

1 **Coevolution of male and female reproductive traits drive cascading**  
2 **reinforcement in *Drosophila yakuba***

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25

26 **ABSTRACT**

27

28           When the ranges of two hybridizing species overlap, individuals may ‘waste’ gametes on  
29 inviable or infertile hybrids. In these cases, selection against maladaptive hybridization can lead  
30 to the evolution of enhanced reproductive isolation in a process called reinforcement. On the  
31 slopes of the African island of São Tomé, *Drosophila yakuba* and its endemic sister species *D.*  
32 *santomea* have a well-defined hybrid zone. *Drosophila yakuba* females from within this zone  
33 show increased postmating-prezygotic isolation towards *D. santomea* males when compared  
34 with *D. yakuba* females from allopatric populations. To understand why reinforced gametic  
35 isolation is confined to areas of secondary contact and has not spread throughout the entire *D.*  
36 *yakuba* geographic range, we studied the costs of reinforcement in *D. yakuba* using a  
37 combination of natural collections and experimental evolution. We found that *D. yakuba* males  
38 from sympatric populations sire fewer progeny than allopatric males when mated to allopatric *D.*  
39 *yakuba* females. Our results suggest that the correlated evolution of male and female  
40 reproductive traits in sympatric *D. yakuba* have associated costs (i.e., reduced male fertility) that  
41 prevent the alleles responsible for enhanced isolation from spreading outside the hybrid zone.

42

## 43 INTRODUCTION

44

45 Reinforcement is the evolutionary process through which prezygotic reproductive isolation  
46 is strengthened by natural selection acting against the production of maladapted, infertile, or  
47 inviable hybrids (Dobzhansky 1937, Coyne and Orr 2004, Pfennig and Pfennig 2012).

48 Reinforcement is expected to drive the evolution of prezygotic isolation in regions where two  
49 closely related species overlap and hybridize (Dobzhansky 1937, Coyne and Orr 2004). This  
50 generates a pattern of “reproductive character displacement,” in which individuals of different  
51 species found in the same area (sympatry) display greater behavioral isolation from one another  
52 than individuals from different areas (allopatry; Brown and Wilson 1956, Rice and Pfennig  
53 2008, Pfennig and Pfennig 2009). Reinforcement is abundant and can be found in a wide range  
54 of taxonomic groups, including fungi (Anderson et al. 1980, Turner et al. 2010, Murphy and Zeyl  
55 2015), animals (Noor 1995, Gerhardt 1994, Pfennig 1998, Rundle and Schluter 1998, Higgie et  
56 al. 2000, Haavie et al. 2004, Jaenike et al. 2006), and plants (Kay and Schemske 2008, Hopkins  
57 and Rauscher 2011, 2012, Hopkins 2013). Therefore, reinforcement could be common during the  
58 ‘completion’ of speciation (Hudson and Price 2014).

59 Data describing specific aspects of the process of reinforcement are becoming  
60 widespread: we now have precise measurements of the strength of reinforcing selection (Hopkins  
61 et al. 2014), know that reinforcement can occur despite gene flow (Sanderson 1989, Servedio  
62 2000, Nosil et al. 2003 Matute 2010b), and have evidence that reinforcement can promote  
63 divergence within species, fostering speciation (Liou and Price 1994, Nosil 2005, Pfennig and  
64 Rice 2014, Nosil and Hohenloe 2012). Still, important aspects of reinforcement remain  
65 unexplored. Particularly we lack information regarding how variation in reproductive isolation is  
66 maintained across a species’ geographic range. The persistence of different levels of  
67 reproductive isolation between populations of a single species in the face of gene flow and  
68 stabilizing selection (Sanderson 1989, Servedio and Kirkpatrick 1997, Servedio and Noor 2003)  
69 poses a conundrum. One possible explanation is that reinforced reproductive isolation (RRI)  
70 favors phenotypes that are selected against in allopatry. In this case, the traits and alleles  
71 involved in reinforcement would not spread across a species’ geographic range but would remain

72 confined to areas of secondary contact (Walker 1974, Caisse and Antonovics 1978, Howard  
73 1993, Pfennig and Pfennig 2009).

74         Recent work suggests that phenotypes favored by reinforcing selection in sympatry are  
75 often disadvantageous in allopatry (Higgie and Blows 2008; Lemmon 2009; Hopkins et al. 2014;  
76 Pfennig and Rice 2014; Kozak et al. 2015). These studies provide evidence that reinforcing  
77 selection acting in sympatry can affect levels of reproductive isolation observed among  
78 conspecific populations: initiating the evolution of reproductive isolation and speciation through  
79 a process referred to as ‘cascading reinforcement’ (Howard 1993; Lemmon 2009; Kozak et al.  
80 2015). To date, all reported cases of cascading reinforcement or of fitness costs associated with  
81 phenotypes involved in RRI come from studies in which premating reproductive isolating  
82 mechanisms are reinforced. For premating reproductive traits, it may be difficult to determine  
83 whether reproductive character displacement in sympatry results from indirect selection against  
84 the production of hybrids or direct selection on traits involved in species recognition (Shaw and  
85 Mendelson 2013).

86         In addition to traits involved in premating isolation, reinforcing selection can act on post-  
87 mating traits (Coyne 1974, e.g., gametic isolation: Lee et al. 1995, Wullschleger et al. 2002,  
88 Springer and Crespi 2007, Turner et al. 2010, Matute 2010 reviewed in Palumbi 2008).  
89 Examples of post-mating traits include proteins involved in interactions between reproductive  
90 tracts and gametes (reviewed in Palumbi 2008). Postmating-prezygotic phenomena have  
91 physiological and behavioral consequences that can influence both female and male fitness  
92 (Pitnick et al 1991, Yapici et al. 1998, Knowles et al. 2004, Markow and O’Grady 2008).  
93 Coevolution of these traits can be rapid when there is sexual conflict: ejaculate traits that directly  
94 increase male fitness can have deleterious effects on female fitness (Bateman 1948, Fowler and  
95 Partridge 1989, Chapman et al. 1995, Arnqvist and Rowe 2002, Wigby et al. 2005, Wigby et al.  
96 2009). Previous experiments in *Drosophila* have demonstrated ongoing coevolution between the  
97 female reproductive tract and male ejaculate (Rice 1996, Holland and Rice 1999, Knowles and  
98 Markow 2001, Bono et al. 2011, Kelleher et al. 2011, Manier et al. 2013). Traits associated with  
99 the female reproductive tract and male ejaculate can therefore become finely tuned within  
100 populations (through continual antagonistic coevolution) but not necessarily across

101 subpopulations within a species (Rice and Hostert 1993, Rice 1996, Manier et al. 2013). This  
102 provides a hypothetical mechanism for how RRI that evolves in sympatry could have cascading  
103 effects on levels of reproductive isolation among conspecific populations.

104 In this study we use the drosophilid flies *Drosophila yakuba* and *D. santomea* to explore  
105 the evolutionary dynamics of reinforcement in *D. yakuba*. *Drosophila yakuba* is a human-  
106 commensal species that is widespread throughout sub-Saharan Africa and has extended its range  
107 to islands in the Gulf of Guinea. On the volcanic island of São Tomé (off the coast of Cameroon  
108 and Gabon), *D. yakuba* occurs at low elevations (below 1,450 m), and is mostly found in open  
109 and semi-dry habitats commonly associated with human settlements (Lemeunier et al. 1986,  
110 Matute et al. 2009). In contrast, *D. santomea*, the sister species of *D. yakuba*, is endemic to the  
111 highlands of São Tomé where it is thought to exclusively breed on endemic figs (*Ficus*  
112 *chlamydocarpa fernandesiana*; Lachaise et al. 2000, Llopart et al. 2005a, b). The two species  
113 come into secondary contact and hybridize in the midlands of the mountain Pico de São Tomé  
114 within a well-defined hybrid zone (Llopart et al. 2005a, b). *Drosophila yakuba* females from this  
115 hybrid zone show higher postmating-prezygotic isolation toward males of *D. santomea* than do  
116 *D. yakuba* females from outside the hybrid zone (Matute 2010a), via an unknown mechanism  
117 acting within the female reproductive tract (Matute 2010a). There is no indication of RRI in *D.*  
118 *santomea*, even though some genetic variance seems to segregate in this species (Matute 2010b).

119 The RRI observed in sympatric *D. yakuba* can also evolve rapidly in experimental  
120 populations derived from allopatric lines (Matute 2010a, b). Matute (2010a, b) imposed strong  
121 selection against hybrids in experimental populations where *D. yakuba* and *D. santomea* were  
122 maintained in sympatry and both behavioral and gametic isolation increase in less than ten  
123 generations. These results show that the genetic variation required for behavioral and gametic  
124 isolation to evolve is present in allopatric populations. Experimental evolution can thus allow us  
125 to test factors that affect the reinforcement of reproductive isolation.

126 Here, we aim to determine whether *D. yakuba* lines that show RRI in female gametic  
127 isolation evolve correlated phenotypes in males, as would be predicted under a scenario of  
128 coevolution between female and male traits. In particular, we studied whether male fertility  
129 varies across an altitudinal transect spanning the range of *D. yakuba* on Pico de São Tomé. We

130 show that natural lines of *D. yakuba* where females have RRI towards *D. santomea* males also  
131 have lower average male fertility in conspecific matings. Additionally, we show that when  
132 enhanced gametic isolation evolves after experimental sympatry with *D. santomea*, lower male  
133 fertility also evolves as a correlated trait. For *D. yakuba* females, enhanced postmating-  
134 prezygotic isolation from *D. santomea* males is advantageous in the hybrid zone, but our results  
135 suggest that lower male fertility outside of areas of potential interbreeding with *D. santomea* is a  
136 correlated cost of reinforced gametic isolation.

## 137 **METHODS**

138

### 139 **Characterization of the hybrid zone**

140 We collected males from the *yakuba* species subcomplex (*D. yakuba*, *D. santomea* and  
141 their reciprocal hybrids) in a transect on the north side of the island of São Tomé starting at sea  
142 level and ending at the *D. yakuba*/*D. santomea* hybrid zone at 1,200m above the sea level. The  
143 collection sites are shown in Supplementary Figure 1. We sampled *Drosophila* males from 17  
144 localities as described in Matute (2015). We counted the number of *D. santomea*, *D. yakuba*, and  
145 both types of reciprocal F<sub>1</sub> hybrids. To quantify levels of hybridization at each locality, we  
146 qualitatively scored the abdominal pigmentation of all the collected individuals that belonged to  
147 the *yakuba* subcomplex of species. We focused only on male flies, as they are much easier to  
148 identify than females. *Drosophila yakuba* males have a dark abdomen, while *D. santomea* males  
149 have a yellow abdomen. F<sub>1</sub> hybrid males from the ♀ *D. yakuba* × ♂ *D. santomea* cross have an  
150 intermediate abdominal pigmentation that is different from pure *D. yakuba* in the A3 and A4  
151 segments (Llopart et al. 2002). F<sub>1</sub> hybrid males from the reciprocal ♀ *D. santomea* × ♂ *D.*  
152 *yakuba* cross have a patch of brown pigmentation in the A2 and A3 segments (Llopart et al.  
153 2002). Counting F<sub>1</sub> males constitutes a conservative estimate of the production of hybrids as it  
154 does not aim to identify individuals from advanced intercrosses.

155

### 156 **Stocks**

157 In addition to collecting hybrid males, we established 344 isofemale lines from putative  
158 *D. yakuba* females collected from 17 different locations along the transect. Although *Drosophila*  
159 *yakuba*, *D. santomea*, and their hybrids have different colored abdomens, the differences  
160 between hybrid females and pure species are subtle (Llopart et al. 2002) and abdomen  
161 pigmentation varies within *D. yakuba* (Matute and Harris 2013). Therefore, we did not use  
162 abdominal pigmentation to distinguish pure species from hybrid females. Instead, we took  
163 advantage of the fact that pure species *D. yakuba* females produce only sterile males when  
164 crossed to *D. santomea* males (Lachaise et al. 2000) to determine if the collected females, and  
165 thus the isofemale lines, were pure species *D. yakuba* or advanced generation *D. yakuba*

166 backcrosses. If the crossed females had ‘pure’ *D. yakuba* genotypes, then they would produce  
167 100% fertile progeny when crossed with *D. yakuba* and 100% sterile progeny when crossed to *D.*  
168 *santomea*. On the other hand if putatively pure *D. yakuba* were in fact of recent hybrid origin,  
169 they would produce sterile male progeny when crossed to either parental (e.g. ~90% if the female  
170 is a F<sub>1</sub> hybrid; see SI for details). Of the 344 collected isofemale lines, we were able to  
171 successfully establish 297 isofemale stocks that fulfilled the criteria of being considered ‘pure’  
172 *D. yakuba* (SI; Supplementary Table 1).

173 For experimental evolution assays (see below), we generated genetically heterogeneous  
174 strains of each species (i.e., synthetic lines) by combining virgin males and females from several  
175 isofemale lines collected on São Tomé outside of the hybrid zone (i.e., all lines were from  
176 allopatric populations). The synthetic *D. santomea* line (*D. santomea* SYN 2005) was generated  
177 by J.A. Coyne by combining six isofemale lines collected in 2005 at the field station Bom  
178 Sucesso (elevation 1,150 m). The synthetic *D. yakuba* line (*D. yakuba* SYN2005) was generated  
179 by combining five isofemale lines collected by J.A. Coyne in 2005 on Pico de São Tomé  
180 (elevation 880m). All stocks were kept in large numbers after they were created. All rearing was  
181 done on standard cornmeal/Karo/agar medium at 24°C under a 12 h light/dark cycle.

182

### 183 **Male fertility: sympatry versus allopatry**

184 We quantified levels of male fertility by counting the number of progeny produced  
185 following crosses between females and males of 20 isofemale lines (all pairwise combinations).  
186 To ensure that these lines were unambiguously sympatric or allopatric ten were collected at the  
187 low elevation end of the transect described above (low elevation = allopatric lines) while the  
188 other 10 were collected in the hybrid zone (high elevation = sympatric lines). These 20 lines  
189 showed no evidence for adaptation to different temperatures (temperature covaries with  
190 elevation) or the evolution of reproductive isolating mechanisms apart from those described  
191 below (see SI for details). Moreover, these lines show only moderate genetic differentiation  
192 (median  $F_{ST} = 0.0503$ ) and no evidence of large chromosomal differences (see SI for details).  
193 These results indicate that allopatric and sympatric lines differ primarily in whether they  
194 occurred in the absence or presence of *D. santomea*.

195 For each of the twenty lines, we collected virgin males and females under CO<sub>2</sub> anesthesia  
196 and kept them in isolation for 3 days in single-sex groups of 20 flies. On day 4, we conducted  
197 no-choice mating trials as previously described (Coyne et al. 2002, Matute and Coyne 2010).  
198 Briefly, we combined a single female and a single male, observed whether the pair mated and, if  
199 so, recorded copulation latency and copulation duration. Females that showed an abnormally  
200 short copulation (< 20 minutes) were discarded as no sperm transfer occurs before that time  
201 (Chang 2004). After 1 hour, we ended the observations and discarded any females that had not  
202 mated. To prevent females from remating, males were removed from each vial by aspiration after  
203 mating was finished. Each mated female was allowed to oviposit for 24 h. We then transferred  
204 the female to a fresh vial and counted the total number of eggs laid. The counting was repeated  
205 daily for 10 days. We scored 10 males per cross for a total of 4,000 males. Crosses were  
206 classified as being one of six possible types: ♀ allopatric × ♂ sympatric, ♀ sympatric ×  
207 ♂ allopatric, ♀ allopatric × ♂ allopatric (different isofemale lines), ♀ sympatric × ♂ sympatric  
208 (different isofemale lines), ♀ allopatric × ♂ allopatric (within isofemale lines), and ♀ sympatric  
209 × ♂ sympatric (within isofemale lines).

210 We analyzed the data using a generalized linear mixed effect model (GLMM) in which  
211 the number of eggs laid by a female (fertility) was the response, the cross type was the fixed  
212 effect (the six cross types defined above), and the identity of the isofemale lines were random  
213 effects ( $female_{line}$  and  $male_{line}$ ). We used the R package ‘lme4’ (function ‘glmer’, Bates et al.  
214 2011) and fitted the model, assuming Poisson distributed error, with the form:

$$fertility \sim cross\ type_i + (1|female_{line}) + (1|male_{line}) + error_i$$

216 To determine whether the type of cross explained a significant proportion of variation in fertility,  
217 we compared the proportion of residual deviance explained by the model described above to one  
218 lacking the fixed effect of cross type using two methods: 1) a likelihood ratio test (‘lrtest’  
219 function of the ‘lme4’ R package) and 2) parametric bootstrapping (‘PBmodcomp’ function in  
220 the ‘pbkrtest’ R package, Halekoh and Højsgaard 2014). Parametric bootstrapping was carried  
221 out using 100 bootstrapped samples. We also used pairwise Tukey contrasts (‘glht’ and ‘linfct’  
222

223 functions of the ‘multcomp’ R package) to determine the types of crosses, if any, differed in  
224 level of fertility.

225

## 226 **Geographic distribution of gametic isolation and male fertility**

227

### 228 *i) Female gametic isolation from D. santomea*

229

230 When mated to *D. santomea* males, sympatric *D. yakuba* females (i.e., from the hybrid  
231 zone) lay fewer eggs than allopatric females (Matute 2010a). We quantified the geographic scale  
232 of female gametic isolation in *D. yakuba* isofemale lines collected along the altitudinal transect  
233 across São Tomé. We used ten lines from each of ten collection locations for a total of 100  
234 isofemale lines. Lines were chosen randomly from the 297 pure *D. yakuba* lines and are listed in  
235 Supplementary Table 1. We collected virgin *D. yakuba* females from each of these 100 isofemale  
236 lines and let them age to four days (as described above) and then mated them to *D. santomea*  
237 SYN2005 males (heterospecific cross). Mated females were kept, and the number of eggs they  
238 produced was scored every 24 hours over the course of ten days. In parallel, we mated *D. yakuba*  
239 females from each isofemale line to males from the same isofemale line and counted the number  
240 of eggs produced by each female (conspecific cross). Heterospecific and conspecific pairings  
241 were monitored in parallel to ensure that mating occurred under the same environmental  
242 conditions. For each line, we scored the number of eggs produced by fifteen females in both  
243 heterospecific and conspecific matings ( $N = 15 \text{ females} \times 100 \text{ lines} = 1,500 \text{ females}$  for each type  
244 of mating). The proportion of eggs produced after heterospecific matings relative to conspecific  
245 matings was taken as an inverse proxy for the magnitude of gametic isolation (i.e., the more eggs  
246 produced after a single heterospecific mating the weaker the gametic isolation; Chang 2004).

247 To analyze whether there were differences in the strength of gametic isolation between  
248 isofemale lines collected from sympatric versus allopatric regions, we fitted GLMMs with  
249 Poisson distributed error using the “glmer” function in the “lme4” R package. We treated the  
250 number of eggs produced in heterospecific and conspecific crosses as separate data sets. The two  
251 GLMMs were therefore constructed as:

252

$$eggs_{Het} \sim presence \setminus absence D. santomea_i + (1|isofemale_{line}) + error_i$$

253

254

and

255

$$eggs_{Con} \sim presence \setminus absence D. santomea_i + (1|isofemale_{line}) + error_i$$

256

257 We assessed significance of the presence \ absence of *D. santomea* on the number of eggs

258 produced using both maximum likelihood tests (LRT, 1 degree of freedom) and parametric

259 bootstrapping (100 bootstrap samples, function ‘PBmodcomp’ in the ‘pbkrtest’ R package;

260 Halekoh and Højsgaard 2014) that compared the two models to two ‘null’ models that lacked the

261 fixed effect of presence \ absence of *D. santomea*.

262

263 *ii) Intraspecific male fertility*

264

265 We next quantified male fertility along the altitudinal transect using the same 100 *D.*

266 *yakuba* isofemale lines used to study female gametic isolation. We crossed males from each of

267 these lines with conspecific females from two tester stocks: one allopatric and one sympatric.

268 *Drosophila yakuba* Täi18 (hereafter referred to as allopatric<sub>Täi18</sub>), is an allopatric isofemale line,

269 collected in the Täi forest on the border between Liberia and Ivory Coast. BOSU1250.5

270 (hereafter referred to as sympatric<sub>BOSU1250.5</sub>) is a *D. yakuba* line collected in 2005 at the heart of

271 the São Tomé hybrid zone and is considered sympatric. We collected virgin males and when they

272 were 4 days old, allowed them to mate to virgin females from either tester stock following the

273 mating procedure described above (no-choice trials). The number of eggs produced over ten days

274 was assessed as a proxy for male fertility with females from different populations. Crosses with

275 allopatric<sub>Täi18</sub> and sympatric<sub>BOSU1250.5</sub> were considered different datasets. Each dataset was

276 analyzed by fitting a GLMM with Poisson distributed error where the number of eggs produced

277 per cross was the response, the origin of the male (whether the isofemale line is sympatric or

278 allopatric) was the fixed effect, and isofemale line was a random effect:

279

$$fertility \sim presence \setminus absence D. santomea_i + (1|isofemale_{line}) + error_i$$

280

281 For both ‘allopatric’ and ‘sympatric’ data sets, the model described above was compared  
282 with a model without the fixed effect of presence \ absence of *D. santomea* using both maximum  
283 likelihood tests (LRT, 1 degree of freedom) and parametric bootstrapping (100 bootstrap  
284 samples, function ‘PBmodcomp’ in the ‘pbkrtest’ R package; Halekoh and Højsgaard 2014).

285

## 286 **Correlation between gametic isolation and male fertility**

287

288 To determine whether the average magnitude of gametic isolation from *D. santomea*  
289 males and average male fertility per isofemale line were correlated across isofemale lines, we  
290 used Spearman’s rank correlation tests implemented in the ‘stats’ package in R (function  
291 ‘cor.test’). Bonferroni correlation tests gave similar results.

292

## 293 **Experimental Sympatry**

294

295 Previous work has shown that strong selection against hybridization can drive the  
296 evolution of enhanced gametic isolation in female *D. yakuba* that are kept in experimental  
297 sympatry with *D. santomea* (Matute 2010a,b). If reinforced gametic isolation and reduced male  
298 fertility are correlated traits, they should evolve together in experimental sympatry. We took an  
299 experimental evolution approach to test this hypothesis and establish whether lower male fertility  
300 between allopatric and sympatric populations of *D. yakuba* could evolve as a correlated trait with  
301 enhanced gametic isolation from *D. santomea* observed in females.

302

303 We kept 23 populations (bottles) of *D. yakuba*, originally derived from a synthetic  
304 allopatric population (*D. yakuba* SYN2005) in experimental sympatry with *D. santomea* (*D.*  
305 *santomea* SYN2005) for ten generations following previously described experimental protocols  
306 (Koopman 1950, Higgin et al. 2000, Blows and Higgin 2002, Matute 2010a). Each bottle  
306 contained 250 *D. yakuba* females, 250 *D. yakuba* males, 250 *D. santomea* females, and 250 *D.*

307 *santomea* males. To set up each successive generation, we collected 250 flies of each sex of *D.*  
308 *yakuba* (identifiable by their abdominal pigmentation) as virgins from the experimental bottles  
309 and transferred them into new bottles. To reconstitute sympatric conditions, 250 *D. santomea*  
310 flies of each sex (collected as virgins from stock bottles) were added to each bottle. All flies were  
311 collected between seven and ten hours after eclosion once the flies had already achieved their  
312 adult pigmentation. Hybrids were recognized by their abdominal pigmentation (intermediate –  
313 yet different – between the two parental species) and were discarded. This procedure was  
314 followed for ten generations. Twenty-three control populations (i.e. bottles) of *D. yakuba* were  
315 maintained in parallel with the same number of conspecifics (500 flies per bottle) but in the  
316 absence of *D. santomea*. The maintenance conditions and population size of *D. yakuba* were the  
317 same between the experimental sympatry and control bottles. The strength of gametic isolation  
318 was measured after ten generations of experimental sympatry using methods described  
319 previously (see “Geographic distribution of gametic isolation and male fertility” above).

320 We compared levels of female gametic isolation from *D. santomea*, male fertility with  
321 sympatric<sup>BOSU1250.5</sup> females, and male fertility with allopatric<sup>Tai18</sup> females after 10 generations of  
322 experimental sympatry. We fitted generalized linear models (GLMs) with Poisson distributed  
323 error (i.e., Poisson regression) in which the magnitude of gametic isolation or male fertility was  
324 the response and the generation (0, or 10) was the fixed effect. To assess the affect of time (i.e.,  
325 generation) on the evolution of gametic isolation or male fertility we used likelihood ratio tests  
326 comparing models including versus excluding this term. Models were fitted using the ‘glm’  
327 function in R. Finally, we used Spearman’s rank correlation to compare whether there was a  
328 correlated response in levels of isolation from *D. santomea* observed in females and isolation  
329 from allopatric *D. yakuba* observed in males across experimental lines.

330

## 331 RESULTS

332

### 333 Characterization of the hybrid zone

334

335 We sampled from across the hybrid zone between *D. santomea* and *D. yakuba* on the  
336 island of Pico de São Tomé to measure the distributions of *D. santomea*, *D. yakuba*, and their  
337 two reciprocal hybrids. The abundances of each of the four genotypes (2 pure species and two F<sub>1</sub>  
338 hybrids) at 17 locations along the altitudinal transect is shown in Figure 1. Hybrids were found in  
339 the same locations as four years prior (Llopart et al. 2005a), suggesting that the hybrid zone has  
340 remained stable since it was first reported. Notably we found a few *D. santomea* males at 700 m  
341 indicating that *D. santomea* males sometimes wander out of the canonical distribution previously  
342 reported for the species.

343

### 344 Male fertility: Sympatry versus allopatry

345

346 We have previously described the reproductive advantage obtained by *D. yakuba* females  
347 that evolved RRI both in nature, and under experimental evolution (Matute 2010a, 2010b). We  
348 hypothesized that there may be an associated cost to RRI that prevents it from spreading to  
349 allopatric populations of *D. yakuba*. We mated males and females of allopatric and sympatric  
350 lines in all 4 possible combinations, and counted how many eggs were produced by each mating  
351 type. We found that males from sympatric areas sire fewer progeny than allopatric males when  
352 mated to allopatric females but not when mated to sympatric females (Figure 2b). Generalized  
353 linear mixed models fitted to the data showed that the type of cross being conducted (the fixed  
354 effect in these models) explained a significant proportion of residual deviance in the number of  
355 eggs produced when compared to a null model lacking this term (LRT:  $\chi^2 = 700.09$ ,  $df = 5$ ,  $P < 1$   
356  $\times 10^{-15}$ ; parametric bootstrapping:  $P = 0.01$ ; Supplementary Table 3). Pairwise comparisons  
357 revealed that the average number of eggs laid in ♂ sympatric x ♀ allopatric crosses (mean =  
358 75.38, standard error [SE] = 0.21) was significantly lower compared to the number of eggs laid  
359 in all other cross types (range of mean [and SE] across other types of cross = 88.79 – 90.23 [0.28  
360 – 0.91]); all  $P < 0.0001$ ; Figure 1; Supplementary Table 4).

361

## 362 **Geographic distribution of gametic isolation and male fertility**

363

364 We characterized the geographic distribution of RRI along an altitudinal transect on Pico  
365 de São Tomé by collecting 10 *D. yakuba* isofemale lines at each of 10 different sites along the  
366 transect (N=100, including the 20 lines we used above) and measuring the strength of gametic  
367 isolation in *D. yakuba* females towards *D. santomea* males. We focused on lines that were  
368 putatively pure *D. yakuba* (rather than lines with potentially admixed ancestry; see SI). We fitted  
369 GLMMs using sympatry with *D. santomea* as a fixed effect and isofemale line as a random  
370 effect. We found that the magnitude of gametic isolation between populations of *D. yakuba* and  
371 *D. santomea* is affected by sympatry with *D. santomea* (LRT:  $\chi^2 = 54.37$ ,  $df = 1$ ,  $P = 1.66 \times 10^{-13}$ ;  
372 parametric bootstrapping:  $P = 0.0099$ ; Figure 3a, black boxes), conforming the expectation  
373 that enhanced gametic isolation is driven by maladaptive hybridization with *D. santomea*.  
374 Female fertility in conspecific matings also differed between allopatric and sympatric lines  
375 (LRT:  $\chi^2 = 86.97$ ,  $df = 1$ ,  $P < 1 \times 10^{-15}$ ; parametric bootstrapping:  $P = 0.0099$ ; Figure 3a, grey  
376 boxes); however, the magnitude of the difference in mean number of eggs produced between  
377 sympatric and allopatric *D. yakuba* females was much lower in conspecific crosses (mean eggs  
378 [SE]: sympatric females = 86.70 [0.17]; allopatric females = 93.19 [0.18]) when compared to  
379 heterospecific crosses (mean eggs [SE]: sympatric females = 34.60 [0.47]; allopatric females =  
380 63.35 [0.60]).

381 We next asked whether male fertility varied among the same lines collected along the  
382 transect. We measured male fertility by mating males from each isofemale line to either  
383 allopatric<sub>Tai18</sub> females or sympatric<sub>BOSU1250.5</sub> females (N=15 crosses per tester line). These two  
384 lines had previously been identified as representative sympatric and allopatric lines (Matute  
385 2010a). The results from these crosses are shown in Figure 3b. We fitted two GLMMs similar to  
386 those used to study the magnitude of gametic isolation observed in females: one for matings with  
387 sympatric<sub>BOSU1250.5</sub> females and one for matings with allopatric<sub>Tai18</sub> females. Male fertility did not  
388 change between regions of sympatry and allopatry when mated to sympatric<sub>BOSU1250.5</sub> females  
389 (LRT:  $\chi^2 = 86.97$ ,  $df = 1$ ,  $P = 1 \times 10^{-15}$ ; parametric bootstrapping:  $P = 0.0099$ ; Figure 3b, grey

390 boxes). By contrast, male fertility differed between allopatric and sympatric regions when males  
391 were mated to allopatric<sub>Tai18</sub> females (LRT:  $\chi^2 = 0.2144$ ,  $df = 1$ ,  $P = 0.64$ ; parametric  
392 bootstrapping:  $P = 0.63$ ; Figure 3b, black boxes). The number of eggs produced by allopatric  
393 females suggests that male fertility is lowest in lines that are sympatric with *D. santomea* (Figure  
394 3b) and that the geographic distribution of reduced male fertility on São Tomé mirrors that of  
395 enhanced gametic isolation.

396

### 397 **Correlation between gametic isolation and male fertility**

398

399 We also examined whether the magnitude of female gametic isolation was correlated  
400 with male fertility in crosses made with allopatric<sub>Tai18</sub> and sympatric<sub>BOSU1250.5</sub> females. We  
401 evaluated the correlation between the average strength of gametic isolation and average male  
402 fertility when mated with allopatric and sympatric females using 25 females and 25 males per  
403 isofemale line. Female gametic isolation was correlated with male fertility with allopatric<sub>Tai18</sub>  
404 females (Spearman's  $\rho = 0.885$ ,  $P < 1 \times 10^{-10}$ ; Figure 4a) but not with sympatric<sub>BOSU1250.5</sub>  
405 females (Spearman's  $\rho = 0.116$ ,  $P = 0.250$ ; Figure 4b). These results illustrate that female  
406 gametic isolation toward heterospecifics in sympatry might evolve at the cost of reduced male  
407 fertility in conspecific allopatric matings.

408

### 409 **Experimental Sympatry**

410

411 Previous experiments have shown that female *D. yakuba* can evolve increased gametic  
412 isolation from *D. santomea* males when evolved under sympatric conditions (Matute 2010a, b).  
413 We repeated this experiment and again found that *D. yakuba* females laid fewer eggs when  
414 mated to heterospecific *D. santomea* males following 10 generations of sympatry than were laid  
415 at generation 0 (Poisson GLM, LRT:  $\chi^2 = 2767.7$ ;  $df = 1$ ;  $P < 1 \times 10^{-10}$ , Figure 5a). The number of  
416 eggs laid following conspecific matings remained the same (LRT:  $\chi^2 = 2.43$ ;  $df = 1$ ;  $P = 0.12$ ;  
417 Figure 5a). We next looked at whether reduced conspecific male fertility with both allopatric<sub>Tai18</sub>  
418 and sympatric<sub>BOSU1250.5</sub> *D. yakuba* evolved as a correlated trait with increased female gametic

419 isolation from *D. santomea*. We found no difference in male fertility among the experimentally  
420 evolved lines when mated to sympatric<sub>BOSU1250.5</sub> females (LRT:  $\chi^2 = 1.06$ ;  $df = 1$ ;  $P = 0.304$ ;  
421 Figure 5b). However, male fertility in matings with allopatric<sub>Tai18</sub> females showed a significant  
422 decrease over ten generations of experimental sympatry (LRT:  $\chi^2 = 743.9$ ;  $df = 1$ ;  $P < 1 \times 10^{-10}$ ,  
423 Figure 5b). In control populations of *D. yakuba* raised in parallel to the experimental populations  
424 but with *D. santomea* absent, we observed no change in female gametic isolation (LRT:  $\chi^2 =$   
425  $0.54$ ;  $df = 1$ ;  $P = 0.462$ ), or male fertility (LRT:  $\chi^2 = 0.89$ ;  $df = 1$ ;  $P = 0.347$ ) over the same 10  
426 generations of evolution. Finally, we observed a significant correlation between levels of female  
427 gametic isolation from *D. santomea* and male fertility with allopatric<sub>Tai18</sub> females across  
428 experimental replicates (Spearman's  $\rho = 0.69$ ;  $P < 1 \times 10^{-10}$ ; Figure 5c) but not with male  
429 fertility in matings with sympatric<sub>BOSU1250.5</sub> females (Spearman's  $\rho = -0.046$ ,  $P = 0.115$ ). These  
430 results provide experimental evidence that when gametic isolation from *D. santomea* males  
431 evolves via reinforcing selection acting on *D. yakuba* females, there is a correlated cost of  
432 postmating prezygotic incompatibility between sympatric males and conspecific females from  
433 allopatric populations.

434

435

436

437

438 **DISCUSSION**

439

440 We have used observation and experimental evidence to show that the reinforcement of  
441 gametic isolation between *D. yakuba* females and *D. santomea* leads to the correlated evolution  
442 of intraspecific postmating prezygotic isolation as the results of reduced conspecific male  
443 fertility. Our results suggest that there is ‘fine-tuning’ between male and female reproductive  
444 traits within populations of *D. yakuba*. Furthermore, we describe a cost associated with RRI in  
445 regions of sympatry with *D. santomea*: when sympatric *D. yakuba* males mate with allopatric *D.*  
446 *yakuba* females, they suffer from decreased fertility. Our finding provides a potential causal  
447 mechanism for the localized effect of reinforcing selection on reproductive isolation: alleles that  
448 underlie female traits that are adaptive in sympatry are associated with alleles that underlie male  
449 traits that are maladaptive in geographic regions outside of hybrid zones.

450 Although enhanced gametic isolation in females is highly beneficial in the presence of *D.*  
451 *santomea* (by reducing maladaptive hybridization) it is associated with conditionally lower male  
452 fertility. This correlated cost, coupled with the fact that enhanced gametic isolation provides no  
453 fitness benefit outside the hybrid zone, likely hampers the spread of the alleles underlying  
454 enhanced RRI into allopatry. Our results shed light on why the observed reinforced gametic  
455 isolation in the *D. yakuba/D. santomea* hybrid zone is confined to areas of secondary contact.  
456 Together, these results provide evidence for cascading reinforcement among populations of *D.*  
457 *yakuba* on the island of São Tomé.

458

459 Alternate explanations for observed patterns of reproductive isolation:

460 Reinforced reproductive isolation is one means by which natural selection can lead to the  
461 completion of speciation (Dobzhansky 1937, Lukhtanov et al 1995, Coyne and Orr 2004).  
462 However, it has remained unclear how the signature of reinforcement in hybrid zones persists  
463 despite migration between allopatric and sympatric populations (Walker 1974, Liou and Price  
464 1994, Servedio and Noor 2003). Strong phenotypic differentiation between sympatric and  
465 allopatric populations can be generated through a variety of processes that do not involve  
466 selection against ‘reinforcing’ alleles in allopatric regions. For example, local adaptation to

467 different environments, geographical structuring of populations, drift, and/or genetic  
468 incompatibilities could all contribute to reductions in gene flow among conspecific populations  
469 and lead to the incidental evolution of reproductive isolation. For the populations and lines of *D.*  
470 *yakuba* we focused on in this study, we addressed these issues and found it unlikely that  
471 phenotypic differentiation between allopatric and sympatric populations results from *i*) local  
472 adaptation to temperature, *ii*) forms of reproductive isolation other than male fertility, *iii*) strong  
473 genetic differentiation among populations at alternate ends of the altitudinal transect, or *iv*)  
474 chromosomal inversions limiting genomic admixture between demes (Hoffman et al. 2004,  
475 Kirkpatrick and Barton 2006) (see SI for details). These results are not surprising given that the  
476 collection sites for the hybrid zone and the allopatric areas are separated by less than 10 km; a  
477 distance some *Drosophila* species can travel overnight (Jones et al. 1981, Coyne et al. 1982,  
478 Coyne and Milstead 1987 but see Timofeef-Ressovsky and Timofeef-Ressovsky 1940; reviewed  
479 in Powell 1997). The fact that we observe some non-zero genetic differentiation among  
480 isofemale lines derived from females sample from opposite ends of the altitudinal transect  
481 suggests that reinforcing selection acting in sympatry – and selection acting in allopatry – could  
482 help drive genetic differentiation among conspecific populations: a supposition that warrants  
483 further investigation.

484

485 Fitness tradeoffs and the cost of reinforcement:

486       The reduced fertility we observed when sympatric males mated to allopatric females  
487 represents a fitness tradeoff between selection acting in regions where *D. yakuba* occurs in  
488 sympatry with *D. santomea* and selection acting in allopatry. Reduced male fertility also implies  
489 indirect costs to females with enhanced gametic isolation in sympatry because their sons will be  
490 less fit than those of allopatric females. The tradeoffs could explain why reinforced gametic  
491 isolation has not spread throughout the whole geographic range of *D. yakuba* on São Tomé. Our  
492 results add to the growing list of examples of traits favored through reinforcement in regions of  
493 sympatry but selected against in allopatry (Lemmon 2009; Hopkins and Rausher 2014; Pfennig  
494 and Rice 2014; Kozak et al. 2015). We do not mean to imply that reduced male fertility is the  
495 only cost associated with enhanced gametic isolation in *D. yakuba* females from the hybrid zone.

496 It is possible that unexplored traits also hamper the spread of enhanced gametic isolation  
497 between sympatric and allopatric regions.

498

499 Coevolution of female and male reproductive traits:

500 At the phenotypic level, our findings highlight the tight link between the female  
501 reproductive tract and male ejaculate. In the case of *D. yakuba* we present here, male  
502 performance depends on the genotype of his mate (Figures 2, 3, 4 and 5). The evolution of  
503 gametic isolation towards *D. santomea* in female *D. yakuba* leads to a co-evolutionary change in  
504 male traits (manifested as reduced fertility with allopatric conspecifics). This result suggests that  
505 there is selectable genetic variation for female and male reproductive traits segregating within  
506 natural populations; a result seen in previous studies (Pitnick and Miller 2000, Miller and Pitnick  
507 2002). A model of coevolution between male and female reproductive traits in *D. yakuba* would  
508 suggest that reinforcing selection can trigger changes in female traits that reduce the production  
509 of maladaptive heterospecific offspring, which in turn leads to coevolutionary changes in  
510 conspecific male traits.

511 Several lines of evidence indicate that the traits involved in the interactions between the  
512 ejaculate and the female reproductive tract are constantly diverging both between and within  
513 species (Manier et al. 2013). First, comparative studies have revealed correlated evolution of  
514 sperm and female reproductive tract morphology in *Drosophila* (Pitnick et al. 1997, 1999).  
515 Specifically, across the whole genus *Drosophila*, sperm length (among other sperm traits) is  
516 highly variable (Joly and Bressac 1994), and there is a strong positive relationship across species  
517 between the length of sperm and the length of the seminal receptacle, the main sperm-storage  
518 organ in the female (Pitnick et al. 1999). Furthermore, experimental evolution of *D.*  
519 *melanogaster* in the laboratory has revealed that increased seminal receptacle length can drive  
520 the evolution of increased sperm length (Pitnick and Miller 2000, Miller and Pitnick 2002). Our  
521 combination of comparative and experimental results suggest that coevolution between the sexes  
522 can drive correlated evolution between sperm and female reproductive tract traits, with evolution  
523 in female traits resulting in selection on corresponding traits in males.

524 At the molecular scale, components of the seminal fluid (such as the accessory gland  
525 proteins, Acps), are known to evolve faster than the rest of the genome (Ram and Wolfner 2007).  
526 Approximately 36% of Acps shared among members of the *D. melanogaster* subgroup (*D.*  
527 *melanogaster*, *D. simulans*, *D. sechellia*, *D. erecta*, and *D. yakuba*) appear to have evolved under  
528 the influence of positive selection (Swanson et al. 2001a; Mueller et al. 2005; Haerty et al. 2007).  
529 Additional studies have shown that Acps evolve rapidly within species (Begun et al. 2000; Kern  
530 et al. 2004; Begun and Lindfors 2005; Schully and Hellberg 2006). Such accelerated rates of  
531 molecular evolution are not unique to male proteins but have also been observed in genes related  
532 to fertilization and female receptors (Andres and Anrqvist 2001, Swanson et al. 2004, Lawniczak  
533 and Begun 2007, Chow et al. 2010). These results suggest that Acps and female receptors, the  
534 molecular underpinnings of male × female post-mating interactions, are prone to rapid evolution  
535 and species harbor sufficient genetic variation in these traits for natural and sexual selection to  
536 act upon.

537

538 Caveats and conclusions:

539 Our results come with at least three caveats. First, it is possible that sympatric females are  
540 of hybrid ancestry. We assessed this issue by measuring sterility of offspring when females were  
541 crossed to either *D. yakuba* and *D. santomea* (see SI for details). However, this assay will only  
542 reveal ‘hybrid’ *D. yakuba* lines that contain alleles that are involved in hybrid sterility with *D.*  
543 *santomea*; it is possible that other parts of the genome have admixed ancestry, although it is  
544 unclear how this might affect male fertility.

545 A second caveat comes from the population cage sizes used for experimental evolution. It  
546 has been shown that high population densities reduce fitness and influence behavior in a variety  
547 of organisms (e.g., Booth 1995, Zachar and Neiman 2013, Matute 2014). We chose to have  
548 cages with the same number of *D. yakuba* individuals, to control for levels of genetic variance  
549 between treatments; however, we did not explore the evolution of reproductive isolation at  
550 different experimental densities.

551 Thirdly, it is worth noting that even though reduced male fertility and enhanced gametic  
552 isolation are correlated traits, we have no evidence that these traits have the same genetic basis or

553 that the alleles controlling the two traits are genetically linked. Since female receptivity  $\times$  male  
554 ejaculate interactions are finely tuned, changes in female phenotypes, such as sperm retention,  
555 might cause concomitant changes in male traits related to ejaculate quality without the need to  
556 invoke genetic linkage. Future work using crosses will need to be done to further elucidate this  
557 connection.

558         It has long been argued that the evolution of reproductive isolation could be constrained  
559 by sexual selection, and that pleiotropic effects of reproductive isolation would either accelerate  
560 or hinder speciation (reviewed in Panhuis et al. 2001, Ritchie 2007). Our data demonstrate that  
561 the evolution of reinforcement is not free of associated costs, and that sexual selection might  
562 oppose reinforcing selection during speciation (Ritchie 2007, Safran et al. 2013). Other cases  
563 have been identified in which reinforcing selection leads to the evolution of traits unfavored by  
564 sexual selection (*Spea multiplicata*: Pfennig 1998; *Drosophila serrata*: Higgie et al. 2000).

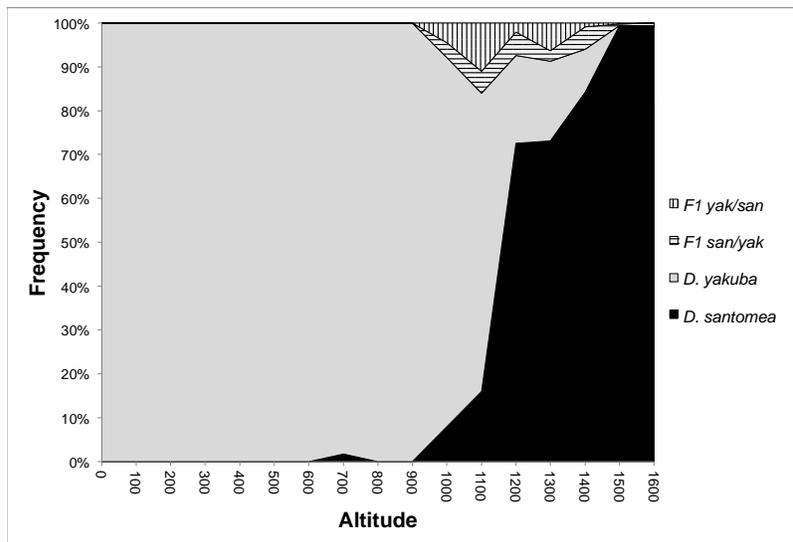
565         Finally, our results contribute to the body of work demonstrating that locally adaptive  
566 traits can have unexpected costs that might hamper their expansion across the entire range of a  
567 species (Littlejohn and Loftus-Hills 1968; Zouros and D'Entremont 1980; Higgie and Blows  
568 2008; Hopkins et al. 2014; Pfennig and Rice 2014; Kozak et al. 2015). When a trait contributes  
569 to reproductive isolation, the pattern of enhanced reproductive isolation in sympatry that is the  
570 hallmark of reinforcement can be explained by selection against the phenotypes involved outside  
571 of the hybrid zone.

572

573 **FIGURES**

574

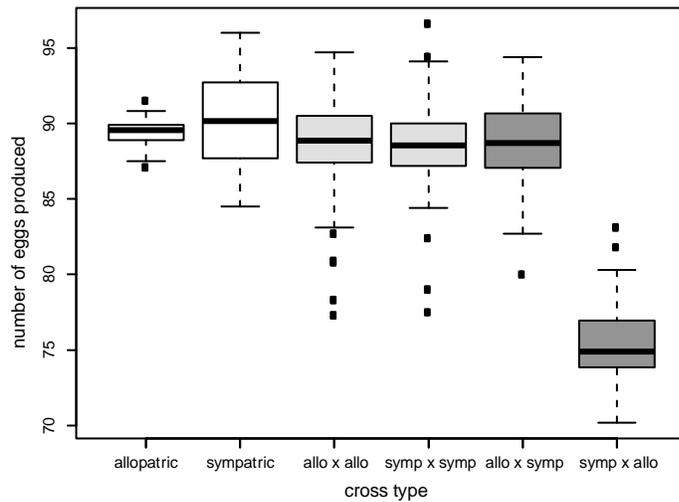
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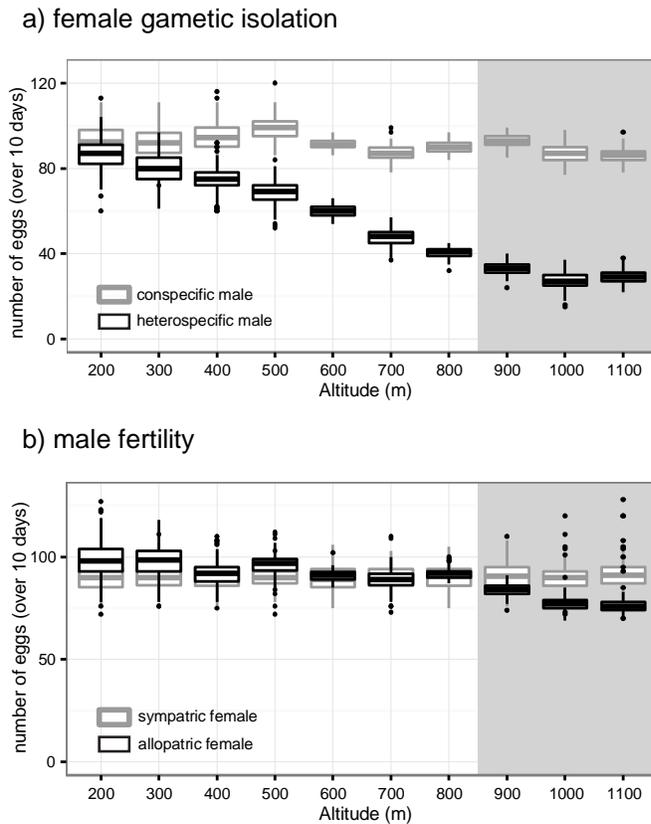
576

577 **FIGURE 1. Abundance of *D. yakuba*, *D. santomea* and their F<sub>1</sub> hybrids along the**  
578 **altitudinal gradient on Pico de São Tomé. Results presented are for F<sub>1</sub> males that were**  
579 **identified based on their abdominal pigmentation.**

580



581  
582 **FIGURE 2. Males from a sympatric population show reduced fertility when mated to**  
583 **allopatric females.** We crossed males and females from 20 isofemale lines in all possible  
584 combinations and counted the number of eggs produced over ten days. Crosses were split into six  
585 categories depending on the identity of the female and the male involved in the cross. The y-axis  
586 shows the average number of eggs produced after 10 days across crosses in each category.  
587 Categories of crosses are (from left to right): males and females from the same allopatric  
588 isofemale line (white box), males and females from the same sympatric isofemale line (white  
589 box), ♂ sympatric × ♀ sympatric from different isofemale lines (light grey), ♂ allopatric × ♀  
590 allopatric from different isofemale lines (light grey), ♂ allopatric × ♀ sympatric lines (dark  
591 grey), and ♂ sympatric × ♀ allopatric lines (dark grey) lines. Sympatric males produced fewer  
592 progeny when mated to allopatric females than any of the other possible crosses.  
593



594

595 **FIGURE 3. Levels of gametic isolation and male fertility across the altitudinal transect.** We

596 measured the levels of gametic isolation and male fertility in 100 lines collected along the

597 altitudinal transect in São Tomé (10 lines per elevation / site). Gray shaded polygons to the right

598 of each panel demarcate the location of the hybrid zone. a) There is no gametic isolation among

599 females when crossed to *D. yakuba* males (conspecific crosses; gray boxes); however, isofemale

600 lines collected from closer to the hybrid zone have lower fertility (i.e. higher gametic isolation)

601 when crossed to *D. santomea* (heterospecific crosses, black boxes). b) Male fertility, measured as

602 the number of eggs produced, is lower for isofemale lines collected from the hybrid zone when

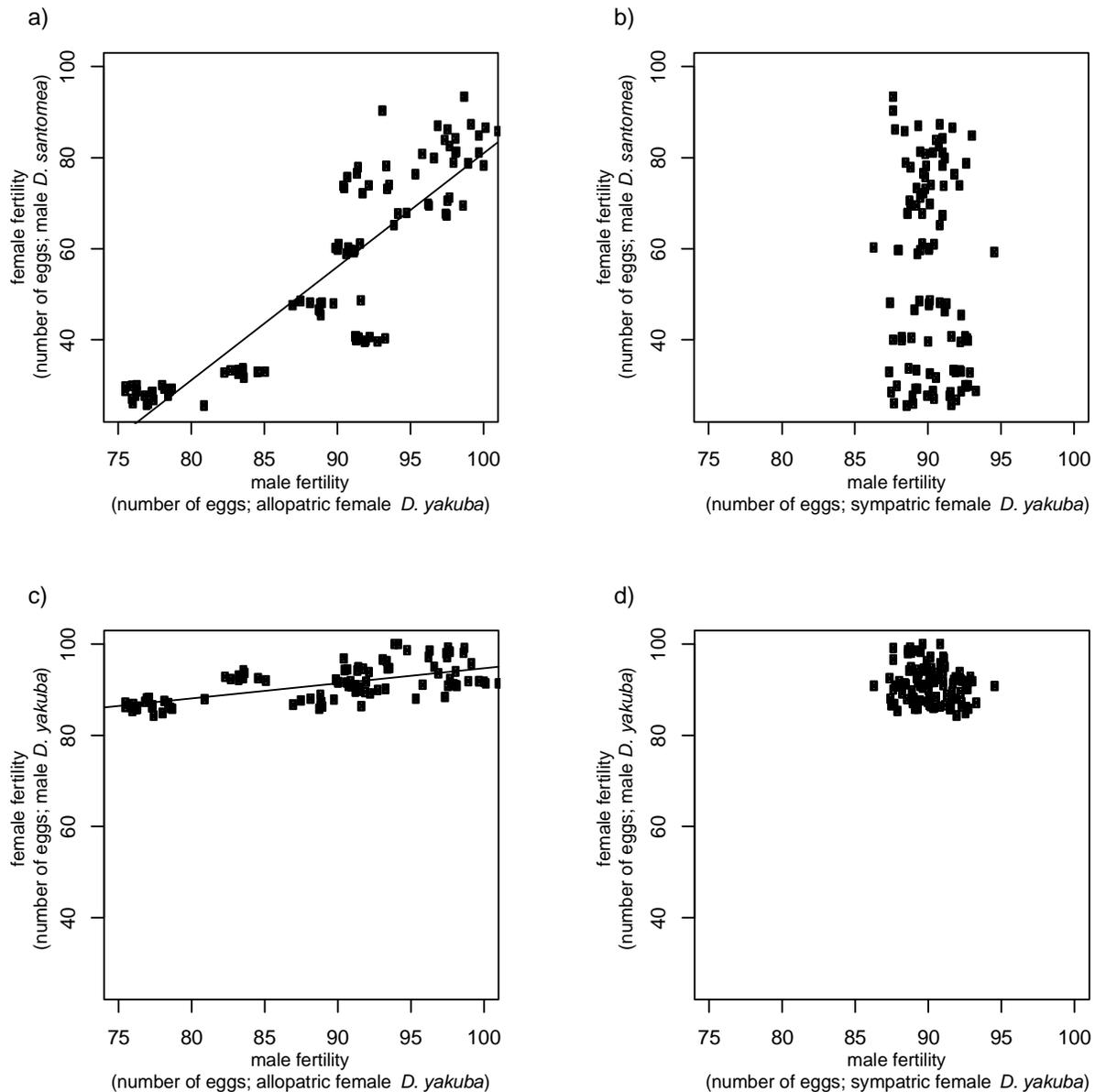
603 mated to female *D. yakuba* from an allopatric isofemale line (black boxes), but not when mated to

604 female *D. yakuba* from a sympatric isofemale line (gray boxes). Males from sympatric

605 populations showed lower fertility when mated to allopatric females, but not when mated to

606 sympatric females.

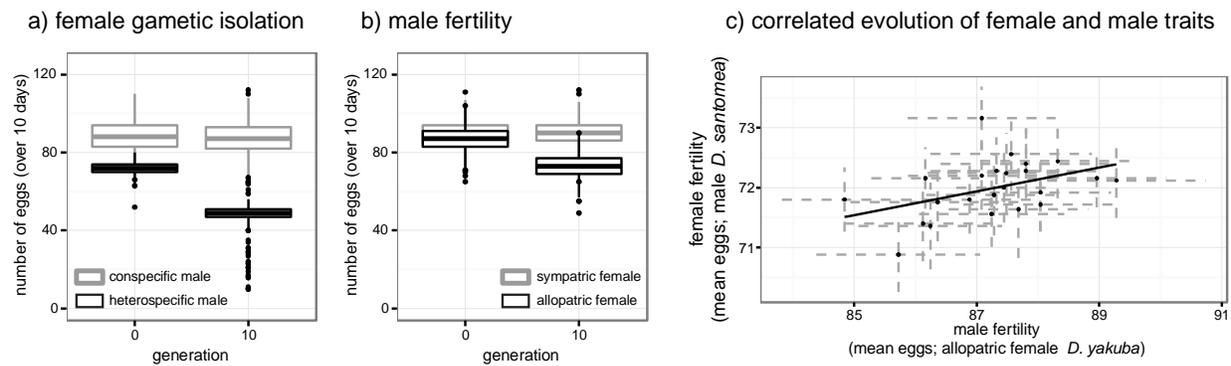
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608

609 **FIGURE 4. Correlations between male fertility and female gametic isolation in natural**  
610 **populations.** a) Male fertility in matings with allopatric females is correlated with the number of  
611 eggs laid by females (female gametic isolation) after heterospecific matings with *D. santomea*  
612 (as an inverse proxy of the strength of gametic isolation) (Spearman's rho = 0.885,  $P < 1 \times 10^{-10}$ ).  
613 b) Male fertility is not correlated female fertility when mated with sympatric females  
614 (Spearman's rho = 0.116,  $P = 0.250$ ). c) The number of eggs laid by females after conspecific

615 matings is also correlated with male fertility in matings with allopatric females (Spearman's rho  
616 = 0.605,  $P < 1 \times 10^{-10}$ ), but not with male fertility in matings with sympatric females (d;  
617 Spearman's rho = -0.149,  $P = 0.140$ ). Lines shown only for significant correlations.  
618



619

620 **FIGURE 5. Experimental evidence that gametic isolation and decreased male fertility**

621 **coevolve after experimental sympatry with *D. santomea*.** a) After 10 generations of

622 experimental sympatry, allopatric *D. yakuba* lines evolved enhanced gametic isolation (black

623 boxes) with no change in conspecific fertility (gray boxes). b) The same evolved lines also

624 showed a decrease in their male fertility when males are mated to an allopatric line of *D. yakuba*

625 (black boxes) but no decrease in their fertility when mated to females from a sympatric line of *D.*

626 *yakuba* (gray boxes). c) Within experimental populations, the degree of female gametic isolation

627 that evolved over 10 generations (“female fertility”; smaller values represent higher levels of

628 isolation) was correlated with levels of male fertility with allopatric *D. yakuba* females. Points in

629 “c)” represent means for each of 23 experimental populations and dashed gray lines represent ±

630 one standard error. Control populations showed no change in gametic isolation from *D.*

631 *santomea*, female fertility with conspecific males, male fertility when males are mated to

632 allopatric *D. yakuba*, or male fertility when mated to sympatric *D. yakuba* (see text for statistics).

633

634

635

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