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4 **Title:** Remating responses are shaped by male post-copulatory manipulation but not

5 reinforcement in *D. pseudoobscura*

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7 RRH: No reinforcement of remating in *D. pseudoobscura*

8 Keywords: speciation; reproductive isolation; allopatry; sympatry; coevolution; sexual conflict

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16 included)

17 **Abstract**

18 Both pre-mating and post-mating prezygotic behaviors have been shown to be the target of
19 reinforcing selection, but it remains unclear whether remating behaviors experience
20 reinforcement, although they can also influence offspring identity and limit formation of
21 hybrids. Here we evaluated evidence for reinforcing selection on remating behaviors in *D.*
22 *pseudoobscura*, by comparing remating traits in females from populations historically allopatric
23 and sympatric with *D. persimilis*. We found that the propensity to remate was not higher in
24 sympatric females, compared to allopatric females, regardless of whether the first mated male
25 was heterospecific or conspecific. Moreover, remating behavior did not contribute to
26 interspecific reproductive isolation among any population; that is, females showed no higher
27 propensity to remate following a heterospecific first mating than they were following a
28 conspecific first mating. Instead, we found that females are less likely to remate after initial
29 matings with unfamiliar males, regardless of species identity, consistent with females being
30 poorly defended against post-copulatory manipulation by males with whom they have not co-
31 evolved. Our results are generally inconsistent with reinforcement on remating traits, and
32 suggest that this behavior is more strongly shaped by the consequences of local antagonistic
33 male-female interactions than interactions with heterospecifics.

34

35 **Introduction**

36 Because hybridization between incompletely isolated species can be costly—in terms of reduced
37 fecundity or offspring survival or fertility—selection is expected to favor traits that reduce the frequency
38 or consequences of these matings in nature (Dobzhansky 1940). This ‘reinforcement’ of incomplete
39 reproductive isolation is thought to play a key role in speciation, especially where there is secondary
40 contact between close relatives (Ortiz-Barrientos et al. 2009). Reinforcement has frequently been
41 examined in the context of selection on premating traits, such as courtship displays or behaviors, which
42 can act to prevent heterospecific matings (e.g., Saetre et al. 1997, Rundle and Schluter 1998). However,
43 post-mating traits could also be subject to reinforcing selection (Servedio and Noor 2003) as could traits
44 that integrate pre- and post-mating responses, such as postmating control of paternity via variable
45 remating rate (Marshall et al. 2002, Kisdi 2003). Control over mate and paternity choice has been shown
46 to evolve rapidly in response to antagonistic coevolution between the sexes (e.g., Rice 1996, Miller and
47 Pitnick 2002, Manier et al. 2013a). Such rapidly evolving reproductive traits can potentially drive
48 divergence between populations and might contribute strongly to reproductive isolation (Parker and
49 Partridge 1998, Rice 1998, Howard 1999, Gavrilets 2000, Panhuis et al. 2001, Martin and Hosken 2003,
50 Ritchie 2007, Howard et al. 2009, Manier et al. 2013b). Therefore both mating and remating behaviors
51 are potentially interesting candidates for examining the evolution of isolating mechanisms between
52 species, including in the context of reinforcement.

53 One key expectation under reinforcement is that populations that are historically sympatric with
54 closely related heterospecifics will show stronger isolation than populations that are historically
55 allopatric (Butlin 1987, Servedio and Noor 2003). This is because only sympatric populations will have
56 experienced selection to avoid producing lower fitness hybrid offspring. Mate choice during the first
57 mating has been observed to show patterns consistent with reinforcement (e.g., Noor 1995, Higgin et al.
58 2000, Saetre et al. 1997, Rundle and Schluter 1998), whereby sympatric females are more discriminating

59 against heterospecifics than are allopatric females. In comparison to initial mate choice, whether
60 remating rates respond to reinforcement is largely unknown (Marshall et al. 2002; but see Matute 2010,
61 and Discussion). Decreasing the time to remating (latency) or increasing the propensity to remate allows
62 females to manipulate paternity, including after mating with a suboptimal male (variously called a
63 ‘rescue effect’ (Fricke et al. 2006) or the ‘trading up’ hypothesis (Byrne and Rice 2005)). Because mating
64 with heterospecifics is generally suboptimal, remating rate could respond to reinforcing selection such
65 that sympatric females increase their propensity to remate with conspecifics following a heterospecific
66 mating (Marshall et al. 2002). It is also possible that exposure to heterospecifics could generally increase
67 remating rates of females in such populations, regardless of first male identity. In comparison, females
68 from populations that are geographically allopatric are not expected to elevate remating responses.

69 Nonetheless, making predictions about remating rate is complex because remating behaviors
70 are the product of both female choice and male manipulation. For example, in *Drosophila*, females are
71 known to exhibit cryptic female choice by controlling number of mates and/or by preferentially using
72 sperm from some male partners (Manier et al. 2010, Lupold et al. 2013, Manier et al. 2013c). However,
73 male *Drosophila* seminal fluid proteins transferred during copulation are known to suppress female
74 remating rate, increase oviposition rate, and reduce lifespan, potentially resulting in net fitness
75 reductions for females (Parker and Partridge 1998, Sirot et al. 2009, and references therein). The
76 resulting antagonistic male-female coevolution acting on these traits can lead females to be poorly
77 defended against males with whom they have not co-evolved (Rice 1998, Parker and Partridge 1998).
78 Under this scenario, for example, allopatric females that are less equipped to defend against
79 heterospecific encounters might exhibit reduced remating rates, even when remating would be
80 individually beneficial.

81 We sought to examine whether remating rates might respond to reinforcing selection in a
82 *Drosophila* species pair that is a canonical example of reinforcement of premating isolation. *Drosophila*

83 *persimilis* and *Drosophila pseudoobscura* are recently diverged (500 kya) sister species with distinct but
84 significantly overlapping ranges (Shaeffer and Miller 1991, Wang et al. 1997, Machado et al. 2002). *D.*
85 *pseudoobscura* has a wide geographic range in North America, stretching west from the Pacific to close
86 to the Mississippi River and far South into Central America; *D. persimilis* has a far narrower range
87 completely sympatric with *D. pseudoobscura* and not extending farther east than the Sierra Nevada and
88 Cascade Mountain ranges (Figure 1). These species exhibit incomplete reproductive isolation and
89 hybridize in the laboratory; natural hybrids, while rare, have been found in the wild (Dobzhansky 1973,
90 Kulathinal et al. 2009). In addition, mate choice patterns consistent with reinforcement have been
91 directly demonstrated in this species pair, whereby allopatric *D. pseudoobscura* females mate at a
92 higher rate with *D. persimilis* males than do *D. pseudoobscura* females from sympatric populations
93 (Noor 1995), although see Anderson and Kim (2005, 2006) for more complex patterns of isolation
94 between sympatric and allopatric populations. Similarly, a recent study evaluating other components of
95 reproductive isolation in this species pair (Castillo and Moyle 2016) found no difference in first mating
96 rates between allopatric and sympatric *D. pseudoobscura* paired with *D. persimilis* males. These previous
97 observations make this species pair particularly interesting for examining whether remating rate might
98 also respond to reinforcing selection.

99 Our primary goal in this study was to evaluate evidence for reinforcing selection on remating
100 behaviors of *D. pseudoobscura* females, using populations historically allopatric and sympatric with *D.*
101 *persimilis*. To do so, we evaluated mating traits in females from three target *D. pseudoobscura*
102 populations: two populations sympatric with *D. persimilis*, and one allopatric population (Figure 1).
103 These populations were a subset of those examined in a larger parallel study of patterns of first mating
104 and conspecific sperm precedence between these species (Castillo and Moyle 2016). Following a first
105 mating with either a heterospecific or a conspecific male, females were given the opportunity to remate
106 with a male from their own population. We assessed whether female remating response depends on

107 identity of the first mated male and, specifically, whether the propensity to remate depends upon
108 female population identity (allopatric/sympatric). If remating behaviors have evolved in response to the
109 presence of heterospecifics, we expected that *D. pseudoobscura* females from sympatric sites would
110 more readily remate if their first mating was with a heterospecific male, consistent with an evolved
111 response to limit the number of hybrid offspring sired from this first mating. An alternative expectation
112 is that females from sympatric populations remate at a higher rate irrespective of first male identity, as
113 a simpler response to potentially suboptimal first matings.

114

115 **Methods**

116 *D. pseudoobscura* and *D. persimilis* collection and maintenance

117 All stocks were reared on standard media prepared by the Bloomington Drosophila Stock
118 Center, and were kept at room temperature (~22C). We used a subset of isofemale lines from a larger
119 panel that were collected in the summers of 2013 and 2014 at three sites (Figure 1). Allopatric *D.*
120 *pseudoobscura* were collected at Zion National Park, UT (kindly provided by N. Phadnis). Sympatric *D.*
121 *pseudoobscura* and *D. persimilis* were collected at two sites: Mt. St. Helena, CA (*D. pseudoobscura*
122 collected by A. Hish/M. Noor, and *D. persimilis* collected by D. Castillo); and, near Meadow Vista and
123 Forest Hill, CA (called here 'Sierra'; Figure 1) (*D. pseudoobscura* and *D. persimilis* collected by D. Castillo).
124 For both sympatric populations, both species were present in field collections and can be considered
125 truly co-occurring/sympatric. Our three focal populations are a subset of four populations used in a
126 parallel study that evaluated evidence for reinforcement on first matings, and on conspecific sperm
127 precedence (Castillo and Moyle 2016). (The current study excludes analysis of an additional allopatric
128 population from Lamoille Canyon, NV). All but one of the 6 isofemale lines from our three populations
129 are shared in common with the other study (MSH3 is not used in Castillo and Moyle 2016), enabling us

130 to compare remating data from both experiments here (see below), as well as reassess the prior first
131 mating result with data obtained from our first mating observations.

132

133 *Mating and Remating assay*

134 To examine remating behaviors in females from our three target *D. pseudoobscura* populations,
135 we used a design in which each female was initially paired with 1 of 5 different types of male (males
136 from each of the 3 *D. pseudoobscura* populations and 2 *D. persimilis* populations) and then, after a
137 refractory period (9 days), given the opportunity to remate with a second male (Supplementary Figure
138 1). The second male was always from the same population as the target female, to ensure females
139 would mate most readily during the second mating. This procedure was performed for each
140 combination of our three female populations and five first male types (15 total cross combinations). Two
141 complete experimental blocks were performed for each cross combination, using two unique isofemale
142 lines from each population. Within each experimental block, a minimum of 8 biological replicates were
143 carried out for each combination of first and second male matings.

144 For each first male pairing, mating behavior was directly observed for 3 hours, and copulation
145 latency (time to start of copulation) and duration (time from start to end of copulation) were recorded.
146 Following the 3-hour observation period, pairs were maintained together for an additional 21 hours, and
147 vials were checked 7 days later for larvae to determine if mating occurred within first 24 hours but
148 outside the initial 3-hour observation window. This allowed us to assess whether female population
149 origin influences mating behavior in the first male mating, and whether this varied according to male
150 population identity. For each second male pairing, female mating behavior was assessed in terms of
151 copulation latency and mating duration within the first 3 hours of pairing. This allowed us to evaluate
152 whether females vary their remating behavior in response to the population and/or species identity of

153 their first mate, in addition to whether these responses differed females from allopatric versus
154 sympatric sites. Finally, differences among isofemale lines in overall propensity to remate following
155 conspecific first matings, was used to confirm that there was heritable genetic variation for this trait
156 within *D. pseudoobscura* (Results). Detailed mating procedures are provided in Supplementary material.

157 After completing at least 8 replicates, we found that copulation duration during the first mating
158 was indistinguishable among all crosses, and copulation latency was either similarly rapid (<10 minutes)
159 in all conspecific pairings, or inconsistently and rarely observed within the first 3 hours in heterospecific
160 pairings (Results). Based on these findings, for the remaining 14 replicates (which primarily focused on
161 heterospecific second pairings) first matings were no longer directly observed for the first 3 hour period,
162 but were instead simply scored for presence/absence of larvae 7 days after co-housing each male-
163 female pair for 24 hours. Regardless of this change for first matings, remating behavior was always
164 assessed as observed copulation, and copulation latency and duration, within the first 3 hours of co-
165 housing.

166 Although Castillo and Moyle (2016) did not observe remating directly, data from that
167 experiment can be used to glean some additional information about remating rates in sympatric versus
168 allopatric females. Similar to the design here, in that study virgin *D. pseudoobscura* females were
169 housed with *D. persimilis* males for 24 hours and then, following a period of 7 days, were given the
170 opportunity to remate with *D. pseudoobscura* males. Progeny after this second mating were scored (*D.*
171 *persimilis* male was marked with a visible marker, and hybrid males are sterile), providing information
172 on whether females remated or not. Females are inferred to have failed to remate if all progeny after
173 second mating were hybrid; that is, if all males were sterile and all females carried the visible mutation).
174 These data were used as an additional test of whether allopatric and sympatric females differed in their
175 propensity to remate (see results).

176 *Statistics*

177 A χ^2 test of independence was used to compare overall *D. pseudoobscura* female mating rates in
178 first pairings with conspecific versus heterospecific males. To make more specific comparisons among
179 groups, we used logistic regression on presence/absence of larvae after mating (mating was considered
180 a binary variable). Logistic regressions were used to assess differences in the mating probabilities of all
181 females during their first matings, and during remating trials, depending upon whether they were
182 initially paired with first males of three classes: males from their own population, males from a different
183 conspecific population, or heterospecific males. Probabilities of mating and of remating were also
184 specifically compared between *D. pseudoobscura* females historically allopatric and sympatric with *D.*
185 *persimilis*. For all logistic regressions, differences between mating types were inferred by examining
186 significance of the regression coefficients. Negative coefficients signified categories where matings were
187 less likely to occur, and positive coefficients signified that mating was more likely to occur.

188 To analyze quantitative copulation latency, we primarily used Cox proportional hazard models in
189 the *survival* package (Therneau 2013) in R, which let us take into account the mating and remating rates
190 as well as probability of mating within our 3 hour observation. For one comparison (allopatric versus
191 sympatric remating latency) we used parametric survival regression (see Supplement for details). We
192 included female genotype in the proportional hazard models to account for correlated observations
193 within a given isofemale line (see Supplemental information for details). Survival curves for a specific
194 mating category were considered different when the coefficient from the model was significantly
195 different than the zero. Negative coefficients signified categories where matings occurred more slowly
196 than baseline, and positive coefficients signified that mating occurred more quickly than baseline.
197 Baseline was always mating involving males from the females own population. Finally, a χ^2 test of
198 independence was used to compare overall *D. pseudoobscura* remating rates between allopatric and
199 sympatric females, using the remating data integrated from Castillo and Moyle (2016).

200

201 **Results**

202 *Initial mate choice contributes to reproductive isolation between species but is not stronger in sympatry*

203 We confirmed that *D. pseudoobscura* females discriminate against *D. persimilis* males; while
204 almost all conspecific matings were successful (164/168), only 25% of heterospecific pairings resulted in
205 mating (93/369), a significant difference in mating propensity ($\chi^2 = 239.70$; $P < 2.2 \times 10^{-16}$). Logistic
206 regressions similarly indicated that the proportion of heterospecific matings was significantly lower than
207 the proportion of first matings either with males a different conspecific population ($\beta = 3.5927$; $P =$
208 8.31×10^{-10}) or with males from the females own population ($\beta = 4.0073$; $P = 7.13 \times 10^{-05}$) (Figure 2). There
209 was no difference in the propensity to mate of females paired with males from their own population
210 versus males from different conspecific populations ($\beta = 0.4146$; $P = 0.722$) (Figure 2), indicating that
211 female choice in conspecific first matings was not sensitive to the population origin of the conspecific
212 male.

213 To test for patterns consistent with reinforcement on first mating, we fit a logistic regression to
214 first mating success according to whether female *D. pseudoobscura* were from a population that was
215 allopatric or sympatric with *D. persimilis*. We found no significant difference between allopatric and
216 sympatric females in probability of mating with a heterospecific first male ($\beta = -0.1079$; $P = 0.623$),
217 consistent with prior observations of mating patterns that used more isofemale lines and one additional
218 allopatric population comparison (Castillo and Moyle 2016). We did not analyze differences in
219 copulation latency between allopatric versus sympatric females in heterospecific first matings because
220 too few of these mating events occurred within the directly observed first 3 hours of cohousing. Only 16
221 of 133 directly observed heterospecific pairings resulted in copulations within the first 3 hours,
222 corresponding to 14% and 15% of sympatric and allopatric female pairings. An additional 20 of the 133

223 directly observed pairings resulted in progeny, but these matings occurred within the subsequent
224 (unobserved) 21 hour period of co-housing.

225

226 *Sympatric females are slower to mate with conspecific males in first matings*

227 If exposure to heterospecifics has resulted in general changes in intrinsic mating behavior,
228 rather than specific responses directed at heterospecific genotypes, allopatric and sympatric females
229 should differ in mating responses to conspecific males. Allopatric and sympatric females did not differ in
230 probability of mating when paired with conspecific males (regardless of their population of origin) ($\beta = -$
231 17.22 ; $P = 0.994$). However, females from sympatric populations took significantly longer to initiate
232 copulation with conspecific males than did allopatric females ($\beta = -0.2693$; $P = 0.0096$). When we
233 simultaneously tested for an effect of sympatry and for the population of origin of the conspecific male,
234 mating latency did not differ according to the specific population of the conspecific male ($\beta = 0.0584$; $P =$
235 0.6717) but the difference between sympatry and allopatry remained ($\beta = -0.2720$; $P = 0.0076$). In
236 other words, sympatric females are slower to initiate copulation, regardless of the population identity of
237 the first conspecific male (i.e. own versus other conspecific population) with which they are paired. It is
238 possible that this is a subtle behavioral response to past selection imposed by heterospecifics: if female
239 *D. pseudoobscura* in sympatry have adapted to encountering heterospecific males, they might be more
240 circumspect in their initial mating decisions in general. This longer latency might contribute to fewer
241 accidental heterospecific matings, especially under less restrictive conditions than those imposed by our
242 lab co-housing experiment.

243

244 *Remating varies depending on the identity of the first mating male, but does not contribute to*

245 *interspecific reproductive isolation or to enhanced isolation in sympatry*

246 To evaluate whether *D. pseudoobscura* females differed in their readiness to remate depending
247 on the identity of the first male they mated with, we compared the frequency of remating and the
248 copulation latency in remating trials following three classes of first mating: with conspecific males from
249 their own population, with conspecific males from a different population, or with heterospecific males.
250 We found that analyses of both mating probability and latency to copulation indicate that remating
251 happens more readily when females first mate with familiar (own population) males, than when initially
252 mated with unfamiliar conspecifics or with heterospecifics. In terms of remating probability, females
253 initially mated to their own population males were significantly more likely to remate compared with
254 females initially mated to a *D. persimilis* male ($\beta = -0.98291$; $P = 0.00555$), although the probability of
255 remating did not differ significantly between females previously mated with conspecific males from
256 their own population versus from a different conspecific population ($\beta = -0.55603$; $P = 0.10471$). In terms
257 of latency to copulation, females first mated with their own male remated more quickly (had shorter
258 latency) than females initially mated with either conspecifics from different populations ($\beta = -0.5213$; $P =$
259 0.02195) or heterospecifics ($\beta = -0.8035$; $P = 0.000526$). (Although trending in this direction, copulation
260 latency was not significantly shorter in females initially mated with conspecifics from a different
261 population versus with heterospecific males (i.e., the confidence intervals on β coefficients overlap).)
262 These observations also indicate there is no generalized female *D. pseudoobscura* response to increase
263 remating following heterospecific first matings.

264 To test for patterns consistent with reinforcement on remating, we assessed whether allopatric
265 versus sympatric females differ in their remating behaviors following first matings with *D. persimilis*
266 males. We found that they did not differ in their probability of remating ($\beta = -0.2851$; $P = 0.5447$), or in
267 how rapidly they remated (parametric survival regression; $\beta = 0.252$; $P = 0.5280$), following a
268 heterospecific first mating. Finally, using a second set of mating data from Castillo and Moyle (2016), we
269 examined the number of females that failed to remate compared to the total number of remating trials

270 scored, and found there was no significant difference in remating rate between females from allopatric
271 versus sympatric populations ($\chi^2=0.1445$; $df=1$, $P=0.7029$). Note that we detected significant differences
272 among *D. pseudoobscura* isofemale lines in their overall propensity to remate following a conspecific
273 first mating (Wald's χ^2 ; $df = 5$; $P = 0.0352$), indicating there is genetic variance for remating behavior
274 available to selection in this species.

275

276 *Allopatric and sympatric females do not differ in remating behavior with conspecifics*

277 To investigate whether allopatric versus sympatric females differ in their intrinsic propensity to remate,
278 we compared remating probability and latency between allopatric and sympatric females that had first
279 mated with conspecifics; we found that they did not differ in their probability of remating ($\beta = 0.1586$, P
280 $= 0.6569$) or in their latency to copulate in remating trials ($\beta = 0.1616$, $P = 0.5840$). When we
281 simultaneously tested for an effect of sympatry and for the population of origin of the first mated
282 conspecific male, allopatric and sympatric females still did not differ in remating latency ($\beta = 0.0961$, $P =$
283 0.7437); however, we did detect a first male population effect, such that remating occurred more
284 rapidly when females had mated first with a conspecific from their own population ($\beta = -0.4975$; $P =$
285 0.0239). This is consistent with our findings that females overall mate quickest following own-male first
286 matings (above). Sympatric and allopatric females did not differ in remating latency following own male
287 matings ($\beta = -0.3492$, $P = 0.1570$).

288

289 **Discussion**

290 In this study our primary goal was to evaluate if sympatric *D. pseudoobscura* females remate
291 more quickly or at a higher rate when previously mated to a heterospecific *D. persimilis*, as expected if

292 remating behavior has responded to reinforcing selection in sympatry. We found no evidence for
293 reinforcement effects on remating, in either probability of remating or in latency to copulation, when
294 females had previously mated to heterospecifics. Sympatric females were also no more likely or faster
295 to remate after conspecific first matings. Therefore our results indicate little evidence that remating
296 behavior in our sympatric populations has responded specifically to reinforcing selection. In addition,
297 our results also imply that our sympatric *D. pseudoobscura* females do not show a generalized change in
298 remating behavior (either an increased general propensity to remate or to remate more quickly) in
299 order to minimize the consequences of suboptimal (especially heterospecific) matings. Our results differ
300 from the only other study (of which we are aware) to compare remating rates between females
301 allopatric and sympatric with a closely-related conspecific species. In it, Matute (2010) found that *D.*
302 *yakuba* females sympatric with *D. santomea* exhibit greater remating rates after heterospecific matings,
303 compared to *D. yakuba* females that are allopatric, a pattern that is consistent with the expectations of
304 reinforcement on remating, but that could also be explained by less direct effects (see below).

305 Given that there is genetic variation for *D. pseudoobscura* female remating behavior (Results),
306 one potential explanation for our findings is that selection on remating behavior is insufficiently strong
307 or consistent to elicit a substantial evolutionary response. That is, if females are only infrequently
308 exposed to the consequences of completed heterospecific matings, then selection on traits that mitigate
309 these consequences could be relatively weak. In our study, only ~14% of *D. pseudoobscura* females
310 mated with *D. persimilis* males within 3 hours of enforced co-housing. In comparison, in Matute's (2010)
311 study that detected enhanced remating in sympatric *D. yakuba* females, ~30% of *D. yakuba* females
312 mated with a *D. santomea* male within a 1 hour observation period (Matute 2010, Table S4), potentially
313 contributing to the different outcomes of that study and our data here. This relatively high first mating
314 rate between *D. yakuba* females and *D. santomea* males should impose stronger selection on sympatric
315 *D. yakuba* to evolve remating habits that reduce the negative effects of heterospecific matings.

316 Alternatively, because female receptivity is also known to be influenced by the number of sperm in
317 storage (the 'sperm effect'; Manning 1962, 1967), *D. yakuba* sympatric females might remate more
318 rapidly because they experience more acute sperm depletion following heterospecific matings (as
319 inferred in Matute 2010), rather than the because of past reinforcing selection for higher remating in
320 response to suboptimal (interspecies) matings. It is difficult to disentangle these two hypotheses
321 without information on remating rates with conspecific males (remating in *D. yakuba* was examined
322 only after heterospecific matings).

323 Alternatively, other forces might be more critical in shaping *D. pseudoobscura* remating
324 behavior than exposure to heterospecifics. In particular, remating behaviors are determined by complex
325 interactions between males and females, some of which might act in ways counter to reinforcing
326 selection imposed by exposure to heterospecifics. There is substantial evidence for sexually antagonistic
327 coevolution acting on remating traits (Parker and Partridge 1998, Arnqvist and Rowe 2005, Crudgington
328 et al. 2005); in these cases, individuals are expected to be well equipped to respond to antagonistic
329 measures employed by others from their own population, but potentially poorly defended against
330 individuals with whom they have not co-evolved.

331 Intriguingly, our observations of remating behavior are consistent with these outcomes of local
332 co-evolution due to sexual antagonism. We found that females mated previously to male conspecifics of
333 their own population remated significantly more quickly and/or more frequently than females
334 previously mated with conspecific males from a different population or with heterospecifics; remating
335 was least frequent after mating with heterospecific males. These observations suggest that increased
336 sexual familiarity results in females better able to combat male post-copulatory manipulation
337 ('molecular coercion'; Parker and Partridge 1998) via the seminal fluid in ejaculate. A similar pattern has
338 been previously observed in Bean Weevils, in which matings involving increasingly more distantly
339 related first males resulted in increasingly reduced rates of female remating; first matings with

340 heterospecific males elicited the greatest post-copulatory egg production and the lowest re-mating rate
341 (Fricke et al. 2006). In both cases, females appear to be more able to resist suppression of remating by
342 local males in comparison to unfamiliar males.

343 In addition to examining remating traits, our experimental design allowed us to reassess
344 evidence of reinforcement in first matings involving these populations. As with a parallel larger study
345 with many of the same isofemale lines (Castillo and Moyle 2016), we found no evidence for
346 reinforcement in first mating between our populations. Discrimination against heterospecific males was
347 not stronger in historically sympatric females, the most straightforward expectation of a response to
348 reinforcing selection. This is curious, as previous studies have detected significantly stronger sexual
349 isolation in sympatric *D. pseudoobscura* females (Noor 1995; Noor and Ortiz-Barrientos 2006). At least
350 two factors could potentially contribute to our observed differences. First, sympatric populations might
351 be polymorphic for high discrimination alleles (as suggested in Barnwell et al. 2008), and we happened
352 to use lines that discriminate differently compared to previous studies. Second, Anderson and Kim
353 (2005, 2006) have argued that gene flow among *D. pseudoobscura* populations has contributed to
354 homogenizing mating discrimination traits between allopatric and sympatric sites. Interestingly, the
355 range of mean heterospecific mating rates for sympatric isofemale lines is broad in both our analysis and
356 in Noor's (1995) study (range=0.22-0.52, 0.16-0.37, respectively). In addition, the sympatric lines in our
357 study have somewhat higher heterospecific mating rates (mean=0.346) compared to Noor's (1995)
358 sympatric lines (mean = 0.252), whereas our allopatric lines mated with heterospecifics at considerably
359 lower rates than Noor's (mean=0.319 versus 0.45 in Noor 1995). These differences suggest indirect
360 evidence that genetic polymorphism within sympatric populations and gene flow/homogenization
361 between *D. pseudoobscura* populations might both contribute to differences between our findings and
362 those in Noor (1995).

363 Regardless of these observations for first matings, our primary analysis of remating suggests
364 that factors such as local sexual coevolution could act counter to reinforcing selection. Even when
365 advantageous for females to manipulate the genetic identity of offspring via remating—such as
366 following matings with heterospecific males—our results suggest that behavioral manipulation of
367 females by male seminal proteins could supersede this response. It has been broadly recognized that
368 sexually antagonistic coevolution and reproductive character displacement can interfere with each
369 other, producing sub-optimal outcomes for one or both processes. Interestingly, this potential tension
370 between intraspecific and interspecific sexual interactions is more often described in terms of
371 reproductive character displacement hampering optimal outcomes of intraspecific sexual selection,
372 rather than the reverse (Ortiz-Barrientos et al. 2009, Pfennig and Pfennig 2012). Here we infer that
373 intraspecific sexual dynamics might instead overwhelm the action of reinforcing selection, producing
374 complex outcomes for remating behaviors within and between species.

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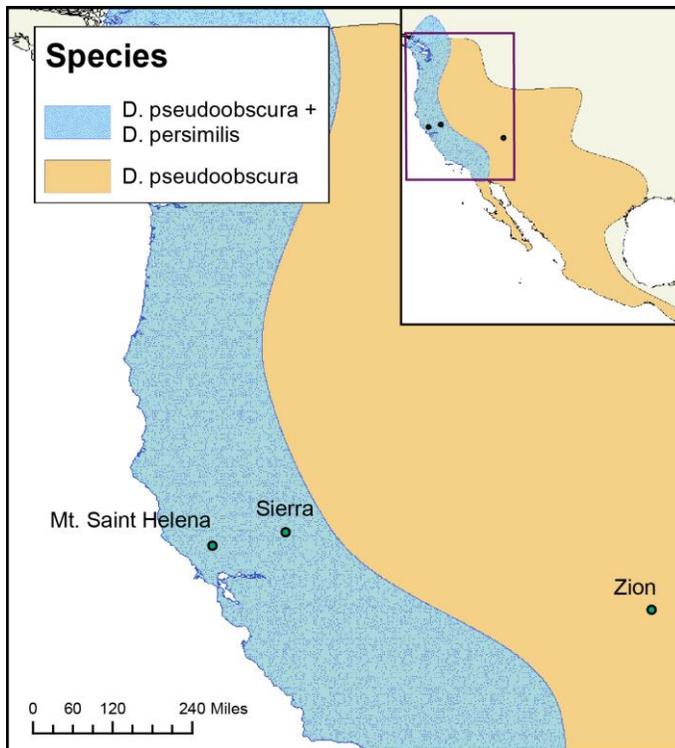
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472 Figure 1: Collection locations for *Drosophila pseudoobscura* and *D. persimilis* study populations. Mt.

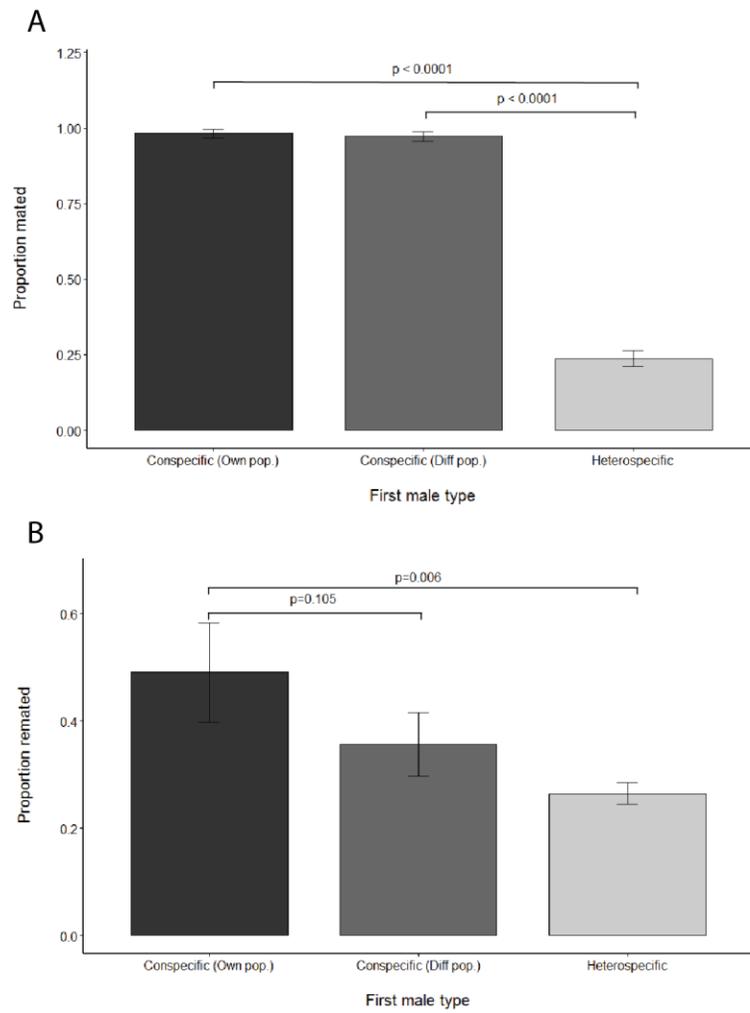
473 Saint Helena and Sierra are sympatric locations (both species); Zion is an allopatric site (*D.*

474 *pseudoobscura* only). Inset: North American range maps for the two species; the range of *D. persimilis* is

475 entirely contained within the broader *D. pseudoobscura* range.

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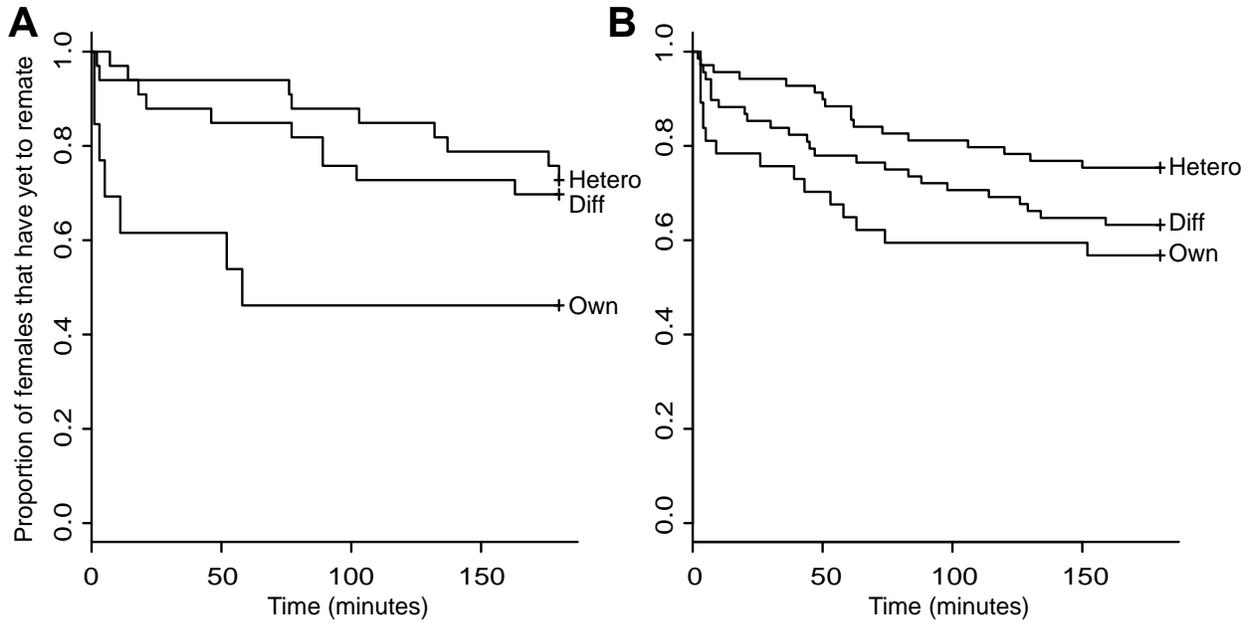


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479 Figure 2: Mating (panel A) and remating (panel B) probabilities of *D. pseudoobscura* females following
480 first matings with males from their own population (Own pop), a different conspecific population (Diff
481 pop), and heterospecific males. P-values are from logistic regressions (see Results).

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485 Figure 3: Survival curves showing remating latencies of allopatric (A) and sympatric female (B) *D.*

486 *pseudoobscura* when mated to different classes of first males: Own (conspecific male from the same

487 population as the female); Diff (conspecific male from a different population); Hetero (heterospecific

488 male). Figure S1 shows survival curves of remating latency separately for each isofemale line.