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4 **Title:** Remating responses are consistent with 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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10 Jeremy S. Davis, Dean M. Castillo, and Leonie C. Moyle*

11 Dept of Biology, Indiana University, Bloomington IN, USA 47405

12 *Corresponding author: lmoyle@indiana.edu

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15 Data archiving: to be archived as supplementary data to the paper, upon publication. (url to be
16 included)

17 **Abstract**

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

37

38 **Introduction**

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

56 One key expectation under reinforcement is that populations that are historically sympatric with
57 closely related heterospecifics will show stronger isolation than populations that are historically
58 allopatric (Butlin 1987, Servedio and Noor 2003). This is because only sympatric populations will have
59 experienced selection to avoid producing lower fitness hybrid offspring. Mate choice during the first
60 mating has been observed to show patterns consistent with reinforcement (e.g., Noor 1995, Higgin et al.
61 2000, Saetre et al. 1997, Rundle and Schluter 1998), whereby sympatric females are more discriminating

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

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

85 coevolutionary arms race, which can vary depending upon the precise details of these male-female
86 interactions (Long et al. 2006, reviewed Tregenza et al. 2006, and see Discussion).

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

105 Our primary goal in this study was to evaluate evidence for reinforcing selection on remating
106 behaviors of *D. pseudoobscura* females, using populations historically allopatric and sympatric with *D.*
107 *persimilis*. To do so, we evaluated mating traits in females from three target *D. pseudoobscura*
108 populations: two populations sympatric with *D. persimilis*, and one allopatric population (Figure 1).

109 These populations were a subset of those examined in a larger parallel study of patterns of first mating
110 and conspecific sperm precedence between these species (Castillo and Moyle 2016). Following a first
111 mating with either a heterospecific or a conspecific male, females were given the opportunity to remate
112 with a male from their own population. We assessed whether female remating response depends on
113 identity of the first mated male and, specifically, whether the propensity to remate depends upon
114 female population identity (allopatric/sympatric). If remating behaviors have evolved in response to the
115 presence of heterospecifics, we expected that *D. pseudoobscura* females from sympatric sites would
116 more readily remate if their first mating was with a heterospecific male, consistent with an evolved
117 response to limit the number of hybrid offspring sired from this first mating. An alternative expectation
118 is that females from sympatric populations remate at a higher rate irrespective of first male identity, as
119 a simpler response to potentially suboptimal first matings.

120

121 **Methods**

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

123 All stocks were reared on standard media prepared by the Bloomington Drosophila Stock
124 Center, and were kept at room temperature (~22C). We used a subset of isofemale lines from a larger
125 panel that were collected in the summers of 2013 and 2014 at three sites (Figure 1). Allopatric *D.*
126 *pseudoobscura* were collected at Zion National Park, UT (kindly provided by N. Phadnis). Sympatric *D.*
127 *pseudoobscura* and *D. persimilis* were collected at two sites: Mt. St. Helena, CA (*D. pseudoobscura*
128 collected by A. Hish/M. Noor, and *D. persimilis* collected by D. Castillo); and, near Meadow Vista and
129 Forest Hill, CA (called here 'Sierra'; Figure 1) (*D. pseudoobscura* and *D. persimilis* collected by D. Castillo).
130 For both sympatric populations, both species were present in field collections and can be considered
131 truly co-occurring/sympatric. Our three focal populations are a subset of four populations used in a

132 parallel study that evaluated evidence for reinforcement on first matings, and on conspecific sperm
133 precedence (Castillo and Moyle 2016). (The current study excludes analysis of an additional allopatric
134 population from Lamoille Canyon, NV). All but one of the 6 isofemale lines from our three populations
135 are shared in common with the other study (MSH3 is not used in Castillo and Moyle 2016), enabling us
136 to compare remating data from both experiments here (see below), as well as reassess the prior first
137 mating result with data obtained from our first mating observations.

138

139 *Mating and Remating assay*

140 To examine remating behaviors in females from our three target *D. pseudoobscura* populations,
141 we used a design in which each female was initially paired with 1 of 5 different types of male (males
142 from each of the 3 *D. pseudoobscura* populations and 2 *D. persimilis* populations). Five day-old virgin
143 females were transferred individually without anesthesia to vials with individual 5-day old virgin males
144 and allowed to mate for 24 hours before the male was removed. Females were then allowed to lay for 9
145 days, a refractory period that pilot trials indicated gives ample time for females to become receptive to
146 males again. Those that produced larvae (and therefore were guaranteed to have mated with the first
147 male) were then given the opportunity to remate with a second, 5-day-old virgin male. The second male
148 was always from the same population as the target female, to ensure females would mate most readily
149 during the second mating. This procedure was performed for each combination of our three female
150 populations and five first male types (15 total cross combinations). Two complete experimental blocks
151 were performed for each cross combination, using two unique isofemale lines from each population.
152 Within each experimental block, a minimum of 8 biological replicates were carried out for each
153 combination of first and second male matings.

154 For each first male pairing, mating behavior was directly observed for 3 hours, and copulation
155 latency (time to start of copulation) and duration (time from start to end of copulation) were recorded.
156 Following the 3-hour observation period, pairs were maintained together for an additional 21 hours, and
157 vials were checked 7 days later for larvae to determine if mating occurred within first 24 hours but
158 outside the initial 3-hour observation window. This allowed us to assess whether female population
159 origin influences mating behavior in the first male mating, and whether this varied according to male
160 population identity. For each second male pairing, female mating behavior was assessed in terms of
161 copulation latency and mating duration within the first 3 hours of pairing. This allowed us to evaluate
162 whether females vary their remating behavior in response to the population and/or species identity of
163 their first mate, in addition to whether these responses differed in females from allopatric versus
164 sympatric sites. Finally, differences among isofemale lines in overall propensity to remate following
165 conspecific first matings, was used to confirm that there was heritable genetic variation for this trait
166 within *D. pseudoobscura* (Results). Detailed mating procedures are provided in Supplementary material.

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

176 To assess whether detected remating differences could be explained by differences in sperm
177 usage and depletion between different cross types, we tracked progeny production of 2 isofemale lines,

178 one allopatric and one sympatric *D. pseudoobscura* strain, across 7 days. As with all first matings,
179 individual virgin females were mated overnight with either a male from their own population, a *D.*
180 *pseudoobscura* male from a different population, or a *D. persimilis* male. Males were removed after 24
181 hours. We found no significant differences in number of progeny produced from own population males,
182 conspecific males from a different population, or heterospecific males, for either allopatric or sympatric
183 isofemale lines, consistent with a previous study that found no evidence for non-competitive gamete
184 isolation contributing to reproductive isolation in this species pair (Lorch and Servedio 2005). Allopatric
185 females produced an average of 86 progeny, which did not differ based on whether they mated with
186 males from their own population versus different population conspecifics ($\beta = 9.400$; $P = 0.603$) or
187 versus heterospecifics ($\beta = 7.487$; $P = 0.609$). Similarly, there was no difference in the number of progeny
188 produced for sympatric females (77 progeny) when mated with males from their own population versus
189 different population conspecifics ($\beta = 2.667$; $P = 0.913$) or versus heterospecifics ($\beta = 7.667$; $P = 0.702$)

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

200 *Statistics*

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

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

224

225 **Results**

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

227 We confirmed that *D. pseudoobscura* females discriminate against *D. persimilis* males; while
228 almost all conspecific matings were successful (164/168), only 25% of heterospecific pairings resulted in
229 mating (93/369), a significant difference in mating propensity ($\chi^2 = 239.70$; $P < 2.2 \times 10^{-16}$). Logistic
230 regressions similarly indicated that the proportion of heterospecific matings was significantly lower than
231 the proportion of first matings either with males from a different conspecific population ($\beta = 3.5927$; $P =$
232 8.31×10^{-10}) or with males from the females own population ($\beta = 4.0073$; $P = 7.13 \times 10^{-05}$) (Figure 2). There
233 was no difference in the propensity to mate of females paired with males from their own population
234 versus males from different conspecific populations ($\beta = 0.4146$; $P = 0.722$) (Figure 2), indicating that
235 female choice in conspecific first matings was not sensitive to the population origin of the conspecific
236 male.

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

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

249

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

251 If exposure to heterospecifics has resulted in general changes in intrinsic mating behavior,
252 rather than specific responses directed at heterospecific genotypes, allopatric and sympatric females
253 should differ in mating responses to conspecific males. Allopatric and sympatric females did not differ in
254 probability of mating when paired with conspecific males (regardless of their population of origin) ($\beta = -$
255 17.22 ; $P = 0.994$). However, females from sympatric populations took significantly longer to initiate
256 copulation with conspecific males than did allopatric females, despite persistent courtship by males ($\beta =$
257 -0.2693 ; $P = 0.0096$). When we simultaneously tested for an effect of sympatry and for the population of
258 origin of the conspecific male, mating latency did not differ according to the specific population of the
259 conspecific male ($\beta = 0.0584$; $P = 0.6717$) but the difference between sympatry and allopatry remained
260 ($\beta = -0.2720$; $P = 0.0076$). In other words, sympatric females are slower to initiate copulation,
261 regardless of the population identity of the first conspecific male (i.e. own versus other conspecific
262 population) with which they are paired. It is possible that this is a subtle behavioral response to past
263 selection imposed by heterospecifics: if female *D. pseudoobscura* in sympatry have adapted to
264 encountering heterospecific males, they might be more circumspect in their initial mating decisions in
265 general. This longer latency might contribute to fewer accidental heterospecific matings, especially
266 under less restrictive conditions than those imposed by our lab co-housing experiment.

267

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

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

270 To evaluate whether *D. pseudoobscura* females differed in their readiness to remate depending
271 on the identity of the first male they mated with, we compared the frequency of remating and the
272 copulation latency in remating trials following three classes of first mating: with conspecific males from
273 their own population, with conspecific males from a different population, or with heterospecific males.
274 We found that analyses of both mating probability and latency to copulation indicate that remating
275 happens more readily when females first mate with familiar (own population) males, than when initially
276 mated with unfamiliar conspecifics or with heterospecifics. In terms of remating probability, females
277 initially mated to their own population males were significantly more likely to remate compared with
278 females initially mated to a *D. persimilis* male ($\beta = -0.98291$; $P = 0.00555$), although the probability of
279 remating did not differ significantly between females previously mated with conspecific males from
280 their own population versus from a different conspecific population ($\beta = -0.55603$; $P = 0.10471$). In terms
281 of latency to copulation, females first mated with their own male remated more quickly (had shorter
282 latency) than females initially mated with either conspecifics from different populations ($\beta = -0.5213$; $P =$
283 0.02195) or heterospecifics ($\beta = -0.8035$; $P = 0.000526$). (Although trending in this direction, copulation
284 latency was not significantly shorter in females initially mated with conspecifics from a different
285 population versus with heterospecific males (i.e., the confidence intervals on β coefficients overlap).)
286 These observations also indicate there is no generalized female *D. pseudoobscura* response to increase
287 remating following heterospecific first matings.

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

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

299

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

301 To investigate whether allopatric versus sympatric females differ in their intrinsic propensity to remate,
302 we compared remating probability and latency between allopatric and sympatric females that had first
303 mated with conspecifics; we found that they did not differ in their probability of remating ($\beta = 0.1586$, P
304 $= 0.6569$) or in their latency to copulate in remating trials ($\beta = 0.1616$, $P = 0.5840$). When we
305 simultaneously tested for an effect of sympatry and for the population of origin of the first mated
306 conspecific male, allopatric and sympatric females still did not differ in remating latency ($\beta = 0.0961$, $P =$
307 0.7437); however, we did detect a first male population effect, such that remating occurred more
308 rapidly when females had mated first with a conspecific from their own population ($\beta = -0.4975$; $P =$
309 0.0239). This is consistent with our findings that females overall mate quickest following own-male first
310 matings (above). Sympatric and allopatric females did not differ in remating latency following own male
311 matings ($\beta = -0.3492$, $P = 0.1570$).

312

313 **Discussion**

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

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

329 Given that there is genetic variation for *D. pseudoobscura* female remating behavior (Results),
330 one potential explanation for our findings is that selection on remating behavior is insufficiently strong
331 or consistent to elicit a substantial evolutionary response. That is, if females are only infrequently
332 exposed to the consequences of completed heterospecific matings, then selection on traits that mitigate
333 these consequences could be relatively weak. In our study, only ~14% of *D. pseudoobscura* females
334 mated with *D. persimilis* males within 3 hours of enforced co-housing, and *D. pseudoobscura* females do
335 not produce fewer progeny when mating with heterospecific males (see methods, and Lorch and
336 Servedio 2005). In comparison, in Matute's (2010) study that detected enhanced remating in sympatric
337 *D. yakuba* females, ~30% of *D. yakuba* females mated with a *D. santomea* male within a 1 hour
338 observation period (Matute 2010, Table S4) and females produce fewer progeny in heterospecific
339 crosses, potentially contributing to the different outcomes of that study and our data here. This

340 relatively high first mating rate between *D. yakuba* females and *D. santomea* males should impose
341 stronger selection on sympatric *D. yakuba* to evolve remating habits that reduce the negative effects of
342 heterospecific matings. Alternatively, because female receptivity is also known to be influenced by the
343 number of sperm in storage (the 'sperm effect'; Manning 1962, 1967), *D. yakuba* sympatric females
344 might remate more rapidly because they experience more acute sperm depletion following
345 heterospecific matings (as inferred in Matute 2010), rather than the because of past reinforcing
346 selection for higher remating in response to suboptimal (interspecies) matings. It is difficult to
347 disentangle these two hypotheses without information on remating rates with conspecific males
348 (remating in *D. yakuba* was examined only after heterospecific matings).

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

357 Intriguingly, our observations of remating behavior are consistent with these outcomes of local
358 co-evolution due to sexual antagonism. We found that females mated previously to male conspecifics of
359 their own population remated significantly more quickly and/or more frequently than females
360 previously mated with conspecific males from a different population or with heterospecifics; remating
361 was least frequent after mating with heterospecific males. These observations suggest that increased
362 sexual familiarity results in females better able to combat male post-copulatory manipulation
363 ('molecular coercion'; Parker and Partridge 1998) via the seminal fluid in ejaculate. A similar pattern has

364 been previously observed in Bean Weevils, in which matings involving increasingly more distantly
365 related first males resulted in increasingly reduced rates of female remating; first matings with
366 heterospecific males elicited the greatest post-copulatory egg production and the lowest re-mating rate
367 (Fricke et al. 2006). In both cases, females appear to be more able to resist suppression of remating by
368 local males in comparison to unfamiliar males.

369 Nonetheless, it should be noted that while our data are consistent with one scenario of sexual
370 conflict, patterns of remating that result from sexual conflict dynamics can be complex, and are not
371 necessarily consistently predictable from population or species crosses (Tregenza et al. 2006). In
372 particular, previous work indicates that the outcome of interactions with less familiar males is
373 dependent on which sex is “ahead” in any particular instance (Long et al. 2006, Tregenza et al. 2006).
374 Our findings are consistent with females being “behind” relative to foreign males, but “ahead” of local
375 males, however alternative patterns can be detected in these kinds of comparisons. For example, Long
376 et al. 2006 performed crosses between six sister laboratory populations that had been isolated for 600+
377 generations and found within-population variation among females in whether they performed better or
378 worse following mating with foreign males compared to local males; they concluded this was due to
379 segregating variation for whether the female was ‘ahead’ or ‘behind’ the specific male genotype to
380 which she was mated. These and other studies indicate that potentially more complex patterns can
381 equally be consistent with conflict scenarios, compared to the one we infer from our observations.
382 Other interpretations of our finding are also possible. For example, the patterns we observed could be
383 due to cryptic female choice for foreign or rare male sperm. This would be especially curious in
384 heterospecific matings, as hybrid inviability makes it strongly disadvantageous for females to
385 preferentially choose sperm from heterospecific males, and for this reason we favor the sexual conflict
386 interpretation. Regardless, our data are clearly inconsistent with reinforcement shaping responses in
387 this trait.

388 In addition to examining remating traits, our experimental design allowed us to reassess
389 evidence of reinforcement in first matings involving these populations. As with a parallel larger study
390 with many of the same isofemale lines (Castillo and Moyle 2016), we found no evidence for
391 reinforcement in first mating between our populations. Discrimination against heterospecific males was
392 not stronger in historically sympatric females, the most straightforward expectation of a response to
393 reinforcing selection. This is curious, as previous studies have detected significantly stronger sexual
394 isolation in sympatric *D. pseudoobscura* females (Noor 1995; Noor and Ortiz-Barrientos 2006). At least
395 two factors could potentially contribute to our observed differences. First, sympatric populations might
396 be polymorphic for high discrimination alleles (as suggested in Barnwell et al. 2008), and we happened
397 to use lines that discriminate differently compared to previous studies. Second, Anderson and Kim
398 (2005, 2006) have argued that gene flow among *D. pseudoobscura* populations has contributed to
399 homogenizing mating discrimination traits between allopatric and sympatric sites. Interestingly, the
400 range of mean heterospecific mating rates for sympatric isofemale lines is broad in both our analysis and
401 in Noor's (1995) study (range=0.22-0.52, 0.16-0.37, respectively). In addition, the sympatric lines in our
402 study have somewhat higher heterospecific mating rates (mean=0.346) compared to Noor's (1995)
403 sympatric lines (mean = 0.252), whereas our allopatric lines mated with heterospecifics at considerably
404 lower rates than Noor's (mean=0.319 versus 0.45 in Noor 1995). These differences suggest indirect
405 evidence that genetic polymorphism within sympatric populations and gene flow/homogenization
406 between *D. pseudoobscura* populations might both contribute to differences between our findings and
407 those in Noor (1995).

408 Regardless of these observations for first matings, our primary analysis of remating suggests
409 that factors such as local sexual coevolution could act counter to reinforcing selection. Even when
410 advantageous for females to manipulate the genetic identity of offspring via remating—such as
411 following matings with heterospecific males—our results suggest that behavioral manipulation of

412 females by male seminal proteins could supersede this response. It has been broadly recognized that
413 sexually antagonistic coevolution and reproductive character displacement can interfere with each
414 other, producing sub-optimal outcomes for one or both processes. Interestingly, this potential tension
415 between intraspecific and interspecific sexual interactions is more often described in terms of
416 reproductive character displacement hampering optimal outcomes of intraspecific sexual selection,
417 rather than the reverse (Ortiz-Barrientos et al. 2009, Pfennig and Pfennig 2012). Here we infer that
418 intraspecific sexual dynamics might instead overwhelm the action of reinforcing selection, producing
419 complex outcomes for remating behaviors within and between species.

420 **Acknowledgements**

421 We would like to thank M. Noor, A. Hish, and N. Phadnis for providing strains used in this experiment,
422 and Donn Castillo for help with collecting strains. Collections were completed with assistance from IU
423 Biology Department travel awards to DMC. Research was supported by Indiana University Dept. of
424 Biology funding to LCM, DMC and JSD.

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427 **References**

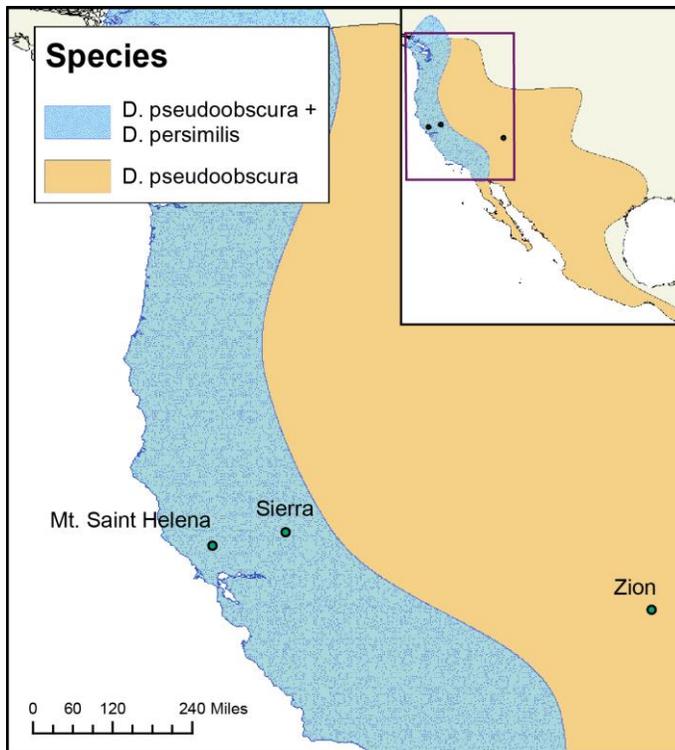
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535 Figure 1: Collection locations for *Drosophila pseudoobscura* and *D. persimilis* study populations. Mt.

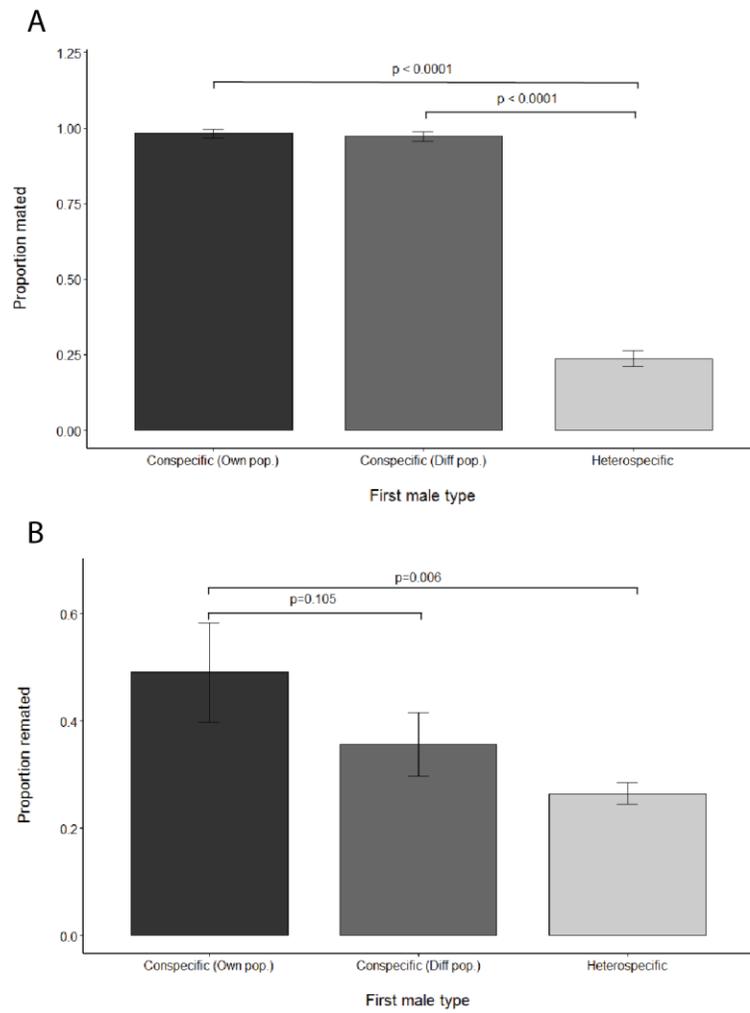
536 Saint Helena and Sierra are sympatric locations (both species); Zion is an allopatric site (*D.*

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

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

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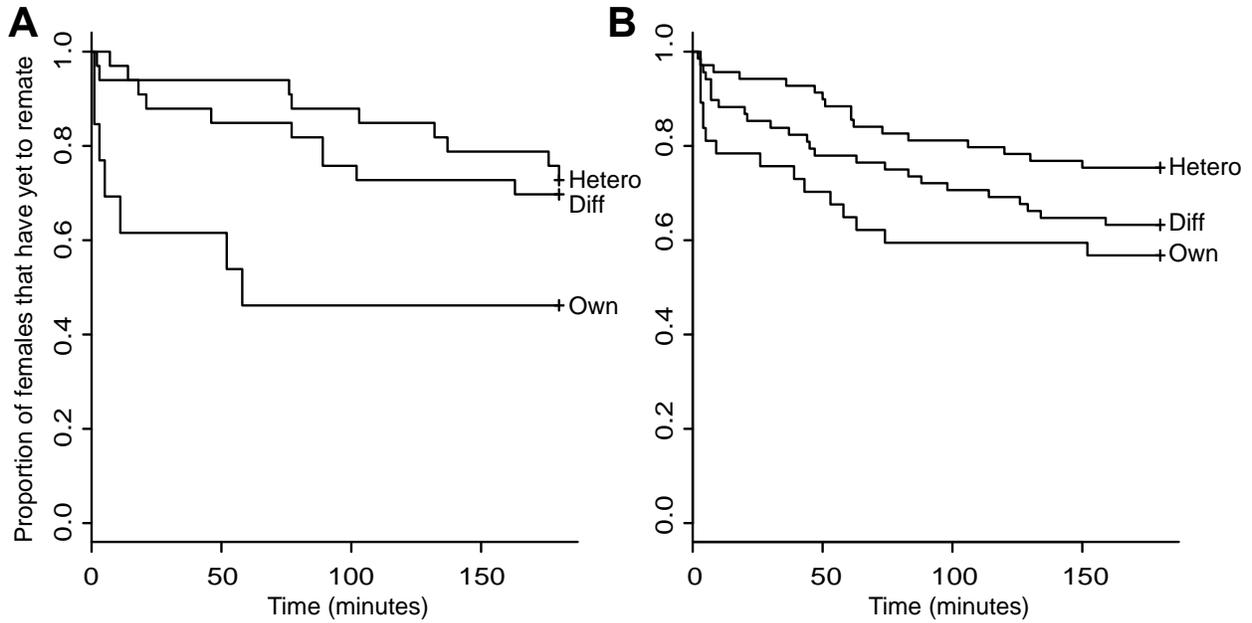


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542 Figure 2: Mating (panel A) and remating (panel B) probabilities of *D. pseudoobscura* females following
543 first matings with males from their own population (Own pop), a different conspecific population (Diff
544 pop), and heterospecific males.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

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

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

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

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