, but uses an exact *F*-distribution instead of an asymptotic χ^2 distribution).

We also used logistic regression 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 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. We 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

Similar to the first-mating data, we first tested whether there was a pattern in CSP consistent with reinforcement, by evaluating whether there was a mean difference in CSP between the allopatric and sympatric populations. 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. Given that the data are not normally distributed we confirmed these results with a Wilcox ranked sum test. To evaluate whether the total phenotypic variation differed between geographical classes of population we compared variance with a Levene-type test (Hui et al. 2008). The specific test we used in the lawstat package is a Kruskal-Wallis modified Brown-Forsythe Levene-type test (our choice of test is described in Supplemental Methods). 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).

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, however, that we still observed significant differences in

pairwise tests between individual allopatric and sympatric populations, for both average and variance measures of CSP and ISC (Supplemental Table 2).

Genetic variation and genotype effects on CSP and ISC

Within each population we were also interested in whether female, male, or female x male genotype predicted variation in the strength of CSP and ISC. Traditionally, this can be tested using a two-way ANOVA with interaction. We instead chose 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. The clustering of residuals, which is a byproduct of the interaction term, can cause the model to look overdispersed. This can be remedied by using quasi-binomial regression, however the Wald Test and Likelihood Ratio tests are no longer applicable in this case (Fox 2008). To circumvent these problems we used a binomial regression in a mixed modeling framework where we could employ parametric bootstrap to test the significance of each variance component (Halekoh and Højsgaard 2014). 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 (ICC) for each. The ICC for the female effect, for example, would be:

$$ICC_F = \frac{\sigma_F^2}{\sigma_F^2 + \sigma_M^2 + \sigma_{MF}^2 + \sigma_T^2 + \frac{\pi^2}{3}}$$

Where *F* represents female variance, *M* represents male variance, *MF* represents the interaction, and *T* represents the identity of the tester male. The $\frac{\pi^2}{3}$ replaces the residual variance for the binomial model with logit link function. In a Gaussian model the ICC can be interpreted as the correlation of residuals for a specific level of a variable averaged across all other variables (high correlation of a residual indicates that variable explains much of the variance in the data). In the case of binomial regression the ICC values are on the log scale, and there is no convenient transformation to proportion scale (Eldridge et al. 2009), so they are only presented as a relative measure of variance explained.

Quantifying sexual selection and variance in male reproductive success

To understand whether the intensity/opportunity for sexual selection has changed among populations we need an estimate of variance in male reproductive success (Wade 1979). 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 males were used as offensive or defensive males so we estimated male fitness as the proportion of progeny sired. We considered tester (defensive) and diallel

block (offensive) males as different classes of males (Wade and Shuster 2004; Shuster et al 2013), to account for the fact that they differed in their frequency in the experiment. Following Shuster et al. (2013) 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 (ex. 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 the variance of reproduction at the level of male genotype so we averaged replicates to generate fitness values for individual genotypes. We made empirical bootstrap confidence intervals of V_{total} for each population to determine how the variation between replicates affected our estimate of variance (Supplemental Methods). Differences in variance estimates can be compared using an *F* test when the variances are normally distributed. The distribution of the V_{total} parameters is unknown so we estimated the significance of differences in male reproductive variance between allopatric and sympatric populations using a bootstrap hypothesis test of the *F* statistic (Supplemental Methods).

RESULTS

No difference 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 between populations ranged

from 46-52% (Lamoille=51.9%, n=179, Zion=52.4%, n=145; Mt. St. Helena=46%, n=200 Sierra=49.5%, n=222), and there was no significant difference in heterospecific mating rate 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). 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 (Table 1; Fig. 1). Only in one of the allopatric populations (Lamoille) did the identity of the *D. persimilis* tester line affect variation in this trait (Table 1; Supplemental Fig 4).

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 (Fig. 2). The mean CSP for the allopatric populations was 0.76 whereas the mean for the sympatric populations was 0.91, and these were significantly different (*t*=-6.5898, df=210.92, *P*<0.001; Wilcox *W*=4427.5, *P*<0.001). In addition, phenotypic variance in CSP (reported here as standard deviations for ease of interpretability) for the allopatric populations was 0.23 (Lamoille) and 0.20 (Zion), and for both of the sympatric populations was 0.13. These differences in variance between the allopatric and sympatric populations were significant (Levene-type test χ^2=22.82, *P*<0.0001; data pooled by geographic region).

Reinforcement has collateral effects on intrapopulation sperm competition

ISC also differed between allopatric and sympatric populations, but in the opposite direction from CSP. The average P2 for ISC in allopatric populations (0.78) was

significantly greater than the average P2 (0.68) of sympatric populations (t=3.738, df=246.55,P=0.0002; Wilcox's W=10280, P=0.0004; Fig. 2). In addition there was more variation in ISC in the sympatric populations than the allopatric populations (Leven-type test χ^2=5.74, P=0.0172); the standard deviation in P2 for allopatry was 0.19 and the standard deviation for P2 in sympatry was 0.23. Given the differences in ISC and CSP across populations we examined the relationship between these two phenotypes across male x female genotype combinations (cells of the diallel crossing design) that only differed in whether they were interacting with a heterospecific or conspecific male. We observed a significant negative relationship between CSP and ISC (Pearson's r=-0.31, P=0.01; Fig. 5)

Genotype effects differ between 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 ICC (i.e. which explained the largest proportion of variance). In some populations the female genotype effect had the largest ICC, while in others the male x female genotype interaction had the largest ICC (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 ICC (usually two to three times greater) than the identity of the GFP tester male.

The opportunity for sexual selection is decreased in sympatry

The opportunity for sexual selection to operate is strongly influenced by the variance in male reproductive success (Wade 1979; Wade and Shuster 2004; Shuster et al 2013). The sympatric populations had significantly lower variance for reproductive success (Table 4) because lower P2 values in sympatry resulted in equal siring success between offensive and defensive males. The variance in reproductive success in the allopatric Lamoille population was significantly greater than both the 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 (Mt. St Helena F=2.65, Bootstrap P=0.003; Sierra F=2.83, Bootstrap P=0.004).

DISCUSSION

Interactions with heterospecifics have the potential to drive divergent sexual selection and the evolution of reproductive isolation, via reproductive character displacement and reinforcement (Higgie and Blows 2007,2008; Pfennig and Pfennig 2009). In this study 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 (Howard 1999). 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 (Castillo and Moyle 2014; Civetta and Finn 2014). Using *Drosophila pseudoobscura* and *D. persimilis* we saw a clear signal of increased conspecific sperm precedence in sympatric populations, consistent with a pattern of reinforcement. The average CSP was higher, and

the overall level of phenotypic variation was lower, specifically in sympatric populations, a pattern expected if there has been recent directional selection acting on CSP in these populations. Sympatric populations also had lower intraspecific sperm competitive ability (lower offensive P2) than allopatric populations. Given the negative correlation between CSP and ISC, we infer that selection for stronger CSP in sympatry has reduced ISC in sympatric populations. The collateral outcome for ISC is less variation in male reproductive success and reduced opportunity for sexual selection.

The pattern of reinforcement that we observed highlights the important role that CSP and sperm competition can play in maintaining species boundaries. Conspecific sperm precedence is known to be a barrier to gene flow in *Drosophila* (Price 1997; Dixon et al 2003; Chang et al 2009) and other taxa (Howard 1999), but its overall importance in nature has been difficult to ascertain (Marshall et al. 2002; Lorch and Servedio 2007). Our results indicate that CSP can strongly contribute to reproductive isolation, specifically in response to reinforcing selection. This, in turn, suggests that other premating barriers preceding CSP are not strong enough to limit the efficacy of selection on CSP (Marshall et al. 2002; Lorch and Servedio 2007).

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 the *Drosophila pseudoobscura* and *D. persimilis* sister pair was used in one of the first studies demonstrating reinforcement on premating barriers (Noor 1995), although studies since this original finding have found more equivocal patterns (Anderson and Kim 2005, 2006; Davis et al in review). 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, consistent with other studies of this species pair (Lorch and Servedio 2005; Davis et al. in review). In comparison, other species pairs with incomplete premating isolation, such as *D. yakuba-santomea* (Coyne et al 2002), have detected evidence for reinforcing selection on postmating barriers, in the form of this non-competitive gametic incompatibility (Matute 2010). Overall, the lack of complete premating isolation and non-competitive gamete isolation appears to have provided an opportunity for CSP to contribute strongly to reproductive isolation in these populations.

In our study we observed not only an increase in mean CSP in sympatric populations but also a decrease in the phenotypic variation—a pattern consistent with strong recent or recurrent selection acting on CSP in sympatry. Models of speciation by sexual selection show that strong divergent selection will erode phenotypic variation (Lande 1981; Kirkpatrick 1982) leaving a signature of reduced variation. Previous studies of reinforcement acting on premating traits focus on the average trait value; while they sometimes qualitatively describe variation in these traits, it is typically not quantified (Jaenike et al. 2006; Matute 2010; Dyer et al. 2014). Our observation of reduced variation specifically in sympatry lends additional support to our inference that CSP has responded to strong selection imposed by heterospecific interactions.

For post-mating prezygotic traits female genotype, male genotype, or the interaction of genotypes have the potential contribute to variation. Female genotypes can contribute via cryptic female choice (Manier et al 2013b), male genotypes can contribute through male-male competition (Clark 2002), and genotype interactions may contribute if genotype x genotype interactions determine the outcome of CSP, similar to what has been

observed for ISC (Clark et al. 1999; Chow et al. 2010). Although studies do not usually quantify/partition the variance in CSP in this way, our design enabled us to do so for both ISC and CSP, to better understand which sex is playing a more critical role in each phenotype. We saw significant male x female genotype interactions for all populations for both CSP and ISC, but only saw significant female genotype effects for CSP. Male genotype effects were not observed for either trait. The strong effect of female genotype on CSP, but not ISC, is consistent with the hypothesis that females face more costs of hybridization (Trivers 1972; Saetre 1997; Bonduriansky 2001) and choice manifests as female control of sperm use patterns (Manier et al 2013; Tyler et al 2013). For CSP, cryptic female choice operates more similarly to premating isolation mechanisms where females are the more "choosy" sex and female effects control the level of reproductive isolation more so than male effects.

One potential consequence of the reproductive trait evolution wrought by reinforcement is collateral changes in the magnitude and efficacy of sexual selection on the same traits, when they share a genetic basis. Previous work has demonstrated a genetic link between CSP and ISC (Castillo and Moyle 2014), and in this study we found both a negative relationship between CSP and ISC, and that ISC was reduced in sympatric populations. Initially we might have hypothesized that selection for CSP would also select for increased P2 among conspecifics, if offensive sperm competitive ability was a general trait that acted regardless of whether the competitor was a conspecific or heterospecific male. Instead these data suggest that selection for increased reproductive isolation affect CSP and ISC in different ways; not only are CSP and ISC observed to be

negatively correlated, but the reduced phenotypic variation seen for CSP in sympatry was not mirrored by reduced phenotypic variation for ISC.

For reproductive isolation to effect sexual selection among conspecifics, we not only need to demonstrate differences in trait means (Higgie and Blows 2008), but also differences in male reproductive success, because variance in reproductive success is what determines the strength/opportunity of sexual selection (Wade 1979). Sperm competition contributes to variance in reproductive success because male genotypes that can disproportionately sire offspring increase their fitness compared to rival males' fitness (Levitan 2008; Pischedda and Rice 2012). Sperm competition is therefore strong when there is greater variance in reproductive success compared to when males have equal probability of siring offspring (sperm competition offensive/defense ability=0.5). 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 reduced differences in sperm competitive ability among competing males. Less variance in male reproductive success translates to less opportunity for sexual selection to act in sympatry compared to allopatry.

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 connection between sexual selection and reproductive isolation is usually conceptualized in terms of sexual selection driving the evolution of reproductive isolation. But a direct genetic connection between these processes implies reproductive isolation also has the reciprocal potential to shape sexual selection (Servedio and Burger 2014). 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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LITERATURE CITED

Anderson, W.W., and Y.K. Kim, 2005 Sexual isolation between sympatric and allopatric populations of *Drosophila pseudoobscura* and *D. persimilis*. Behavior Genetics 35: 305-312.

Anderson, W.W., and Y.K. Kim, 2006 A further analysis of sexual isolation between sympatric and allopatric populations of *Drosophila pseudoobscura* and *D. persimilis* - Rejoinder to

Noor and Ortiz-Barrientos. Behavior Genetics 36: 328-330.

Barnwell, C.V., and M.A.F. Noor, 2008 Failure to replicate two mate preference QTLs across multiple strains of *Drosophila pseudoobscura*. Journal of Heredity 99: 653-656.

Begun, D.J., P. Whitley, B.L. Todd, H.M. Waldrip-Dail, A.G. Clark. 2000. Molecular population genetics of male accessory gland proteins in *Drosophila*. Genetics. 156, 1879–1888.

Bjork, A., W.T. Starmer, D.M. Higginson, C. J. Rhodes, and S. Pitnick. 2007. Complex interactions with females and rival males limit the evolution of sperm offence and defence. Proceedings of the Royal Soceity B 274: 1779–1788.

Bonduriansky, R. 2011. Sexual selection and conflict as engines of ecological diversification. The American Naturalist 178:729-745.

Carney, S.E., M.B. Cruzan, and M.L. Arnold. 1994. Reproductive interactions between hybridizing irises: analyses of pollen tube growth and fertilization success. American Journal of Botany 81:1169–1175.

Castillo, D.M., and L.C. Moyle. 2014. Intraspecific sperm competition genes enforce post-mating species barriers in *Drosophila*. Proceedings of the Royal Society B 281.

Civetta, A., and A.G. Clark. 2000. Correlated effects of sperm competition and postmating female mortality. Proceedings of the National Academy of Science of the USA 97:13162-13165.

Civetta, A., and S. Finn. 2014. Do candidate genes mediating conspecific sperm precedence affect sperm competitive ability within species? A test case in *Drosophila* G3 4:1701-1707.

Chang, A.S. 2004. Conspecific sperm precedence in sister species of *Drosophila* with overlapping ranges. Evolution 58:781-789

Chow, C. Y., M. F. Wolfner, and A. G. Clark. 2010. The genetic basis for male x female interactions underlying variation in reproductive phenotypes of *Drosophila*. Genetics 186:1355-U1451.

Clark, A. G., D. J. Begun, and T. Prout. 1999. Female x male interactions in *Drosophila* sperm competition. Science 283:217-220.

Coyne, J. A., S.Y. Kim, A.S. Chang, D. Lachaise, and S. Elwyn. 2002. Sexual isolation between two sibling species with over- lapping ranges: *Drosophila santomea* and *Drosophila yakuba*. Evolution 56:2424–2434.

Dixon, S.M., J.A. Coyne, and M.A.F. Noor. 2003. The evolution of conspecific sperm precedence in *Drosophila*. Molecular Ecology 12:1179–1184.

Dobzhansky, T. 1951. Genetics and the Origin of Species. Columbia University Press New York NY.

Dyer, K.A., B.E. White, J.L. Sztepanacz, E.R. Bewick, and H.D. Rundle. 2014. Reproductive character displacement of epicuticular compounds and their contribution to mate choice in *Drosophila subquinaria* and *Drosophila recens*. Evolution 68:1163-1175.

Eberhart, W.G. 1996. Female control: sexual selection by crypitc female choice. Princeton University Press, Princeton, NJ.

Eldridge, S.M., O.C. Ukoumunne, and J.B. Carlin. 2009. The intra-cluster correlation coefficient in cluster randomized trials: a review of definitions. International Statistical Review 77:378-394.

Fiumera, A.C., B L. Dumont and A.G. Clark. 2006. Natural variation in male-induced 'cost-of-mating' and allele-specific association with male reproductive genes in *Drosophila melanogaster*. Philosophical Transactions of the Royal Soceity B 361: 355–361.

Fox, J. 2008. Applied regression analysis and generalized linear models 2nd Ed. Sage Publications, Inc. Thousand Oaks, CA.

Halekoh, U., and S. Højsgaar. 2014. A Kenward-Roger approximation and parametric bootstrap methods for tests in linear mixed models - The R Package pbkrtest. Journal of Statistical Software 59:1-30.

Hauser, T.P., R. Jorgensen, and H. Ostergard. 1997. Preferential exclusion of hybrids in mixed pollinations between oilseed rape (*Brassica napus*) and weedy *B. campestris* (Brassicaceae). American Journal of Botany 84:756–762.

Hewitt, G.M., P. Mason, and R. Nichols. 1989. Sperm precedence and homogamy across a hybrid zone in the alpine grasshopper *Podisma pedestris*. Heredity 62:343–353

Higgie, M., and M.W. Blows. 2007. Are traits that experience reinforcement also under sexual selection? The American Naturalist 170:409-420.

Higgie, M., and M.W. Blows. 2008. The evolution of reproductive character displacement conflicts with how sexual selection operates within a species. Evolution 62:1192-1203.

Holtzman, S., D. Miller, R. Eisman, H. Kuwayama, T. Niimi, and T. Kaufman. 2010 Transgenic tools for members of the genus *Drosophila* with sequenced genomes. Fly 4:349-362.

Howard, D.J . 1993. Reinforcement: origins, dynamics, and the fate of an evolutionary

hypothesis *in* R.G. Harrison, ed. Hybrid Zones and Evolutionary Process. Oxford University Press, Oxford, pp. 46–69

Howard, D. J. 1999. Conspecific sperm and pollen precedence and speciation. Annual Review of Ecology and Systematics 30: 109-132.

Hui, W., Y.R. Gel, and J.L. Gastwirth. 2008. lawstat: an R package for law, public policy and biostatistics. Journal of Statistical Software 28.

Jaenike, J., K.A. Dyer, C. Cornish, M.S. Minhas. 2006. Asymmetrical reinforcement and *Wolbachia* infection in *Drosophila*. Plos Biology 4:1852-1862.

Janetos, A.C. 1980. Strategies of female mate choice: a theoretical analysis. Behavioral Ecology and Sociobiology 7:107-112.

Kay, K. M. 2006. Reproductive isolation between two closely related hummingbird-pollinated Neotropical gingers. Evolution 60: 538-552.

Kirkpatrick, M. 1982. Sexual selection and the evolution of female choice. Evolution 36:1-12.

Kirkpatrick, M., and V. Ravigne. 2002 Speciation by natural and sexual selection: Models and experiments. The American Nautralis 159:S22-S35.

Klips, R.A. 1999. Pollen competition as a reproductive isolating mechanism between two sympatric *Hibiscus* species (Malvaceae). American Journal of Botany 86:269–272.

Kozak, G., M.G. Rolan, C. Rankhorn, A. Falater, E.L. Berdan, and R.C. Fuller. 2015. Behavioral isolation due to cascade reinforcement in *Lucania* killifish. The American Naturalist 185:491-506.

Lesnoff, M., and R. Lancelot. 2012. aod: Analysis of Overdispersed Data. R package

version 1.3.

Levitan, D.R. 2008. Gamete traits influence the variance in reproductive success, the intensity of sexual selection, and the outcome of sexual conflict among congeneric sea urchins. Evolution 62:1305-1316.

Lorch, P.D., and M.R. Servedio 2005. Postmating-prezygotic isolation is not an important source of selection for reinforcement within and between species in *Drosophila pseudoobscura* and *D. persimilis*. Evolution 59:1039-1045.

Lorch, P.D., and M.R. Servedio. 2007. The evolution of conspecific gamete precedence and its effect on reinforcement. Journal of Evolutionary Biology 20:937-949.

Lupold, S., S. Pitnick, K.S. Berben, C.S. Blengini, J.M. Belote, and M.K. Manier. 2013. Female mediation of competitive fertilization success in *Drosophila melanogaster*. Proceedings of the National Academy of Sciences of the USA 110: 10693-10698.

Manier, M.K., J.M. Belote, S. Lüpold, K.S. Berben, O. Ala-Honkola, W.F. Collins, and S. Pitnick. 2013a. Rapid diversification of sperm precedence traits and processes among three sibling *Drosophila* species. Evolution 67: 2348-2362

Manier, M.K., S. Lupold, J.M. Belote, W.T. Starmer, K.S. Berben *et al.*, 2013b Postcopulatory sexual selection generates speciation phenotypes in *Drosophila*. Current Biology 23: 1853-1862.

Markow TA, O'Grady PM. 2005 Drosophila: A guide to species identification and use. Academic Press, Waltham Mass.

Marshall, J. L., M. L. Arnold and D. J. Howard, 2002 Reinforcement: the road not taken. Trends in Ecology & Evolution 17: 558-563.

Matute, D. R. 2010. Reinforcement of gametic isolation in Drosophila. PLoS Biology 8: 3.

Metz, E.C. R. E. Kane, H. Yanagimachi and S. R. Palumbi. 1994. Fertilization between closely related sea urchins is blocked by incompatibilities during sperm-egg attachment and early stages of fusion. The Biological Bulletin 187:23–34.

Mullen, S.P., T.C. Mendelson, C. Schal, and K.L. Shaw. 2007. Rapid evolution of cuticular hydrocarbons in a species radiation of acoustically diverse Hawaiian crickets (Gryllidae: Trigonidiinae: Laupala). Evolution 61: 223-231

Noor, M.A.F. 1995. Speciation driven by natural selection in *Drosophila*. Nature 375: 674-675.

Nosil, P., B.J. Crespi, and C.P. Sandoval. 2003. Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement. Proceedings of the Royal Soceity London B 270:1911-1918.

Panhuis, T.M., R. Butlin, M. Zuk and T. Tregenza, 2001 Sexual selection and speciation. Trends in Ecology & Evolution 16: 364-371.

Peterson, M.A., E.L. Larson, M. Brassil, K.J. Buckingham, D Juarez, et al. 2011. Cryptic gametic interactions confer both conspecific and heterospecific advantages in the *Chrysochus* (Coloptera: Chrysomleidae) hybrid zone. Genetica 139:663-676.

Pfennig KS, Pfennig DW. 2009. Character displacement: ecological and reproductive responses to a common evolutionary problem. Quarterly Review of Biology 84:253-276.

Pischedda, A. and W. R. Rice. 2012. Partiotioning sexual selection into its mating success and fertilization success components. Proceedings of the National Academy of Sciences of the USA 109:2049-2053.

Porretta, D., and S. Urbanelli. 2012. Evolution of premating reproductive isolation among conspecific populations of the sea rock-pool beetle *Ochthebius urbanelliae* driven by reinforcing natural selection. Evolution 66:1284-1295.

Price, C.S.C. 1997 Conspecific sperm precedence in *Drosophila*. *Nature* 388:663-666

Price, C.S., C.H. Kim, J. Posluszny, J.A. Coyne. 2000. Mechanisms of conspecific sperm precedence in *Drosophila*. Evolution 54:2028-2037

Prout, T., and A.G. Clark. 1996. Polymorphism in genes that influence sperm displacement. Genetics 144:401-408.

Ramsey, J., H.D. Bradshaw Jr., and D.W. Schemske. 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). Evolution 57:1520-1534.

Reynolds, J.D., and M.R. Gross. 1990. Costs and benefits of female mate choice: is there a lek paradox? The American Naturalist 136:230-243.

Rieseberg, L.H. 1995. Interspecific pollen competition as a reproductive barrier between sympatric species of *Helianthus* (Asteraceae). Am . J . Bot. 82, 515–519

Rugman-Jones, P.F., and P.E. Ready. 2007. Conspecific sperm precedence in *Callosobruchus subinnotatus* (Coleoptera: Bruchidae): mechanisms and consequences. Proceedings of the Royal Society of London B 274:983-988.

Saetre, G.-P., M. Král, and S. Bures. Differential species recognition abilities of males and females in a flycatcher hybrid zone. Journal of Avian Biology 28:259-263.

Servedio, M.R. 2001. Beyond reinforcement: the evolution of premating isolation by direct selection on preferences and postmating, prezygotic incompatibilities. Evolution 55:1909–1920.

Servedio, M. R., and M. A. F. Noor. 2003. The role of reinforcement in speciation: Theory and data. Annual Review of Ecology Evolution and Systematics 34: 339-364.

Servedio, M.R., and R. Burger. 2014. The counterintuitive role of sexual selection in species maintenance and speciation. Proceedings of the National Academy of Science of the USA 111:8113-8118.

Shuster, S.M., W.R. Briggs, and P.A. Dennis. 2013. How multiple mating by females affects sexual selection. Philosophical Transactions of the Royal Soceity B 368:20120046.

Sirot LK, LaFlamme BA, Sitnik JL, Rubinstein CD, Avila, FW, Chow CY, Wolfner MF. 2009. Molecular social interactions: *Drosophila melanogaster* seminal fluid proteins as a case study in *Socio-Genetics*, (ed. M.B. Sokolowski), pp. 23-56. Waltham, Mass: Academic Press.

Tinghitella, R.M., E.G. Weigel, H. Head, and J.W. Bougman. 2013. Flexible mate choice when mates are rare and time is short. Ecology and Evolution 3:2820-2831.

Trivers, R.L. 1972. Parental investment and sexual selection *in* B. Campbell, ed., Sexual selection and the Descent of Mane. Aldine, Chicago pp. 137-179.

Tyler, F., X.A. Harrison, A. Bretman, T. Veen, R. Rodriguez-Munoz, and T Trezenga. 2013. Multiple post-mating barriers to hybridization in field crickets. Molecular Ecology 22:1640-1649.

Wade, M.J. 1979. Sexual selection and variance in reproductive success. The American Naturalist 114:742-747.

Wade, M.J., and S.M. Shuster. 2004. Sexual selection: harem size and the variance in male reproductive success. The American Naturalist 164: E83-E89.

Williams, J .H., Jr., W.E. Friedman, and M.L. Arnold. 1999. Developmental selection within the angiosperm style: using gamete DNA to visualize interspecific pollen competition. Proceedings of the National Academy of Sciences of the USA 96:9201–9206

Wong, A., M.C. Turchin, M.F. Wolfner, and C.F. Aquadro. 2008 Evidence for positive selection on *Drosophila melanogaster* seminal fluid protease homologs.Molecular Biology and Evolution 25:497-506.

Zhang, R., A.G. Clark, and A.C. Fiumera. 2012. Natural genetic variation in male reproductive genes contributes to non-transitivity of sperm competitive ability in *Drosophila melanogaster*. Molecular Ecology 22:1400-1415.

Table 1. Genetic variation in probability of mating with heterospecifics across populations as determined by a Wald's test on the logistic regression (Left) and variation in heterospecific mating due to identify of *D. persimilis* tester male (Right).

	Female Genotype		*D. persimilis* tester male	
Population	χ^2 test statistic df=3	*P*-value	χ^2 test statistic df=3	*P*-value
Lamoille (A)	14.0	**0.003**	8.1	**0.044**
Zion (A)	7.7	0.053	1.1	0.78
Mt. St. Helena (S)	22.6	**<0.001**	4.2	0.25
Sierra (S)	40.4	**<0.001**	0.15	0.99

Table 2. The genotype effects predicting CSP in a model that included the *D. persimilis* tester male. The maximum likelihood estimate (ML est.) and intraclass correlation (ICC) are reported as point estimates from the full model from the original data. The Likelihood ratio test statistic (LR) was compared to the parametric bootstrap LR test distribution which generated the bootstrap *P*-value.

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

Table 3. The genotype effects predicting ISC in a model that included the GFP *D. pseudoobscura* tester male. The maximum likelihood estimate (ML est.) and intraclass correlation (ICC) are reported as point estimates from the full model from the original data. The Likelihood ratio test statistic (LR) was compared to the parametric bootstrap LR test distribution which generated the bootstrap *P*-value.

	Lamoille (A)				Zion (A)			
Effect	ML est.	ICC	LR	*P*-Value	ML est.	ICC	LR	*P*-Value
Female	0.0668	0.018	0.825	0.2131	0.3003	0.074	3.202	0.0647
Male	0.0000	0.000	0.000	0.5037	0.0405	0.010	0.170	0.3721
M x *F*	**0.2098**	**0.057**	**29.932**	**0.0023**	**0.3056**	**0.076**	**22.47**	**0.0022**
GFP-M	**0.0879**	**0.024**	**23.88**	**0.0010**	**0.0835**	**0.020**	**12.21**	**0.0011**
	Mt. St. Helena (S)				Sierra (S)			
Effect	ML est.	ICC	LR	*P*-Value	ML est.	ICC	LR	*P*-Value
Female	0.0000	0.00	0.000	1.0000	0.0000	0.00	0.000	0.3744
Female	0.0184	0.005	0.096	0.4120	0.0000	0.00	0.000	1.0000
M x *F*	**0.2195**	**0.060**	**52.44**	**0.0019**	**0.4139**	**0.111**	**70.85**	**0.0021**
GFP-M	**0.0825**	**0.022**	**35.24**	**0.0010**	0.0077	0.002	0.902	0.0886

Table 4. The variance in male reproductive success for allopatric (Lamoille, Zion) and sympatric (Mt. St. Helena, Sierra) populations of *D. pseudoobscura* estimated from ISC data. Empirical bootstrap intervals were calculated for each population.

Population	Empirical Mean	Lower Bootstrap CI	Upper Bootstrap CI
Lamoille	0.066	0.052	0.085
Zion	0.090	0.070	0.117
Mt. St. Helena	0.033	0.022	0.056
Sierra	0.031	0.020	0.053

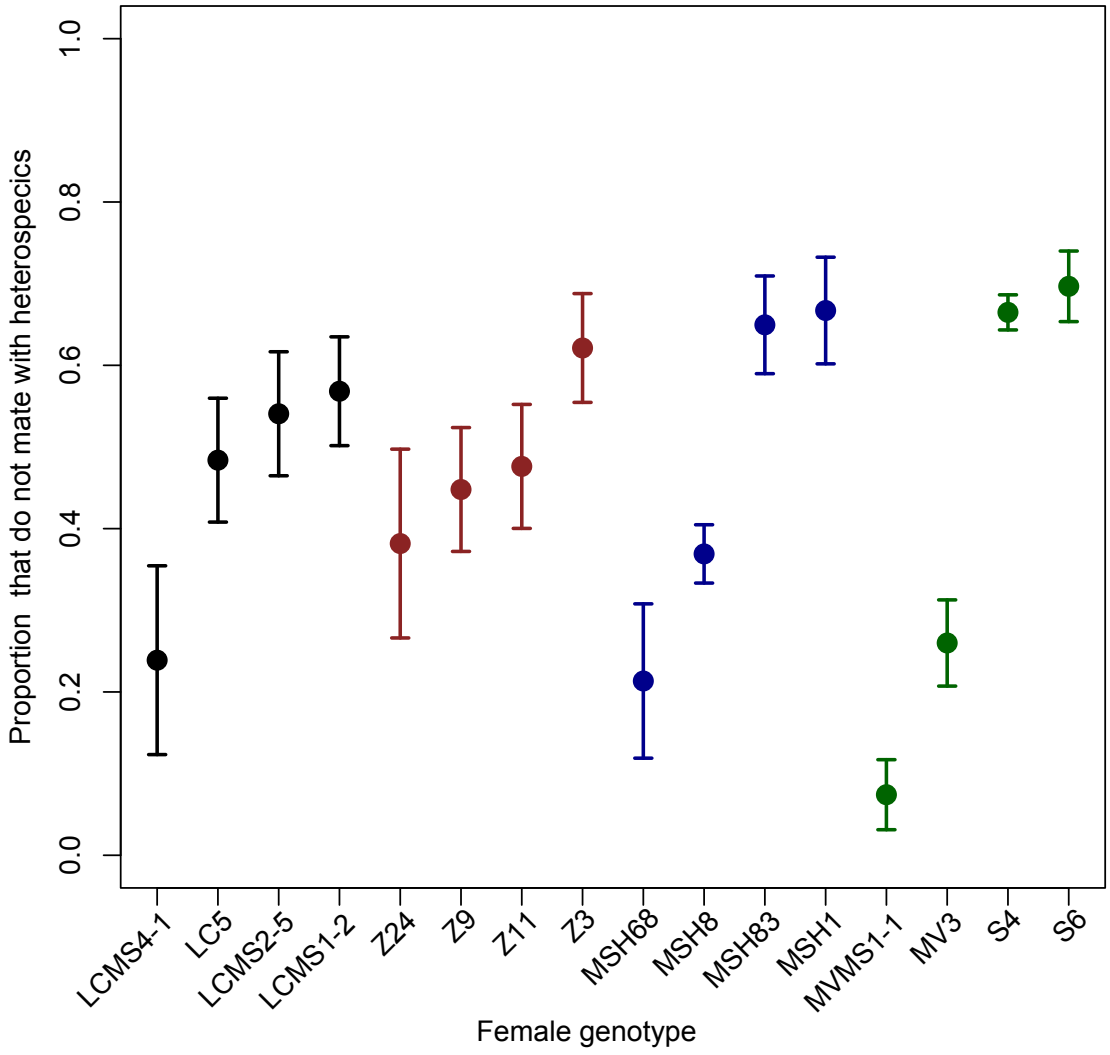

Figure 1. Genetic variation in the probability of *D. pseudoobscura* females to mate with *D. persimilis* males. The average proportion that did not mate represents the magnitude of reproductive isolation for this trait. The average proportion was calculated from each female genotype being tested with each of four *D. persimilis* tester males.

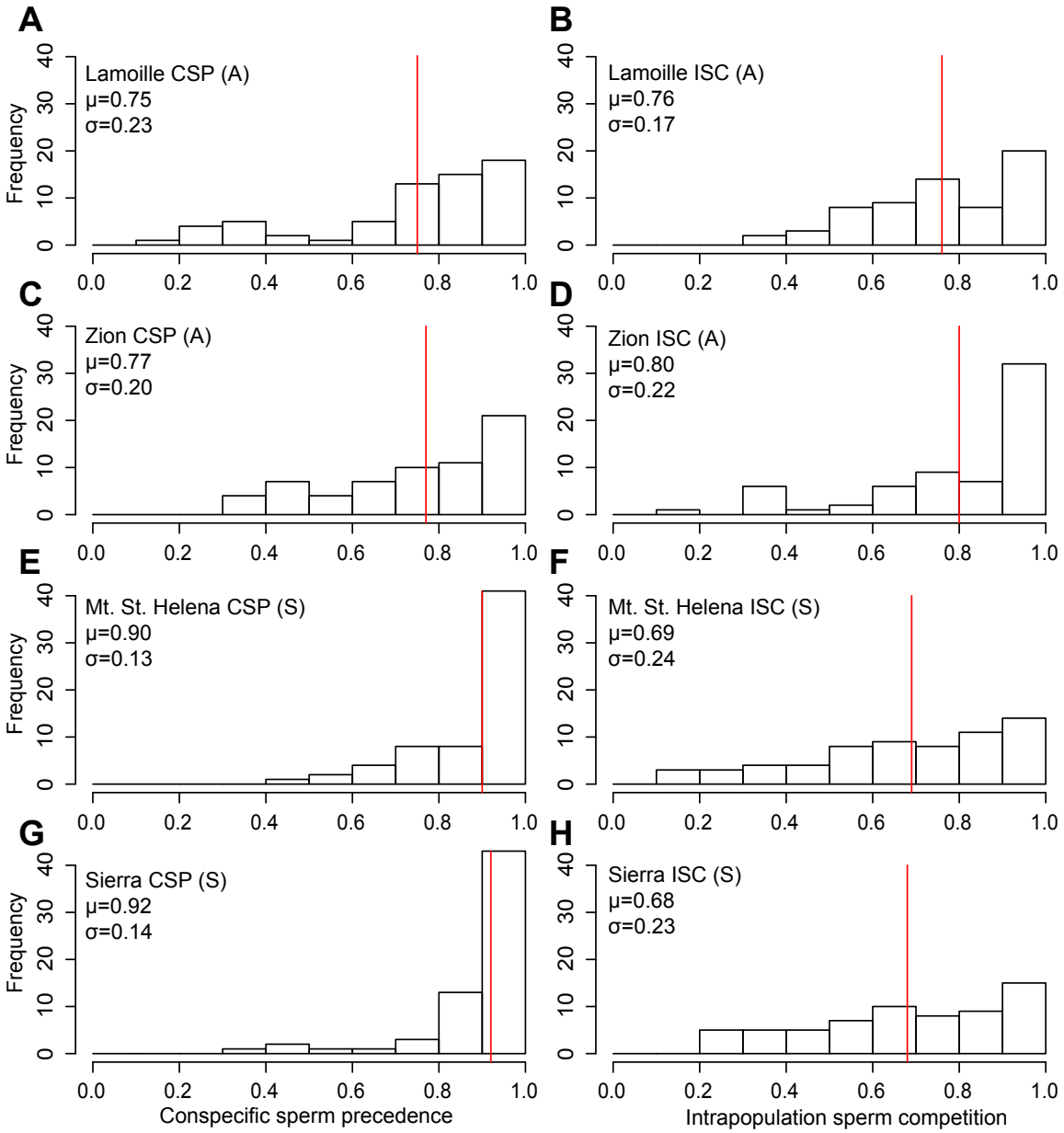

Figure 2. Phenotypic distributions for CSP and ISC across all four populations demonstrating differences in mean and variance between allopatric and sympatric populations A) CSP for Lamoille-Allopatry, B) ISC for Lamoille-Allopatry, C) CSP for Zion-Allopatry, D) ISC for Zion-Allopatry, E) CSP for Mt. St. Helena-Sympatry, *F*) ISC for Mt. St. Helena-Sympatry, G) CSP for Sierra-Sympatry, and H) ISC for Sierra-Sympatry. The red line in each distribution represents the mean.

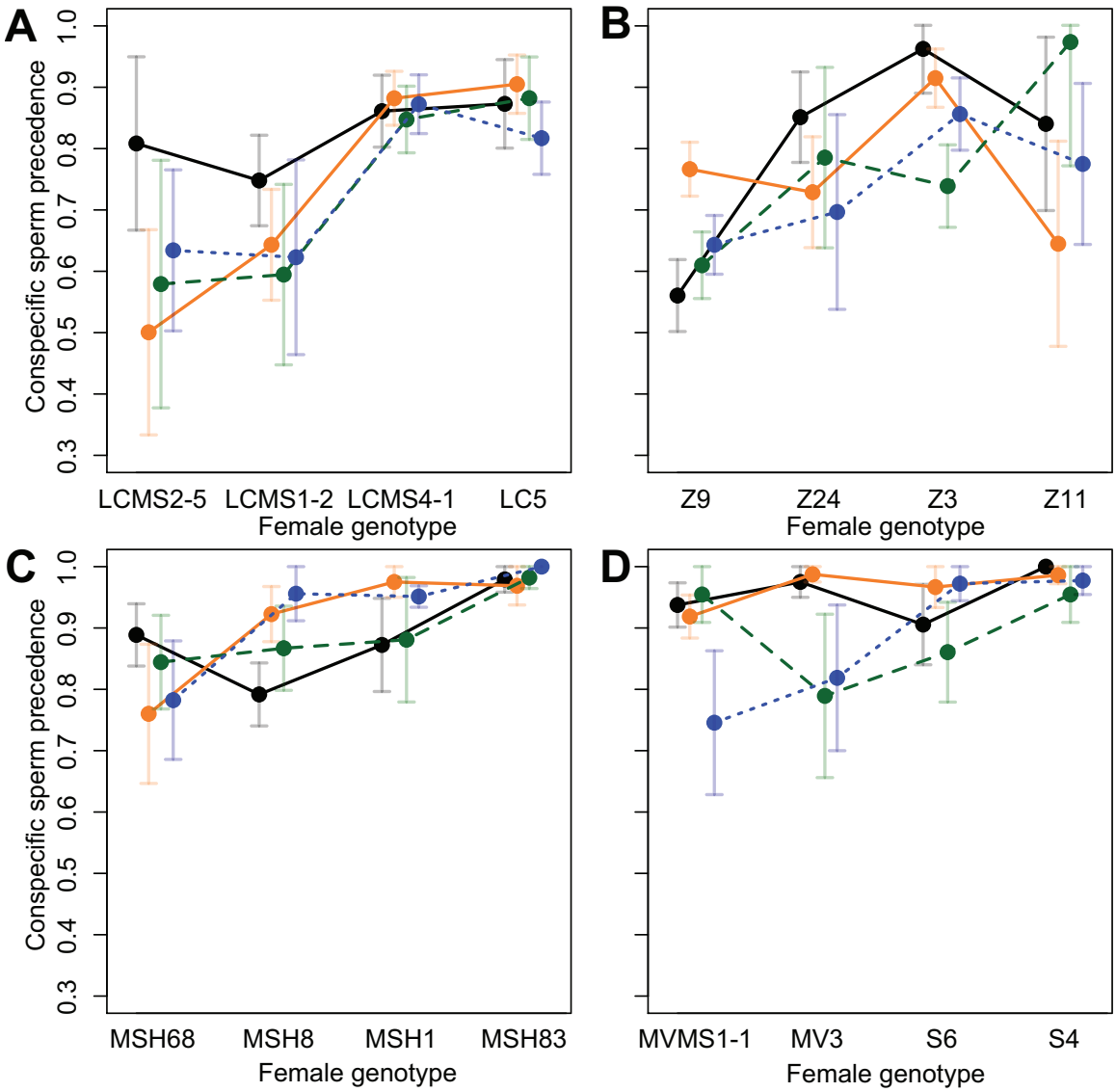

Figure 3. The average conspecific sperm precedence (CSP) in each population demonstrating differences in female genotype and male-female genotype interactions A) Lamoille-Allopatry, B) Zion-Allopatry, C) Mt. Dt. Helena-Sympatry, and D) Sierra-Sympatry. Each point represents a specific male-female genotype combination. Female genotypes are ordered by mean CSP, and male genotypes are in the same color for each female genotype. Colors were re-used between populations, but second male genotypes were unique to each population.

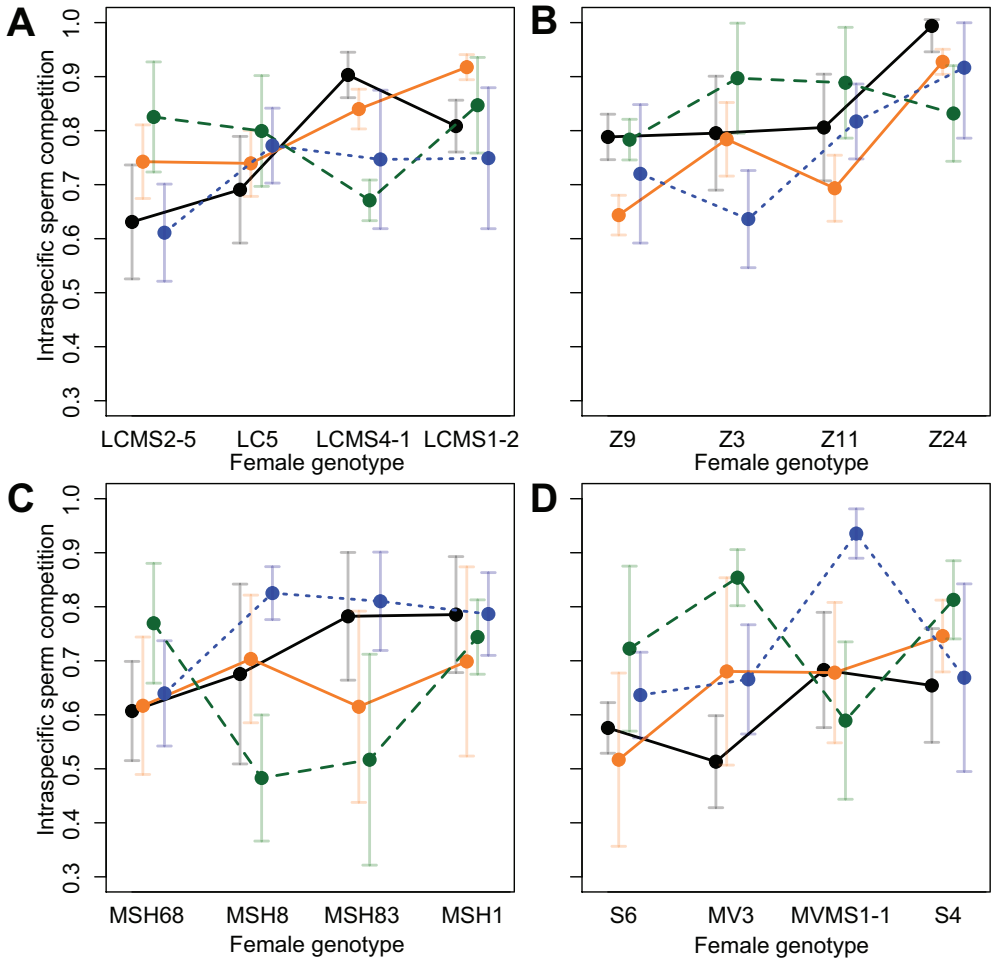

Figure 4. The average intrapopulation sperm competition (ISC) in each population demonstrating differences in female genotypes and population averages A) Lamoille-Allopatry, B) Zion-Allopatry, C) Mt. Dt. Helena-Sympatry, and D) Sierra-Sympatry. Each point represents a specific male-female genotype combination. Female genotypes are ordered by mean CSP, and male genotypes are in the same order for each female genotype.

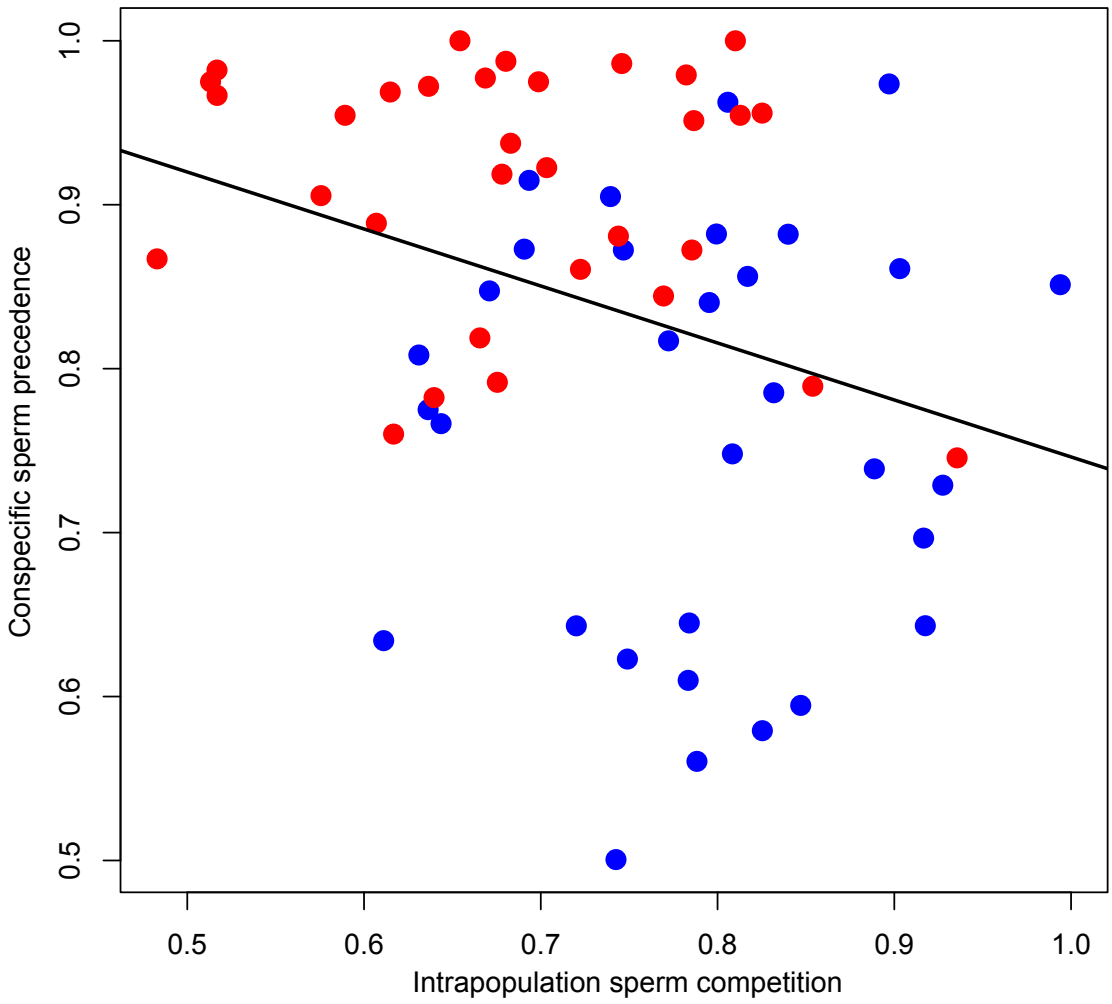

Figure 5. The negative correlation between intrapopulation sperm competition (ISC) and conspecific sperm precedence (CSP) across all four populations with each point representing a male x female genotype combination. Blue points are from allopatric populations and red points are from sympatric populations