1 **TITLE:** Conspecific sperm precedence is reinforced but sexual selection weakened in
2 sympatric populations of *Drosophila*

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SUMMARY: Sexual selection is well recognized as a driver of reproductive isolation between lineages. However, selection for increased reproductive isolation could reciprocally change the outcomes of sexual selection, when these processes share a genetic basis. Direct selection for reproductive isolation occurs 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. We tested whether i) CSP responded to reinforcing selection, and ii) this response in sympatric populations altered intraspecific sperm competition (ISC) and the strength of sexual selection, with the sister species *Drosophila pseudoobscura* and *D. persimilis*. We used sperm competition experiments to evaluate differences in CSP and ISC between two sympatric and two allopatric populations of *D. pseudoobscura*. Using multiple genotypes for each population allowed us to estimate not only patterns of phenotype divergence, but also the opportunity for sexual selection within each population. Consistent with a pattern of reinforcement, the sympatric populations had higher mean CSP. Moreover, ISC was altered in sympatric populations, where we observed decreased average offensive sperm competitive ability against conspecific males, allowing less opportunity for sexual selection to operate within these populations. 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.

46 **Introduction**

47 When closely-related species comes into contact, the presence of heterospecifics
 48 can influence sexual interactions and therefore alter patterns of selection on reproductive
 49 traits. In cases where these species have the potential to interbreed, selection can favor
 50 divergence in sexual traits to avoid costs of heterospecific mating, a type of reproductive
 51 character displacement commonly called reinforcement [1-3]. The frequency at which
 52 reinforcement contributes to speciation is still under debate [3-4] although several recent
 53 examples provide strong evidence for reinforcement acting on mating traits [5-10].
 54 Regardless, the mating trait changes that evolve in response to reinforcement can have
 55 collateral effects on intraspecific sexual dynamics [6]. This can in turn alter the
 56 magnitude and efficacy of sexual selection specifically within populations exposed to
 57 heterospecifics. These potential reciprocal interactions between sexual selection and
 58 reproductive isolation remain relatively untested [6-7], but can have important
 59 consequences for how we interpret evolution of sexual traits and interactions. For
 60 example, patterns of reproductive trait evolution in rapid radiations, where sexual
 61 selection is thought to be the primary driver, may be misinterpreted if they do not take
 62 into account species interactions.

63 For reinforcement and sexual selection to reciprocally affect the evolution of
 64 sexual traits, these traits must be involved in both processes and share a genetic basis.
 65 Currently the best example of a shared genetic basis for sexual selection and reproductive
 66 isolation comes from *Drosophila* sperm competition genes, several of which have been
 67 shown to mediate both sexual selection through intraspecies sperm competition (ISC) and
 68 reproductive isolation via conspecific sperm precedence (CSP) [11]. 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 overwhelmingly focused on pre-mating traits, postcopulatory prezygotic traits including CSP can also be the target of reinforcement [15-17]. 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

92 (male-male [29-30] and male-female: [31-33]. Both are expected to maintain high
 93 variance in the affected traits and, indeed, sperm competition genes are often highly
 94 variable both in terms of molecular and phenotypic variation [30, 33-34]. In contrast,
 95 under models of speciation by sexual selection—where isolation is generated by strong
 96 disruptive selection between populations and directional selection within a population
 97 [35-36]—genetic variance of traits that act as barriers to reproduction is expected to be
 98 reduced and the overall trait mean shifted. The net effect of selection imposed by
 99 intrapopulation sexual interactions and by reinforcement can together produce phenotypic
 100 and genetic variation in sperm competition traits/genes that is different from the optimal
 101 variation when sexual selection acts alone.

102 One way these potentially antagonistic optima could play out is when
 103 reinforcement-mediated changes in the mean and variance of sperm competition traits
 104 alter the opportunity for sexual selection among conspecifics [7]. Sperm competition
 105 contributes to variance in reproductive success because male genotypes that can
 106 disproportionately sire offspring increase their fitness compared to the fitness of rival
 107 male conspecifics [37-38]. Strong sperm competition leads to greater opportunity for
 108 sexual selection because there is greater variance in reproductive success compared to
 109 scenarios where males have equal probability of siring offspring. This generates two
 110 alternative predictions of the possible effects of reinforcement on sexual selection. First,
 111 the response to strong directional selection from reinforcement on sperm competition
 112 traits could lead to greater siring ability in intrapopulation sperm competition, increasing
 113 variance in reproductive success and opportunity for sexual selection. Alternatively,

114 strong directional selection could reduce phenotypic variation so that competitive ability
115 is equalized among males, thus reducing the opportunity for sexual selection.

116 The strategy we used to evaluate the interaction between selection for increased
117 reproductive isolation (i.e. reinforcement) and sexual selection acting on sperm
118 competition genes was to estimate variation between genotypes in CSP and ISC in
119 parallel. Both CSP and ISC are measures of postcopulatory offensive sperm competition,
120 estimated by allowing females to mate sequentially with two different male genotypes
121 and scoring the paternity of the resulting progeny. Here our focus was on second-male or
122 ‘offensive’ siring success. This is typically referred to as ‘P2’ and captures the ability of
123 the second mated male to sire offspring by displacing or disabling the sperm of the first
124 male. For our experiments the first male was either heterospecific (to estimate CSP) or
125 conspecific (to estimate ISC) tester male. By comparing the relative competitive success
126 of replicate male lines against a common set of either heterospecific and conspecific male
127 tester genotypes, we could estimate post-copulatory CSP and ISC in parallel in the same
128 experiment. Using this design we also estimated which genotype effects (male genotype,
129 female genotype, or the interaction) might shape CSP and ISC. Females experience the
130 most cost of heterospecific matings [39-41] and could control CSP via cryptic female
131 choice [42], thus we would expect strong female genotype effects on CSP. This contrasts
132 with previous studies of ISC where both male and female genetic effects, and their
133 interaction were significant effects [31,33]. Unlike ISC the phenotypic and genetic
134 variance for CSP has not been empirically explored and their similarity to ISC is
135 currently unknown.

136 In this study, we examine evidence for reinforcement of CSP among populations
137 of *Drosophila pseudoobscura* that are allopatric or sympatric with their closely related
138 sister species *D. persimilis*, and evaluate the potential consequences of these
139 heterospecific interactions for ISC and sexual selection within *D. pseudoobscura*
140 populations. One of the first clear empirical demonstrations of reinforcement on pre-
141 mating isolation was described in this species pair [43]. This finding suggests that
142 heterospecific interactions and matings are frequent and sustained over evolutionary time
143 and can act as a substantial selective agent on reproductive traits in this system. Here we
144 determine whether there is evidence that heterospecific interactions have selected for
145 increased CSP, by comparing this barrier among populations of *D. pseudoobscura* that
146 are allopatric or sympatric with *D. persimilis*. A pattern of stronger CSP specifically in
147 sympatry is consistent with reinforcement; moreover, because postcopulatory traits are
148 less likely to be directly affected by environmental conditions, this pattern is unlikely to
149 be explained by alternative phenomena, such as ecological selection, that could also
150 explain character displacement in sympatry (see Discussion). Using a consistent design
151 across all populations we could also estimate premating reproductive isolation in the
152 same experiment, and compare its strength in sympatry and allopatry. Second, we
153 evaluate whether selection for strong CSP in sympatry has affected ISC, and thereby
154 post-copulatory sexual selection, as might occur when CSP and ISC have shared genetic
155 architecture. Throughout, we test for differences in trait variation across a set of distinct
156 genotypes which allows us to specifically evaluate which sex is playing a more critical
157 role in determining variation in heterospecific and conspecific postcopulatory
158 interactions.

159 RESULTS

160 *No difference between allopatric and sympatric populations in premating isolation*

161 Because our experimental assessment of CSP involved first mating with a
 162 heterospecific *D. persimilis* male, we were able to estimate the magnitude of premating
 163 isolation in each *D. pseudoobscura* population in our experiment. We did not find
 164 evidence for a pattern consistent with reinforcement of premating isolation mediated by
 165 female mate preference. The average probability of heterospecific matings ranged from
 166 46-52% between populations, and did not differ between allopatric and sympatric
 167 populations (χ^2 test of independence: $\chi^2=1.185$, $df=1$, $P=0.2763$; Wald's Test: $\chi^2=1.9$,
 168 $df=4$, $P=0.75$; Table 1). In pairwise tests between each allopatric and sympatric
 169 population we also failed to reject the null hypothesis. Though we did not detect a signal
 170 of reinforcement there was ample genetic variance in heterospecific mating rate between
 171 female genotypes available for selection within each population (Fig. 1; Supplemental
 172 Table 1). Only in one of the populations (Lamoille, which is allopatric) did the identity of
 173 the *D. persimilis* tester line affect variation in premating isolation (Supplemental Table
 174 1).

175

176 *Reinforcement acts on conspecific sperm precedence*

177 Unlike premating isolation, we observed a pattern consistent with reinforcement for
 178 conspecific sperm precedence (CSP). Specifically, in sympatry we find both greater
 179 average CSP ($t=-6.5898$, $df=210.92$, $P<0.001$; Wilcox $W=4427.5$, $P<0.001$) and less
 180 phenotypic variation in this trait (Levene-type test $\chi^2=22.82$, $P<0.0001$) when data were
 181 pooled by geographic region (allopatry versus sympatry) (Table 1; Fig. 2A). These

182 differences in both the average and variance of CSP were also observed in pairwise tests
183 between individual allopatric and sympatric populations (Supplemental Table 2).

184

185 *Reinforcement has collateral effects on intrapopulation sperm competition*

186 ISC also differed between allopatric and sympatric populations, in both mean and
187 variance (Table 1; Fig 2B). First, mean offensive ability for ISC was significantly lower
188 in sympatric populations ($t=3.738$, $df=246.55$, $P=0.0002$; Wilcox's $W=10280$, $P=0.0004$).

189 This contrasts with the observed increase in offensive CSP in sympatric populations.

190 Second, there was more variation in ISC in the sympatric populations compared to the
191 allopatric populations (Leven-type test $\chi^2=5.74$, $P=0.0172$). Given the differences in ISC

192 and CSP across populations, we used the mean CSP and ISC phenotype for each male x

193 female genotype combination within a population (i.e., each cell within the diallel

194 crossing design) to examine the pattern of relationship between the two phenotypes

195 across the four populations. We observed a significant negative relationship between CSP

196 and ISC (Pearson's $r=-0.31$, $P=0.01$; Fig. 5). Since each male or female genotype is

197 represented in multiple combinations we controlled for non-independence using a linear

198 mixed effect model, and confirmed that the negative slope of the relationship was

199 significant as indicated by a confidence interval that did not overlap zero (Profiled CI = -

200 0.451, -0.028).

201

202 *Female genotype effects contribute to CSP and male x female genotype effects explain*

203 *both CSP and ISC*

204 Of male, female, and male x female genotype effects that could contribute to explaining
 205 the variance in CSP, we found that three out of the four populations had a significant
 206 female genotype effect on CSP (Table 2; Fig 3), and all populations had a significant
 207 male x female genotype interaction effect. The *D. persimilis* tester male line was also
 208 significant in three out of four populations. There was no consistent pattern among
 209 populations in which effect had the largest intraclass correlation (i.e. which explained the
 210 largest proportion of variance; see Methods); in some populations the female genotype
 211 effect had the largest intraclass correlation, while in others the male x female genotype
 212 interaction had the largest intraclass correlation (Table 2). In contrast, for ISC in all four
 213 populations we only observed significant male x female genotype interaction and a
 214 significant effect of the first-tester male genotype (Table 3; Fig. 4). In every case, the
 215 male x female genotype effect had a larger intraclass correlation (usually two to three
 216 times greater) than the identity of the specific tester male genotype within each *D.*
 217 *pseudoobscura* population.

218

219 *The opportunity for sexual selection is decreased in sympatry*

220 Our design allowed us to describe the reproductive success of males in terms of offensive
 221 (second male) and defensive (first-tester male) success. We found that the sympatric
 222 populations had significantly lower variance for reproductive success compared to the
 223 allopatric populations (Figure 6; Supplemental Table 4). The variance in reproductive
 224 success across all male genotypes (both offensive and defensive) in the allopatric
 225 Lamoille population was significantly greater than both sympatric populations (Mt. St
 226 Helena $F=1.96$, Bootstrap $P=0.003$; Sierra $F=2.08$, Bootstrap $P=0.008$), as was the

227 variance in reproductive success in the allopatric Zion population compared to the
 228 sympatric populations (Mt. St Helena $F=2.65$, Bootstrap $P=0.003$; Sierra $F=2.83$,
 229 Bootstrap $P=0.004$). This reduced variance in reproductive success in sympatry is a
 230 product of lower offensive sperm competition values in sympatry, that result in equalized
 231 differences in the siring success between offensive and defensive males.

232

233 DISCUSSION

234 Interactions with heterospecifics have the potential to drive divergent sexual selection
 235 and the evolution of reproductive isolation, via reproductive character displacement and
 236 reinforcement [6-7,44]. Using *D. pseudoobscura* and *D. persimilis*, we assessed whether
 237 there was evidence for reinforcement of species barriers in sympatry via elevated female
 238 preference or conspecific sperm precedence, traits that are known to contribute to
 239 reproductive isolation across numerous taxa [2]. Premating isolation is historically
 240 considered to be a strong barrier to isolation between these species, and one that
 241 reinforcing selection has acted on [43], but we saw no evidence for reproductive
 242 character displacement for this trait. In contrast we saw a clear signal of increased CSP in
 243 sympatric populations, consistent with a pattern of reinforcement. Specifically, the
 244 average CSP was higher, and the overall level of phenotypic variation was lower, in
 245 sympatric populations, a pattern consistent with recent or recurrent directional selection
 246 acting on CSP in these populations. We further asked whether reinforcement could have
 247 collateral effects on intraspecific sperm competition and sexual selection, given that these
 248 two traits are mechanistically and genetically linked [11,45]. We found that sympatric

249 populations also had lower ISC ability (lower offensive ability) than allopatric
250 populations, consistent with weakened sexual selection in sympatry.

251 Our results indicate that CSP can strongly contribute to reproductive isolation in
252 response to reinforcing selection. While CSP is known to be a barrier to gene flow in
253 *Drosophila* [12-13] and other taxa [2], its overall importance in nature has been difficult
254 to ascertain [14,16]. Moreover, previous studies of reinforcement sometimes qualitatively
255 describe variation in the target premating traits, but trait variance is typically not
256 quantified [5,9,17] even though models of speciation by sexual selection predict that
257 strong divergent selection will erode phenotypic variation in selected traits [46-47]. Our
258 observations of both increased mean CSP and reduced variation specifically in sympatry
259 provide compelling support for the inference that CSP has responded to strong selection
260 imposed by heterospecific interactions, and underscores the important role that CSP can
261 play in maintaining species boundaries.

262 The pattern of reproductive character displacement that we observed for CSP is
263 consistent with reinforcement, but other factors have been proposed to account for
264 reproductive character displacement including differential fusion [48] or ecological
265 differences that have collateral effects on mating traits [44,49]. Differential fusion
266 predicts that strong reproductive isolation evolves between species in allopatry and
267 merely prevents species collapse upon secondary contact, so that sympatric species
268 incidentally appear to have stronger isolation [50-51]. If differential fusion operates at the
269 deme/lineage level within a population we would expect the sympatric CSP values to be a
270 subset of allopatric CSP values [17]. This is not the case, however, because the sympatric
271 values of CSP are systematically higher than in allopatry (Figure 2). Regardless, the

272 differential persistence of demes/lineages with strong CSP in sympatry would
 273 nevertheless be consistent with selection from standing variation leading to reinforcement
 274 [52,53]. Similarly, several lines of evidence argue that systematic ecological differences
 275 between allopatry and sympatry are unlikely to explain our observed postcopulatory
 276 differences. Although both sympatric populations are located in California, they are
 277 ecological distinct (collected from two different mountain ranges) in terms of numerous
 278 ecological factors [54]. Indeed, habitat variation between sympatric populations of *D.*
 279 *pseudoobscura* has led to differences in inversion frequencies maintained by ecological
 280 forces that differ in these locations [55]. Moreover, the ecological differences across the
 281 whole range of *D. pseudoobscura* are largely continuous, rather than uniquely
 282 differentiating regions of allopatry and sympatry/co-occurrence with *D. persimilis*. Given
 283 the ecological diversity between populations we do not expect a consistent direction of
 284 natural selection acting on either the sympatric or allopatric populations. Arguably more
 285 important, there are no established mechanisms whereby external ecological factors are
 286 expected to have a direct effect on the strength of sperm competition consistent with our
 287 observed pattern. Indirect effects of diet and nutrition can affect sperm competition
 288 outcomes [56-57], but should not persist in the lab environment. Moreover, if ecological
 289 mechanisms existed there is no reason to expect they would act in the specific direction
 290 we observed here. Given this, while the ecological alternative to reinforcement might be
 291 plausible for some premating phenotypes, it is unlikely to explain the postcopulatory
 292 phenotypes that we examine here.

293 Our second major inference is that the response to reinforcing selection observed
 294 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, as well as reduced variance in post-copulatory fitness based on ISC estimates. Sperm competition strongly contributes to sexual selection in *D. pseudoobscura* where multiple mating is frequent in wild caught females [58], and male mating success, including sperm competition, is a major component of selection in natural populations [59]. The observed reduction in offensive sperm competition differs from both of our *a priori* expectations. One *a priori* hypothesis was that selection for increased CSP in sympatry would select for increased offensive sperm competitive ability among conspecifics, if offensive ability were a general trait that acted regardless of whether the competitor was a conspecific or heterospecific male. In contrast, we observed that ISC, was lower for sympatric populations compared to allopatric populations; that is, average offensive ability was closer to 0.5, indicating a greater equalization in sperm competitive ability among competing males. Our other *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. This observation is also inconsistent with an alternative explanation-that selection for weaker ISC in sympatry indirectly increased CSP. This alternative is more generally implausible as it requires that there has been selection specifically to reduce ISC, solely in sympatry. 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.

317 For reinforcing selection to influence and interfere with sexual selection, the
 318 selection favoring increased CSP must outweigh selection acting to maximize ISC. One
 319 way CSP could have a larger effect on fitness than ISC is via a higher selective premium
 320 specifically for females. Weaker CSP results in substantial fitness deficits for females
 321 because of reproductive investment in low or no fitness hybrids, whereas weaker ISC
 322 likely has a comparatively marginal effect on female fitness outcomes. Regardless, the
 323 strength of reinforcing selection on CSP depends on the frequency of heterospecific
 324 matings. Several lines of evidence suggest that heterospecific mating rates are common
 325 between these species. First, from our data we observe a large range in the frequency
 326 with which *D. pseudoobscura* females accept *D. persimilis* males in no-choice
 327 experiments, with some genotypes on average accepting *D. persimilis* 90% of the time.
 328 Second, while no estimates for heterospecific mating rate exist from natural populations,
 329 rare F1 progeny have been identified from wild collections [60]. Third, genetic evidence
 330 suggests there has been post-speciation gene flow (i.e., evidence of movement of alleles
 331 between species) between *D. pseudoobscura* and *D. persimilis* [61-62]. Notably, these
 332 estimates of realized gene flow will systematically underestimate the rate of
 333 heterospecific matings, because they will only capture events that result in F1 progeny
 334 that themselves then successfully reproduced; for example, given the presence of strong
 335 CSP, many heterospecific matings may never produce hybrid progeny.

336 We were able to test the hypothesis that females face more costs of hybridization
 337 [39-41] and that choice manifests as female control of sperm use patterns [63-65] by
 338 contrasting the genotype effects (male, female, and male x female genotype effects)
 339 between CSP and ISC. 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. Significant female genotype effects for CSP suggest that 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 [66].

Strong female genotype effects on CSP are also consistent with the current knowledge of postcopulatory sexual selection in the obscura group. Both *D. pseudoobscura* and *D. persimilis* produce two sperm morphs: longer fertilizing eusperm and shorter non-fertilizing parasperm. In *D. pseudoobscura*, the female reproductive tract is spermicidal and higher proportions of parasperm help protect eusperm from these negative effects [67]. Females in sympatric populations may have evolved more effective spermicide against heterospecific males at a cost of spermicidal effectiveness with conspecific males. In this case reproductive isolation would be mediated by cryptic female choice and heterospecific male-female compatibility. This hypothesis may also be consistent with our finding that the *D. persimilis* male genotype contributed significantly to observed variation in CSP.

Reinforcement acting on CSP suggests that other prezygotic barriers that act before 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 equally strong in sympatry and 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

363 more variable patterns [68-70; but see 71]. Our observation of a strong response in CSP
364 also suggests that the populations of *D. pseudoobscura* and *D. persimilis* we examined
365 are not strongly isolated by non-competitive (gametic) isolation, in agreement with
366 inferences from other studies of this specific species pair [70,72]. Though we lack data on
367 CSP from earlier collections in this species pair, our observations here might suggest that
368 the relative contribution of barriers to reproduction has changed in sympatry over time,
369 from premating isolation to CSP. Both gene flow between sympatry and allopatry, or a
370 cost to female premating preferences, might explain this shift over time. Depending on
371 the levels of gene flow among sympatric and allopatric populations, strong premating
372 isolation in sympatry could be lost due to “swamping effects” of allopatric gene flow [73]
373 or could lead to greater species wide reproductive isolation [74-75]. Our data suggest that
374 it’s unlikely that gene flow from sympatry into allopatry created greater reproductive
375 isolation in allopatry (thereby reducing the signal of reinforcement) because the average
376 allopatric premating isolation in our experiment is similar to previous reports [43]. This
377 suggests that reduced premating isolation has emerged in sympatry, but it is difficult to
378 disentangle the effects of gene flow from the cost of female choice as causes of this
379 reduction. Both processes could contribute to the large variance we see for female
380 preference in sympatry compared to the more uniform level of premating isolation in
381 allopatry (Fig 1). The probability that strong female preference have been lost in
382 sympatry also depends on the frequency of this trait and any associated costs of
383 choosiness. When *D. pseudoobscura* stocks are kept in the absence of heterospecific
384 interactions female preference against heterospecifics decreases with longer periods of
385 experimental allopatry, suggesting that it may be costly to maintain this trait [76]. In

386 either case, the reduction in the strength of premating isolation in sympatry suggests that
387 this barrier to reproduction may only generate transient patterns of reinforcement.

388 Overall, our data suggest that strong reinforcing selection for reproductive
389 isolation can have consequences for sexual selection and sexual interactions, in these
390 important postmating sperm competition traits. The direction of this interaction provides
391 an interesting inversion to standard expectations about the connection between sexual
392 selection and speciation. Sexual selection is often thought of as a driver of sexual
393 characteristics whose evolutionary divergence then contributes to reproductive isolation.
394 But a direct genetic connection between these processes implies reproductive isolation
395 also has the reciprocal potential to shape sexual selection [77]. Based on our observations
396 of higher mean but lower variance in CSP in sympatry, a negative correlation between
397 CSP and ISC, and reduced variance in reproductive success via ISC among sympatric
398 conspecific males, we infer that strong selection for reproductive isolation within
399 populations exposed to heterospecific species has reduced the efficacy of sexual selection
400 in these populations, a collateral effect of reinforcing selection that has not previously
401 been demonstrated.

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412

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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	<i>P</i> -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
<i>D. persimilis</i>	0.3413	37.49	0.0013	0.082
Zion (A)				
Effect	ML est.	LR	<i>P</i> -Value	Intraclass Corr.
Female	<i>0.2683</i>	<i>2.72</i>	<i>0.05632</i>	0.067
Male	0.0000	0.00	0.4190	0.00
M x F	0.3315	16.30	0.00238	0.0833
<i>D. persimilis</i>	0.0865	6.44	0.0068	0.0217
Mt St. Helena(S)				
Effect	ML est.	LR	<i>P</i> -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
<i>D. persimilis</i>	0.0000	0.00	0.9851	0.000
Sierra (S)				
Effect	ML est.	LR	<i>P</i> -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
<i>D. persimilis</i>	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	<i>P</i> -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	<i>P</i> -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	<i>P</i> -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	<i>P</i> -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

681

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

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

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Figure 3. Conspecific sperm precedence (CSP) for all male-female genotype

701

combinations in each population demonstrating a significant effect of female genotype

702

and male-female genotype interaction on the outcome of CSP. A) Lamoille-Allopatry, B)

703

Zion-Allopatry, C) Mt. Dt. Helena-Sympatry, and D) Sierra-Sympatry. Each point

704

represents a specific male-female genotype combination. Error bars are $\pm$ one standard

705

error. Female genotypes are ordered by mean CSP. Each color represents a single male

706

genotype for each population. Colors were re-used between each population panel, but

707

actual second male genotypes were unique to each population.

708

709

710

711 **Figure 4.** Intrapopulation sperm competition (ISC) for all male-female genotype

712 combination in each population demonstrating a significant male-female genotype

713 interaction on the outcome of ISC. A) Lamoille-Allopatry, B) Zion-Allopatry, C) Mt. Dt.

714 Helena-Sympatry, and D) Sierra-Sympatry. Each point represents a specific male-female

715 genotype combination. Error bars are $\pm$ one standard error. Female genotypes are

716 ordered by mean ISC. Each color represents a single male genotype for each population.

717 Colors were re-used between each population panel, but actual second male genotypes

718 were unique to each population.

719

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

724

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.

733 MATERIALS AND METHODS

734 Wild type fly stocks

735 All stocks were reared on standard media prepared by the Bloomington Drosophila Stock
736 Center, and were kept at room temperature (~22C). We used a set of isofemale lines
737 collected from four natural populations in the summers of 2013 and 2014. Allopatric *D.*
738 *pseudoobscura* were collected at Zion National Park, UT (kindly provided by N. Phadnis)
739 and Lamoille Canyon, NV (collected by D. Castillo). Sympatric *D. pseudoobscura* and *D.*
740 *persimilis* were collected at two sites: Mt. St. Helena, CA (*D. pseudoobscura* collected by
741 A. Hish/M. Noor and D. Castillo, and *D. persimilis* collected by D. Castillo); and, near
742 Meadow Vista and Forest Hill, CA (called here ‘Sierra’; *D. pseudoobscura* and *D.*
743 *persimilis* collected by D. Castillo). For both sympatric populations, both species were
744 present in field collections and can be considered truly co-occurring/sympatric.

745

746 Conspecific sperm competition assay

747 Sperm competition assays generally involve mating an individual female sequentially
748 with two distinct male genotypes. In all experimental crosses between species, females
749 were paired first with a *D. persimilis* male and second with a *D. pseudoobscura* male; that
750 is, the assays are evaluating the “offensive” sperm competitive ability of conspecific
751 males to displace heterospecific sperm (equivalent to ‘P2’, or second male siring ability;
752 [78]). We focused on “offensive” sperm competition because *D. pseudoobscura* females
753 do not remate with *D. persimilis* males if they have first mated with a conspecific,
754 therefore we cannot evaluate “defensive” sperm competition in this cross. In this
755 experiment we partitioned the variance in CSP due to male genotype, female genotype,

756 and the male x female genotype interaction using a “diallel-like” crossing design, which
 757 is commonly used for this purpose [31,33; Supplemental Fig 1). A diallel cross is a
 758 mating scheme commonly used to estimate the genetic effects, additive genetic variance,
 759 and heritability, of quantitative traits by crossing all parental genotypes in all possible
 760 combinations [79]. Our design is “diallel-like” because we did not use progeny from the
 761 diallel to estimate heritability. We completed separate CSP experiments for each of our
 762 four *D. pseudoobscura* collection locations (Sympatric= Sierra and Mt. St. Helena,
 763 Allopatric= Zion and Lamoille). For each population we used a 4x4x4 design: four *D.*
 764 *pseudoobscura* female genotypes from that population, four *D. persimilis* genotypes as
 765 first males (“tester males”), and four *D. pseudoobscura* male genotypes as second males
 766 from the same population as females. Each 4x4x4 combination was replicated once (n=64
 767 unique cross combinations for each population). If CSP is important for reproductive
 768 isolation in sympatry it should be consistently strong across multiple heterospecific
 769 genotypes. Accordingly, rather than rely on a single *D. persimilis* genotype, we aimed to
 770 use multiple wild-collected *D. persimilis* tester male lines for our experiments. Of these,
 771 two *D. persimilis* lines were collected at the same time and in the same traps as the *D.*
 772 *pseudoobscura* strains at the Sierra location and another two at the Mt. St. Helena
 773 location.
 774 Virgin individuals were collected and aged 7 days prior to the initiation of an
 775 experimental block. One day before mating, *D. persimilis* tester males were isolated
 776 individually [80]. The following day, females were individually added (without
 777 anesthesia) to a vial containing a tester male and were co-housed for 24 hours, after
 778 which time the tester male was removed. We kept females housed individually in these

779 vials for 7 days before second mating (similar to [80]). After 7 days we inspected all vials
 780 for the presence of larvae to determine if females had mated with the first *D. persimilis*
 781 tester males. This was used to evaluate evidence for differences in successful first
 782 matings (pre-mating isolation) among allopatric and sympatric populations, rather than
 783 observing matings directly, as there is high variance in time to copulation in this
 784 heterospecific pairing [70]. Only females that had mated (i.e. had produced larvae within
 785 7 days) were retained for the remainder of the CSP experiment.

786 For the second mating, each individual female was paired with one of the four *D.*
 787 *pseudoobscura* male genotypes from her own population to determine the strength of
 788 CSP. These second males were also isolated one day before the introduction of the
 789 female. Seven days after mating with the first male, females were transferred, without
 790 anesthesia, to the vial containing the second male. Individual pairs were co-housed for 24
 791 hours and the male was removed on the second morning. The female was kept for five
 792 days (transferring after 2 days to avoid overcrowding of larvae). All progeny produced in
 793 the five-day window after the second mating were collected; from these progeny a
 794 maximum of 10 males and 10 females, randomly chosen from the total group of progeny,
 795 were used to score CSP (P2) as described below.

796

797 Intrapopulation sperm competition assay

798 The design for intrapopulation sperm competition (ISC) assay mirrored the experimental
 799 design for CSP except that, rather than a *D. persimilis* tester male, the first male was a *D.*
 800 *pseudoobscura* tester male derived from the same population as the *D. pseudoobscura*
 801 female and second male genotypes in the trial. For each population we used a 4x2x4

802 design: four *D. pseudoobscura* female genotypes, two *D. pseudoobscura* GFP genotypes
803 as first males and 4 *D. pseudoobscura* male genotypes as second males. The same female
804 x second male genotypes were used in ISC and CSP experiments. Each combination was
805 replicated twice (n=64 for each population, with 32 unique cross combinations). This
806 allowed us to have a total sample size per population that matched the CSP experiment
807 (64 replicates per population, 256 replicates across all populations).

808 The details of the mating scheme (virgin collection, aging of individuals, isolation of
809 individuals, etc.) are identical to the CSP experiment. We did not observe matings
810 directly, but the average refractory period for *D. pseudoobscura* is 4 days [81], so we are
811 confident that on average only a single mating occurred in the 24 hour co-housing
812 timeframe. Each individual female was randomly assigned one of the two *D.*
813 *pseudoobscura* first male (tester) genotypes to determine the strength of P2 (second male
814 siring ability) by our four focal second male genotypes, against these tester male
815 genotypes. The female was kept for five days after the second mating (transferring after 2
816 days to avoid overcrowding of larvae). All progeny produced in the five-day window
817 after the second mating were collected and scored.

818

819 Generating visibly-marked tester males for quantifying CSP and ISC

820 To allow efficient progeny scoring, paternity was scored with the aid of visible markers
821 in both CSP and ISC experiments. This required us to generate marked male tester lines
822 with wild-caught *D. persimilis* (for CSP tester males) and *D. pseudoobscura* (for ISC
823 tester males) lines from each study population.

824 For CSP, to introduce a visible marker into wild-type wild-collected *D. persimilis* males
825 from our sympatric sites, we introgressed an X-linked marker (“short” or sh) from a *D.*
826 *pseudoobscura* line, into four of our collected *D. persimilis* genotypes (Supplemental Fig
827 2). These four *D. persimilis* tester males originated from isofemale lines collected at the
828 Sierra and Mt St. Helena locations and were used to evaluate the mean strength and
829 variation in CSP for all four *D. pseudoobscura* populations in the CSP experiment. We
830 first crossed these *D. persimilis* sh mutant males to females from each of the four wild-
831 type *D. persimilis* isofemale lines (keeping each tester genotype separate throughout this
832 process). This produced F1 daughters heterozygous for the sh allele, that were
833 backcrossed to wild type males from the same wildtype isofemale line. From the BC1
834 progeny we retained sh males, and these were backcrossed to the original *D. persimilis*
835 isofemale line to generate BC2s (Supplemental Fig 2). This process of alternating males
836 and females for each backcross generation within each *D. persimilis* isofemale line was
837 completed until the BC12. The alternation of male/female during backcrossing was
838 necessary because recombination only occurs in females, but to retain the marker we had
839 to select for sh males every second generation. After the BC12, the progeny within each
840 BC isofemale line were interbred to create males and females homozygous for the sh
841 allele. We did not directly evaluate how much of the sh line genome was introgressed in
842 each case, however, *D. pseudoobscura* and relatives have a much higher recombination
843 rate than *D. melanogaster* [82], and previous introgression lines between these species
844 have eliminated unwanted regions after 4 generations of backcrossing [83].
845 For ISC experiments, the marked tester males were created by introgressing a green
846 fluorescent protein marker (GFP) into 2 wild type *D. pseudoobscura* strains per location

(therefore 8 strains in total, using wild-collected isofemale lines that were not used as female or second male genotypes for the ISC experiments). The original GFP strain was obtained from the UCSD stock center (14011-0121.166) the creation of which is described in Holtzman et al. [84]. We chose this marker because it is dominant [11]. We chromosomally mapped the GFP insertion of the original GFP strain to the second chromosome using a multiply marked (MM) strain, which contains visible recessive markers on all of the major chromosomes (y;gl;or;inc kindly provided by N. Phadnis, University of Utah). This mapping was completed in order to ensure the GFP insertion was not on the 3rd chromosome which, in *D. pseudoobscura*, contains large inversions that would have inhibited recombination of the marker into the wild-type backgrounds of our *D. pseudoobscura* isofemale lines.

The original GFP line was created in a stock that carried the X-linked white mutation. To eliminate the white allele from the population, in the parental cross we crossed the WT line with the GFP carrying male, and then used only F1 males with wild-type X chromosomes (no white mutation) to backcross in this initial generation. For the remaining eight backcross generations, we used females to allow recombination. We then chose 10 sibling pairs for each genotype to ensure the GFP marker was homozygous. These sub-lines were inbred for two generations. In the second generation we testcrossed the founder pair of individuals of each sub-line to ensure they were homozygous for the GFP marker. We recovered 2-4 lines that were homozygous for the GFP marker for each genotype. We then combined inbred lines that had originated from the same isofemale genotype to reduce any potential effects of inbreeding depression that might have arisen during marker introgression.

870

871 Scoring conspecific sperm precedence

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

881 Because female hybrids are fertile in these crosses, the *sh* allele was used to
882 differentiate the female progeny of heterospecific versus conspecific males and therefore
883 to score CSP from female offspring. Since the *sh* allele is recessive we could not score F1
884 females directly, but instead scored their offspring for the presence of the *sh* allele. If an
885 F1 female was hybrid (and carrying the *sh* allele from the *D. persimilis* male) we would
886 expect half of her sons and half of her daughters to have the *sh* phenotype. We previously
887 confirmed that the half segregation held for known hybrid progeny. For each cross, ten
888 F1 females (that could be hybrid or purebred) were housed individually with a *D.*
889 *pseudoobscura* male that also carried the *sh* allele (UCSD stock center *Dpse co;sh* 14011-
890 0121.13). We chose a *D. pseudoobscura* male for these crosses to increase the number of
891 progeny to score since *D. pseudoobscura* females (and therefore any purebred female
892 progeny in our experiment) exhibit premating isolation with *D. persimilis* males; hybrid

893 females do not demonstrate a mating preference. After a week the parental individuals
 894 were cleared from the vials and the vials were retained to score progeny. As progeny
 895 eclosed they were scored for the presence of sh allele. Any F1 female that produced sh
 896 progeny was considered hybrid. We required each F1 female to produce at least 10
 897 progeny to be used in scoring CSP.

898 Our measure of CSP was then the number of purebred progeny out of the total
 899 number of F1 individuals scored for a particular cross. If all progeny produced in a cross
 900 were scored as hybrid, we did not use this replicate in our analyses because we could not
 901 ensure that a second mating had taken place. Note that the frequency of this failure to
 902 remate following a first mating does not differ between populations [70]. Every CSP
 903 estimate was based on at least 10 scored progeny and, for the majority of the crosses, we
 904 scored close to 20 individuals. In addition, to ensure that CSP estimated here does not
 905 simply reflect stronger fecundity stimulation by conspecific males, in a pilot experiment
 906 we determined that there was no difference in progeny production in heterospecific vs.
 907 conspecific matings for any of the allopatric or sympatric populations, consistent with
 908 previous work [70,72]. There was also no correlation between the total number of
 909 progeny scored for CSP and the magnitude of CSP, and the number of progeny scored
 910 did not differ between populations. These observations suggest that there are no
 911 postzygotic survivorship barriers in hybrids between these species that would
 912 systematically differ between sympatric and allopatric populations, confounding our
 913 estimate of CSP.

914

915 Scoring intrapopulation sperm competition

916 We scored all progeny that eclosed in the five days after the second mating for the
 917 presence/absence of the GFP phenotype. Our measure of sperm competition (P2) for ISC
 918 was then the number of wild-type (non-GFP) progeny out of the total number of progeny
 919 scored for a particular cross. If all progeny produced in a cross were GFP, we did not use
 920 this replicate because we could not ensure that a second mating had taken place. (As with
 921 CSP, the proportion of females that did not remate was not significantly different
 922 between populations). Individuals were scored as they eclosed, using a Leica M205FA
 923 Stereo Microscope that has an Hg fluorescent lamp attached and GFP filter. Individuals
 924 were anesthetized and the ocelli were examined for GFP signal as described in Castillo
 925 and Moyle [11].

926

927 Statistical analyses

928 All analyses were completed in R v 3.01.

929 Differences in the probability of first mating with heterospecifics

930 We evaluated evidence for a pattern consistent with reinforcement acting on first mating
 931 (simple prezygotic isolation) in two ways. First, we used a χ^2 test of independence to test
 932 the null hypothesis that the mating rate with heterospecifics was the same for alternative
 933 geographic scenarios (allopatric vs. sympatric), after combining both allopatric and both
 934 sympatric populations for this single comparison (pairwise tests among individual
 935 populations gave the same result). Second, because χ^2 tests might lack power, and since
 936 mating events can be coded as a binary variable (0 for did not mate, 1 for successful
 937 mating), we used a logistic regression model with all four populations represented by a
 938 categorical variable using the glmer function. We then tested whether there were any

939 differences in heterospecific mating between populations by conducting a Wald's test
940 (using the `wald.test` function from the `aod` package; [85]).

941 To evaluate whether there was significant variation within each population (i.e.,
942 among isofemale line genotypes) in the probability of mating with a heterospecific, we
943 used logistic regression. We first fit a full model where the probability of mating with a
944 heterospecific depended on the isofemale line, the *D. persimilis* tester line, and the male x
945 female genotype interaction, and tested significance of these effects using a Wald's test.
946 Because there was no significant interaction for any population, we fit a reduced model
947 that only contained the effects of isofemale line and *D. persimilis* tester line without the
948 interaction, and report these models in the results.

949 Differences in mean and variance of CSP and ISC between populations

950 We evaluated evidence for a pattern in CSP consistent with reinforcement, by evaluating
951 whether the allopatric and sympatric populations had a mean difference in CSP or
952 whether they differed in variance. For analyses of mean differences, we pooled the two
953 allopatric populations because there was no significant difference in mean CSP between
954 them (Allopatry $t = -0.45064$, $df = 123.62$, $P = 0.653$) and pooled the two sympatric
955 populations for the same reason (Sympatry $t = -0.86678$, $df = 125.87$, $P = 0.3877$). We
956 tested the hypothesis that the mean CSP differed between geographic scenarios using a
957 Welch's t -test that accounts for unequal variances between samples, and (given that the
958 data are not normally distributed) we also confirmed these results with a Wilcoxon
959 ranked sum test. To evaluate differences in variance, we again pooled the allopatric and
960 sympatric populations because the variance was equivalent between allopatric
961 populations ($\chi^2 = 0.031899$, $P = 0.8585$), and between sympatric populations ($\chi^2 =$

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 [86]. The specific test we used in the lawstat is a Kruskal-Wallis modified Brown-Forsythe Levene-type test. The Brown-Forsythe test is based on the absolute deviations from the median, which retains statistical power for many types of non-normal data [87]. Kruskal-Wallis tests are rank-based tests. We used the Kruskal-Wallis modification because the variance in proportion data derived from binomial data does not accurately reflect variance in the original data [88].

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 $\chi^2 = 0.949$, $P = 0.3316$; Sympatric variance $\chi^2 = 0.0796$, $P = 0.7782$). 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).

979

980 Genetic variation and genotype effects on CSP and ISC

981 Within each population we assessed whether female, male, or female x male
982 genotype predicted variation in the strength of CSP and ISC. While this can be tested
983 using a two-way ANOVA with interaction, the assumptions of ANOVA, including
984 normally distributed residuals and heterogeneity in the distribution of the residuals, are

typically violated by binomial data such as our sperm competition data [89-90]. We instead chose binomial regression, as this more naturally models our count/binomial data. The model is of the form

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

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 (σ_α^2 , σ_β^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 [91]. 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. 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 the case of binomial

1007 regression the ICC values are on the log scale, and there is no convenient transformation
1008 to proportion scale [92], so they are presented here as a relative measure of variance
1009 explained.

1010

1011 Quantifying sexual selection and variance in male reproductive success

1012 To evaluate whether the intensity/opportunity for sexual selection differs among

1013 populations we require an estimate of variance in male reproductive success [93]. In a

1014 natural population most males can gain fitness through offensive (P1) and defensive (P2)

1015 sperm competition, so the best estimate for variance in reproductive success would be

1016 total progeny produced. In our experiment we did not score lifetime progeny production,

1017 and specific male genotypes were either used as offensive or defensive males only. As

1018 such we estimated male fitness as the proportion of progeny sired, taking into

1019 consideration that we had two distinct classes of males—tester first (defensive) males and

1020 second (offensive) males--that may differ in their frequency and variance in fitness in the

1021 experiment. Following Shuster et al. [94] we define total variance in male reproductive

1022 success as the sum of within and between male class variance

$$1023 \quad V_{total} = (f_{P1})(V_{P1}) + (f_{P2})(V_{P2}) + (\bar{X}_{P2} - \bar{X}_{P1})^2(f_{P2})(f_{P1})$$

1024 The two terms on the left hand of the equation represent the within class variance (for

1025 example, V_{P1} is the variance in sperm competitive success between tester males and f_{P1} is

1026 the frequency of tester males used in the experiment). The last term represents the

1027 between class variance.

1028 We were interested in reproductive variance at the level of male genotype so we averaged

1029 biological replicates to generate mean fitness values for each individual genotype. We

1030 used empirical bootstrap confidence intervals to estimate error that may have been a
1031 product of averaging over replicates [95-96]. For the bootstrap procedure we sampled 16
1032 data points, with replacement, from the 16 original empirical replicates for each genotype
1033 (32 for defensive males). We then averaged these data points and calculated V_{total} as
1034 described above. We completed 1000 bootstrap replicates for each population. We
1035 constructed the 95% confidence interval using the bootstrap difference $\delta^* = V_{total} -$
1036 V_{total}^* where * represents each bootstrap replicate. The interval is then $[V_{total} -$
1037 $\delta_{0.05}^*, V_{total} - \delta_{0.95}^*]$.
1038 The confidence intervals for the Zion population did not overlap with the confidence
1039 intervals for either sympatric population and can be considered significantly different at
1040 the 0.05 level (Supplemental Table 4). The Lamoille population confidence intervals
1041 overlapped with the sympatric populations, but overlap in confidence intervals does not
1042 mean parameters are not statistically different [97]. This is because confidence intervals
1043 calculated for independent parameters cannot replace a comparative test of the
1044 differences between two parameters. Therefore, we conducted bootstrap hypothesis
1045 testing [95-96] to determine whether differences in V_{total} between populations were
1046 significant, specifically by calculating bootstrap F statistics. The F statistic is a ratio of
1047 any two variance parameters, for example $F = V_{total,pop1} / V_{total,pop2}$. We compared the
1048 V_{total} in pairwise comparisons following standard bootstrap methods, where bootstrap
1049 samples are generated under the null hypothesis, and then this distribution is compared to
1050 the empirically observed statistic. For our scenario, the null hypothesis was that there was
1051 no differences in V_{total} between populations. Therefore we sampled, with replacement,
1052 offensive and defensive genotypes after pooling data from both populations. We then

1053 randomly assigned each value to one of the two populations. This generated a bootstrap
 1054 replicate with approximately equal variance between the populations. We then could
 1055 calculate $F = V_{total, pop1} / V_{total, pop2}$ for each replicate. The bootstrap p-value is then
 1056 calculated by comparing the bootstrap statistic (F^*) to the observed statistics (F) using
 1057 $p^*(F) = \frac{1}{B} \sum_{j=1}^B I(F^* > F)$. $I()$ is an indicator function that is equal to 1 when the
 1058 argument is true (bootstrap statistic > observed statistic), and 0 when false. B is the
 1059 number of bootstrap replicates (1000 per population comparison).