1   **Coevolution of male and female reproductive traits drive cascading**

2   **reinforcement in *Drosophila yakuba***

3

4   Aaron A. Comeault[1], Aarti Venkat[2] and Daniel R. Matute[1]

5

6   [1]Biology Department, University of North Carolina, Chapel Hill, North Carolina

7   250 Bell Tower Drive, Genome Sciences Building

8   Chapel Hill, NC

9   27510, USA

10

11   [2]Department of Human Genetics

12   The University of Chicago

13   920 East 58th Street

14   Chicago, IL

15   60637, USA.

16

17   Correspondence:

18   Biology Department, University of North Carolina, 250 Bell Tower Road, Chapel Hill, 27599.

19   Tel: 919-962-2077

20   Fax: 919-962-1625

21   E-mail: dmatute@email.unc.edu

22

24   Running title: reinforcement in *Drosophila yakuba*

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 Pfenning

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 drosphilid 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_1 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_1 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_1 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_1 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_1 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 24ºC 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 in the absence or presence of *D. santomea*.

195   For each of the twenty lines, we collected virgin males and females under CO_2 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:

215

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

216

217   To determine whether the type of cross explained a significant proportion of variation in fertility,

218   we compared the proportion of residual deviance explained by the model described above to one

219   lacking the fixed effect of cross type using two methods: 1) a likelihood ratio test ('lrtest'

220   function of the 'lmtest' R package) and 2) parametric bootstrapping ('PBmodcomp' function in

221   the 'pbkrtest' R package, Halekoh and Højsgaard 2014). Parametric bootstrapping was carried

222   out using 100 bootstrapped samples. We also used pairwise Tukey contrasts ('glht' and 'linfct'

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 females × 100 lines = 1,500 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

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

253

254   and

255

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

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$_{Täi18}$), 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$_{BOSU1250.5}$) 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$_{Täi18}$ and sympatric$_{BOSU1250.5}$ were considered different datasets. Each dataset was

276   analyzed by fitting a GLMM with Poison 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

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

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   We kept 23 populations (bottles) of *D. yakuba*, originally derived from a synthetic

303 allopatric population (*D. yakuba* SYN2005) in experimental sympatry with *D. santomea* (*D.*

304 *santomea* SYN2005) for ten generations following previously described experimental protocols

305 (Koopman 1950, Higgie et al. 2000, Blows and Higgie 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$_{BOSU1250.5}$ females, and male fertility with allopatric$_{Täi18}$ 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_1

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

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$_{Täi18}$ females or sympatric$_{BOSU1250.5}$ 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$_{BOSU1250.5}$ females and one for matings with allopatric$_{Täi18}$ females. Male fertility did not

388   change between regions of sympatry and allopatry when mated to sympatric$_{BOSU1250.5}$ 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$_{Täi18}$ 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$_{Täi18}$ and sympatric$_{BOSU1250.5}$ 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$_{Täi18}$

404   females (Spearman's rho = 0.885, $P < 1\times10^{-10}$; Figure 4a) but not with sympatric$_{BOSU1250.5}$

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; $P < 1\times10^{-10}$, 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$_{Täi18}$

418   and sympatric$_{BOSU1250.5}$ *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$_{BOSU1250.5}$ females (LRT: $\chi^2 = 1.06$; df = 1; $P = 0.304$;

421   Figure 5b). However, male fertility in matings with allopatric$_{Täi18}$ females showed a significant

422   decrease over ten generations of experimental sympatry (LRT: $\chi^2 = 743.9$; df = 1; $P < 1\times10^{-10}$,

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$_{Täi18}$ females across

428   experimental replicates (Spearman's rho = 0.69; $P < 1\times10^{-10}$; Figure 5c) but not with male

429   fertility in matings with sympatric$_{BOSU1250.5}$ 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 $\times$ 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
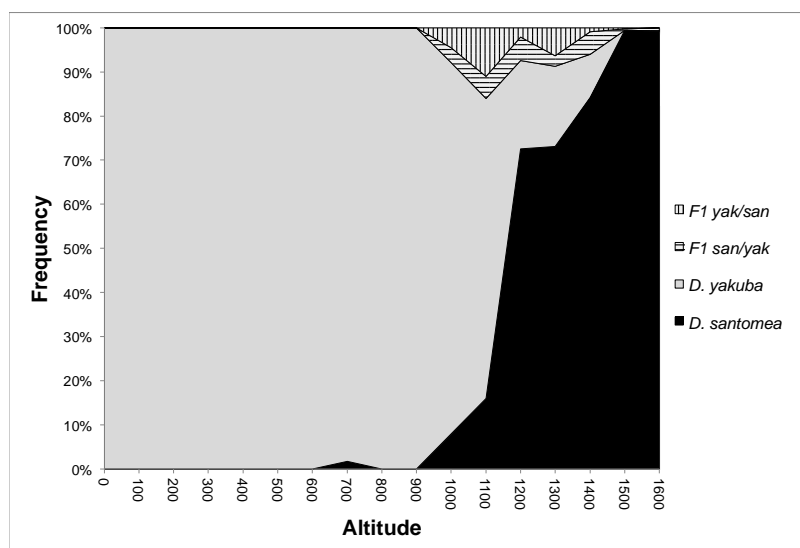
571   of the hybrid zone.

572

573 **FIGURES**

574

575

576

577 **FIGURE 1. Abundance of *D. yakuba*, *D. santomea* and their F_1 hybrids along the**

578 **altitudinal gradient on Pico de São Tomé.** Results presented are for F_1 males that were

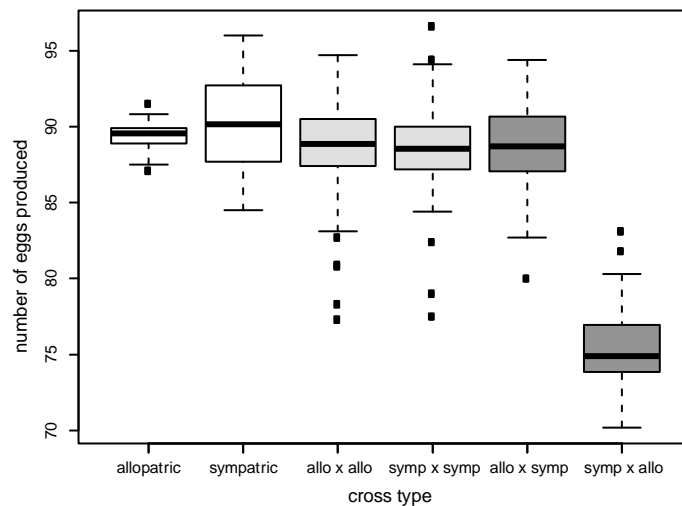579 identified based on their abdominal pigmentation.

580

581

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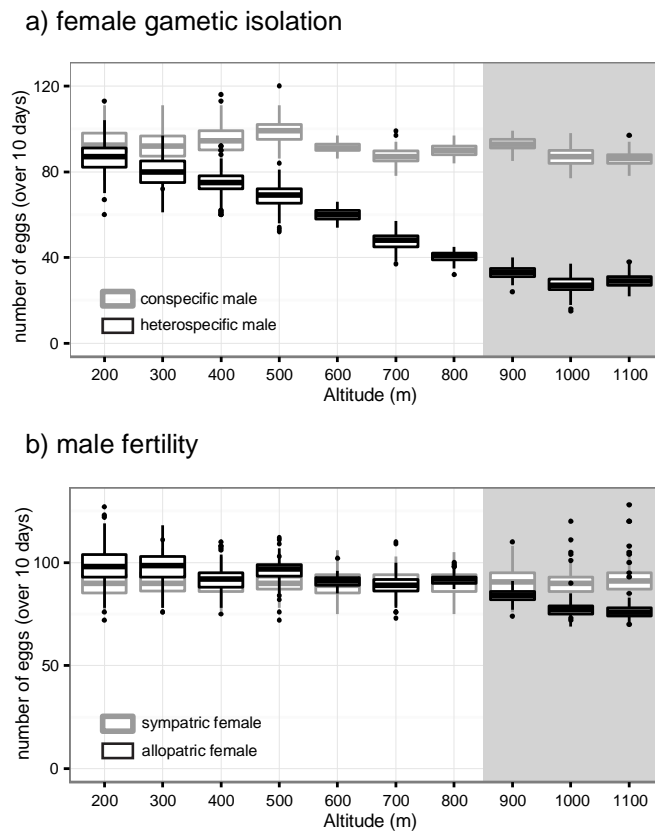
593

594

FIGURE 3. Levels of gametic isolation and male fertility across the altitudinal transect. We measured the levels of gametic isolation and male fertility in 100 lines collected along the altitudinal transect in São Tomé (10 lines per elevation / site). Gray shaded polygons to the right of each panel demarcate the location of the hybrid zone. a) There is no gametic isolation among females when crossed to *D. yakuba* males (conspecific crosses; gray boxes); however, isofemale lines collected from closer to the hybrid zone have lower fertility (i.e. higher gametic isolation) when crossed to *D. santomea* (heterospecific crosses, black boxes). b) Male fertility, measured as the number of eggs produced, is lower for isofemale lines collected from the hybrid zone when mated to female *D. yakuba* from an alloparic isofemale line (black boxes), but not when mated to female *D. yakuba* from a sympatric isofemale line (gray boxes). Males from sympatric populations showed lower fertility when mated to allopatric females, but not when mated to sympatric females.
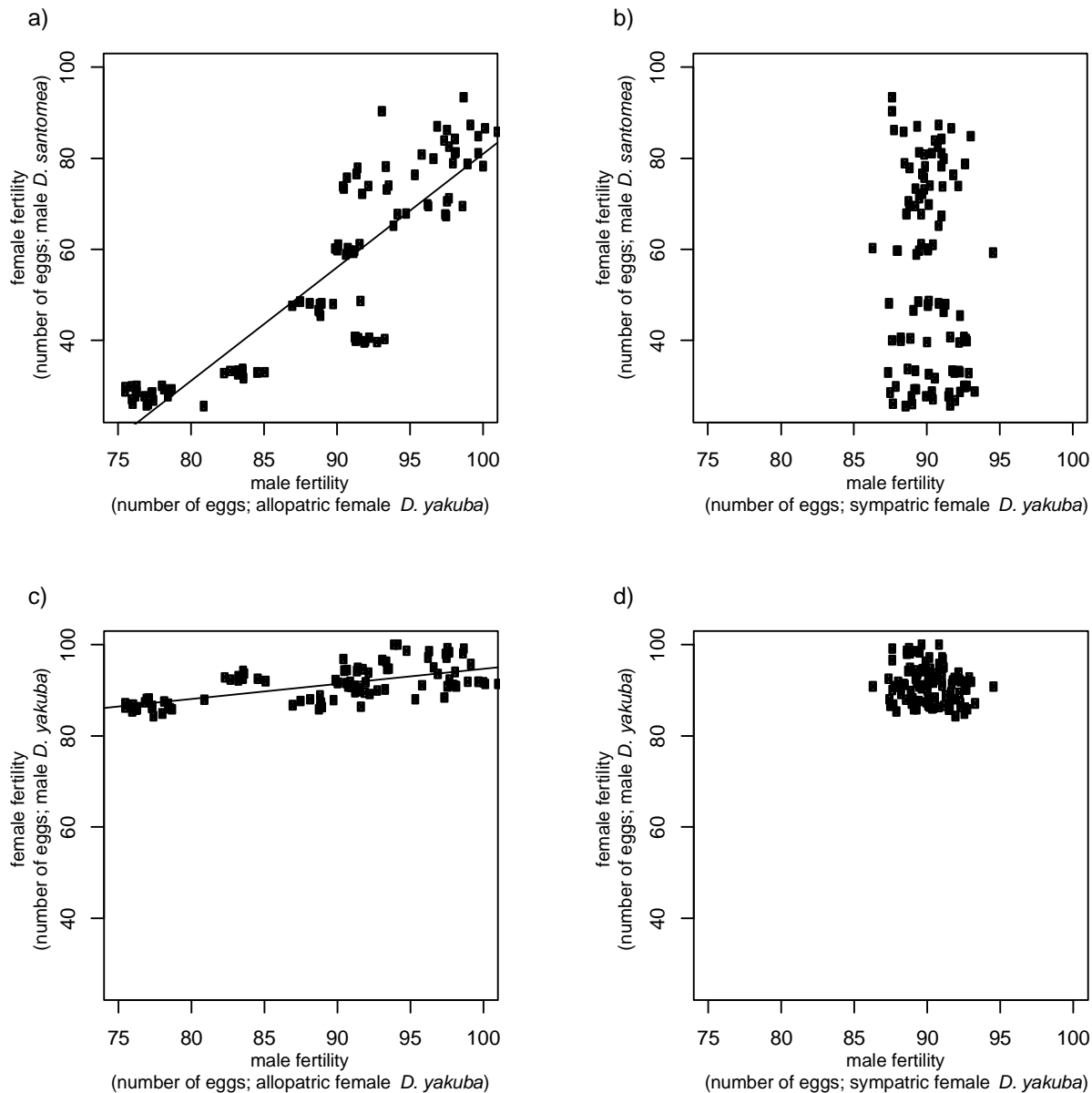
607

608

FIGURE 4. **Correlations between male fertility and female gametic isolation in natural populations.** a) Male fertility in matings with allopatric females is correlated with the number of eggs laid by females (female gametic isolation) after heterospecific matings with *D. santomea* (as an inverse proxy of the strength of gametic isolation) (Spearman's rho = 0.885, P < 1 ×10^{-10}).
b) Male fertility is not correlated female fertility when mated with sympatric females (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 ×10^{-10}), but not with male fertility in matings with sympatric females (d;

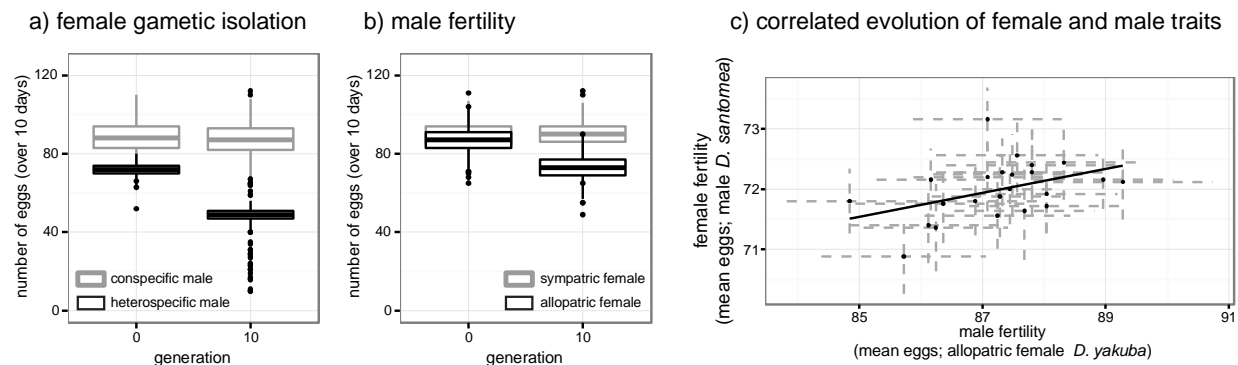617   Spearman's rho = -0.149, P = 0.140). Lines shown only for significant correlations.

618

FIGURE 5. Experimental evidence that gametic isolation and decreased male fertility coevolve after experimental sympatry with *D. santomea*. a) After 10 generations of experimental sympatry, allopatric *D. yakuba* lines evolved enhanced gametic isolation (black boxes) with no change in conspecific fertility (gray boxes). b) The same evolved lines also showed a decrease in their male fertility when males are mated to an allopatric line of *D. yakuba* (black boxes) but no decrease in their fertility when mated to females from a sympatric line of *D. yakuba* (gray boxes). c) Within experimental populations, the degree of female gametic isolation that evolved over 10 generations ("female fertility"; smaller values represent higher levels of isolation) was correlated with levels of male fertility with allopatric *D. yakuba* females. Points in "c)" represent means for each of 23 experimental populations and dashed gray lines represent ± one standard error. Control populations showed no change in gametic isolation from *D. santomea,* female fertility with conspecific males, male fertility when males are mated to allopatric *D. yakuba,* or male fertility when mated to sympatric *D. yakuba* (see text for statistics).

REFERENCES

Anderson J.B., Korhonen K., Ullrich R.C. (1980) Relationships between European and North American biological species of *Armillaria mellea*. *Experimental Mycology* **4**: 87-95.

Andolfatto, P., Davison, D., Erezyilmaz, D., Hu, T.T., Mast, J., Sunayama-Morita, T., Stern D.L. 2011 Multiplexed Shotgun Genotyping for Rapid and Efficient Genetic Mapping. *Genome Res.* **21**: 610-617.

Andrés, J. A. and Arnqvist, G. 2001. Genetic divergence in the seminal signal-receptor system in houseflies: the footprints of sexually antagonistic coevolution? *Proc. R. Soc. B*. **268**: 557-563.

Arnqvist, G. and L. Rowe. 2002. Antagonistic coevolution between the sexes in a group of insects. *Nature* **415**: 787-789.

Barton, N.H., and Turelli, M. 1989. Evolutionary quantitative genetics: How little do we know? *Ann. Rev. Genet*. **23**: 337–370.

Barton, N., and Keightley P.D. 2002. Understanding quantitative genetic variation. *Nat. Rev. Genet*. **3**: 11–20.

Bateman A.J. 1948. Intra-sexual selection in *Drosophila*. *Heredity*, **2**: 349–368.

Bates, D., Maechler, M., and Bolker, B. 2011. lme4: Linear mixed-effects models using S4 classes. R package version 0.999375-39. http://CRAN.R-project.org/package=lme4

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

Begun, D. J., and Lindfors, H. A. 2005. Rapid evolution of genomic *Acp* complement in the *melanogaster* subgroup of *Drosophila*. *Mol. Biol. Evol*. **22**: 2010-2021.

Booth, D. J. 1995. Juvenile groups in a coral-reef damselfish: density-dependent effects on individual fitness and population demography. *Ecology* **76:** 91-106.

Blows, M.W., and Higgie, M. 2002 Evolutionary experiments on mate recognition in the *Drosophila serrata* species complex. *Genetica* **116**: 239–250.

Blows, M.W., and Higgie M. 2003. Genetic constraints on the evolution of mate recognition

665   under natural selection. *Am. Nat.* **161**: 240–253.

666   Blows, M.W, and Hoffmann, A.A. 2005 A reassessment of genetic limits to evolutionary

667   change. *Ecology* **86**: 1371–1384.

668   Bono, J.M., Matzkin, L.M., Kelleher, E.S., and Markow T.A. 2011. Post-mating

669   transcriptional changes in the reproductive tracts of con- and heterospecifically mated

670   *Drosophila mojavensis* females. *Proc. Nat. Acad. Sci. U.S.A.* **108**: 7878-7883.

671   Brown, W. L., and Wilson, E. O. 1956. Character displacement. *Systematic Zoology*, **5**: 49-

672   64.

673   Caisse, M., and Antonovics, J. 1978. Evolution in closely adjacent plant populations. IX.

674   Evolution of reproductive isolation in clinal populations. *Heredity* **40**: 371-384.

675   Chang, A.S. 2004. Conspecific sperm precedence in sister species of *Drosophila* with

676   overlapping ranges. *Evolution* **58**: 781–789.

677   Chapman T., Liddle L.F., Kalb J.M., Wolfner M.F., and Partridge L. 1995. Cost of mating in

678   *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature*

679   **373**: 241–244.

680   Chow, C. Y., Wolfner, M. F., and Clark, A. G. 2010. The genetic basis for male × female

681   interactions underlying variation in reproductive phenotypes of *Drosophila*. *Genetics*

682   **186**: 1355-1365.

683   Coyne, J. A. 1974. The evolutionary origin of hybrid inviability. *Evolution* **28**: 505–506.

684   Coyne, J.A., Boussy, I.A., Prout, T., Bryant, S., Jones, J.S., and Moore J.A. 1982. Long-

685   distance migration in *Drosophila*. *Am. Nat.* **119**: 589–595.

686   Coyne, J.A., and Milstead, B. 1987. Long-distance migration of *Drosophila*. 3. Dispersal of

687   *D. melanogaster* alleles from a Maryland orchard. *Am. Nat*. **130**: 70–82.

688   Coyne, J.A., and Orr, H.A. 1989. Patterns of speciation in *Drosophila*. *Evolution* **43**: 262–

689   281.

690   Coyne, J.A., Kim, S.Y., Chang, A.S., Lachaise, D., and Elwyn, S. 2002. Sexual isolation

691   between two sibling species with overlapping ranges: *Drosophila santomea* and

692   *Drosophila yakuba*. *Evolution* **56**: 2424–2434.

693   Coyne, J.A., and Orr, H.A. 2004. Speciation. Sunderland, Mass., Sinauer Associates.

694   Dobzhansky, T. 1937. Genetics and the Origin of Species. Columbia Univ. Press, New York.

695   Fowler K., and Partridge L. 1989. A cost of mating in female fruitflies. *Nature* **338**: 760–761.

696   Gavin‐Smyth, J., and Matute, D. R. 2013. Embryonic lethality leads to hybrid male

697   inviability in hybrids between *Drosophila melanogaster* and *D. santomea. Ecol. Evol.*,

698   **3**: 1580-1589.

699   Gerhardt, H.C. 1994. Reproductive character displacement of female mate choice in the grey

700   treefrog, *Hyla chrysoscelis. Anim. Behav.* **47**: 959–969.

701   Haavie, J., Borge, T., Bures, S., Garamszegi, L.Z., Lampe, H.M., Moreno, J., Qvarnström,

702   A., Török, J., and Sætre, G.P. 2004. Flycatcher song in allopatry and sympatry–

703   convergence, divergence and reinforcement. *J. Evol. Biol.* **17**: 227–237.

704   Haerty, W., Jagadeeshan, S., Kulathinal, R. J., Wong, A., Ram, K. R., Sirot, L. K., Levesque,

705   L., Artieri, C.G., Wolfner, M.F., Civetta, A. and Singh, R. S. 2007. Evolution in the fast

706   lane: rapidly evolving sex-related genes in *Drosophila. Genetics* **177**: 1321-1335.

707   Halekoh, U., and Højsgaard, S. 2014. A Kenward-Roger Approximation and Parametric

708   Bootstrap Methods for Tests in Linear Mixed Models - The R Package pbkrtest. *J. Stat.*

709   *Softw.* **59**: 1-30.

710   Higgie, M., Chenoweth, S., and Blows, M.W. 2000 Natural selection and the reinforcement

711   of mate recognition. *Science* **290**: 519–520.

712   Higgie, M., and Blows M.W. 2007. Are traits that experience reinforcement also under

713   sexual selection? *Am. Nat.* **170**: 409–420.

714   Higgie, M., and Blows, M.W. 2008. The evolution of reproductive character displacement

715   conflicts with how sexual selection operates within a species. *Evolution* **62**: 1192–1203.

716   Higgie, M., Chenoweth, S., and Blows, M. W. 2000. Natural selection and the reinforcement

717   of mate recognition. *Science* **290**: 519-521.

718   Hine, E., McGuigan, K., and Blows, M.W. 2011. Natural selection stops the evolution of

719   male attractiveness. *Proc. Natl. Acad. Sci. USA* **108**: 3659–3664.

720   Hoffmann, A.A., Sgro, C.M., and Weeks, A.R. 2004. Chromosomal inversion polymorphisms

721   and adaptation. *Trends Ecol. Evol.* **19**: 482-488.

722   Holland, B., and Rice, W. R. 1999. Experimental removal of sexual selection reverses

723   intersexual antagonistic coevolution and removes a reproductive load. *Proc. Natl. Acad.*

724   *Sci. USA* **96**: 5083-5088.

725   Hopkins, R., and Rausher, M.D. 2011. Identification of two genes causing reinforcement in

726   the Texas wildflower, *Phlox drummondii. Nature* **469**: 411-415.

727   Hopkins, R., and Rausher, M. D. 2012. Pollinator-mediated selection on flower color allele

728   drives reinforcement. *Science* **335**: 1090-1092.

729   Hopkins, R. 2013. Reinforcement in plants. *New Phytologist* **197**: 1095–1103.

730   Hopkins, R., Guerrero, R., Rausher, M. D., and Kirkpatrick, M. 2014. Strong Reinforcing

731   Selection in a Texas Wildflower. *Curr. Biol. 24: 1995-1999*

732   Howard, D.J. 1993. Reinforcement: origin, dynamics, and fate of an evolutionary hypothesis,

733   in Hybrid zones and the evolutionary process (Harrison, R. G., ed.), pp.46–69, New

734   York: Oxford University Press.

735   Hudson, R.R., Slatkin, M., and Maddison, W.P. 1992. Estimation of levels of gene flow from

736   DNA sequence data. *Genetics* **132**: 583–589.

737   Hudson, E. J., and Price, T. D. 2014. Pervasive reinforcement and the role of sexual selection

738   in biological speciation. *J. Heredity* **105**: 821-833.

739   Jaenike, J., Dyer, K.A., Cornish, C., and Minhas, M.S. 2006 Asymmetrical reinforcement

740   and *Wolbachia* infection in *Drosophila*. *PLoS Biol* **4**: e325.

741   Joly, D., and Bressac, C. 1994. Sperm length in Drosophilidae (Diptera): estimation by testis

742   and receptacle lengths. *International Journal of Insect Morphology and Embryology* **23**:

743   85-92.

744   Jones, J.S., Bryant, S.H., Lewontin, R.C., Moore, J.A., and Prout, T. 1981. Gene flow and the

745   geographical distribution of a molecular polymorphism in *Drosophila pseudoobscura*.

746   *Genetics* **98**: 157–178.

747   Kay, K.M., and Schemske, D.W. 2008. Natural selection reinforces speciation in a radiation

748   of Neotropical rainforest plants. *Evolution* **62**: 2628-2642.

749   Kelleher, E.S., Clark, N.L. and Markow, T.A. 2011. Female reproductive protease evolution

750   suggests sexual conflict in geographically isolated races of *Drosophila mojavensis*.

751   *Genetics* **187**:865-876.

752   Kern, A. D., Jones, C. D., and Begun, D. J. 2004. Molecular population genetics of male
753   accessory gland proteins in the *Drosophila simulans* complex. *Genetics* **167**: 725-735.

754   Kirkpatrick, M., and Barton, N. 2006. Chromosome inversions, local adaptation, and
755   speciation. *Genetics* **173**: 419–434.

756   Knowles, L.L., Hernandez, B.B., and Markow, T.A. 2004. Exploring the consequences of
757   postmating-prezygotic interactions between the sexes. *Proc. R. Soc. Lond. B* **271**: S357–
758   S359.

759   Koopman, K.F. 1950. Natural selection for reproductive isolation between *Drosophila*
760   *pseudoobscura* and *Drosophila persimilis*. *Evolution* **4**: 135–148.

761   Kozak, G. M., Roland, G., Rankhorn, C., Falater, A., Berdan, E. L., and Fuller, R. C. 2015.
762   Behavioral isolation due to cascade reinforcement in *Lucania* killifish. *Am. Nat.* **185**:
763   491-506.

764   Lachaise, D., Harry, M., Solignac, M., Lemeunier, F., Bénassi, V., and Cariou, M.L. 2000.
765   Evolutionary novelties in islands: *Drosophila santomea*, a new *melanogaster* sister
766   species from São Tomé. *Proc. R. Soc. Lond. B.* **267**: 1487–1495.

767   Lawniczak M.K., and Begun, D.J. 2007. Molecular population genetics of female-expressed
768   mating-induced serine proteases in *Drosophila melanogaster*. *Mol Biol Evol* **24**: 1944–
769   1951.

770   Lee, Y.H., Ota, T., and Vacquier, V.D. 1995. Positive selection is a general phenomenon in
771   the evolution of abalone sperm lysin. *Mol. Biol. Evol.* **12**: 231–238.

772   Lemeunier, F., David, J.R., Tsacas, L., and Ashburner, M. 1986. The *melanogaster* species
773   group, *In* The Genetics and Biology of *Drosophila*, Vol. 3, edited by Ashburner M.,
774   Carson, H.L., Thompson, J.N. Academic Press, New York, NY, USA.

775   Lemmon, E. M. 2009. Diversification of conspecific signals in sympatry: geographic overlap
776   drives multidimensional reproductive character displacement in frogs. *Evolution*
777   63:1155–1170.

778   Lemmon, A. R., Smadja, C., and Kirkpatrick, M. 2004. Reproductive character displacement
779   is not the only possible outcome of reinforcement. *J. Evol. Biol.* **17**: 177-183.

780   Levin, D. A., and Kerster, H. W. 1967. Natural selection for reproductive isolation in *Phlox*.

781   *Evolution* **21**: 679-687.

782   Levin, D. A., and Schaal, B. A. 1970. Corolla color as an inhibitor of interspecific

783   hybridization in *Phlox*. *Am. Nat.* **104**: 273-283.

784   Li, H., and Durbin, R. 2009 Fast and accurate short read alignment with Burrows-Wheeler

785   Transform. *Bioinformatics* **25**: 1754-1760.

786   Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis,

787   G., Durbin, R. and the 1000 Genome Project Data Processing Subgroup. 2009. The

788   Sequence alignment/map (SAM) format and SAMtools. *Bioinformatics* **25**, 2078-2079.

789   Liou, L.W. and Price, T.D. 1994. Speciation by reinforcement of premating

790   isolation. *Evolution* **48**: 1451–1459.

791   Littlejohn, M. J., and Loftus-Hills, J. J. 1968. An experimental evaluation of premating

792   isolation in the *Hyla ewingi* complex (Anura: Hylidae). *Evolution* **22**: 659-663.

793   Llopart, A., Lachaise, D., and Coyne, J.A. 2005a. An anomalous hybrid zone in *Drosophila*.

794   *Evolution* **59**: 2602-2607.

795   Llopart, A., Lachaise, D., Coyne, J.A. 2005b. Multilocus analysis of introgression between

796   two sympatric sister species of *Drosophila*: *Drosophila yakuba* and *D. santomea*.

797   *Genetics* **171**: 197–210.

798   Lukhtanov, V. A., Kandul, N. P., Plotkin, J. B., Dantchenko, A. V., Haig, D., and Pierce, N.

799   E. 2005. Reinforcement of pre-zygotic isolation and karyotype evolution in *Agrodiaetus*

800   butterflies. *Nature* **436**: 385-389.

801   Manier M.K., Belote, J.M., Lüpold, S., Berben, K.S., Ala-Honkola, O., Collins, W.F., and

802   Pitnick, S. 2013. Rapid diversification of sperm precedence traits and processes among

803   three sibling *Drosophila* species. *Evolution* **67**: 2348-2362.

804   Markow, T.A., and O'Grady, P. 2008. Reproductive ecology of *Drosophila*. *Functional*

805   *Ecology* **22**: 747–759.

806   Matute, D.R., Novak, C.J., Coyne, J.A. 2009 Temperature-based extrinsic reproductive

807   isolation in two species of *Drosophila*. *Evolution* **63**: 595-612.

808   Matute, D.R. 2010a Reinforcement of gametic isolation in *Drosophila*. *PLoS Biol*. **8**:

809   e1000341.

810 Matute, D.R. 2010b Reinforcement can overcome gene flow during speciation in *Drosophila*.
811   *Curr Biol* **20**: 2229-2233.

812 Matute, D.R., and Coyne, J.A. 2010. Intrinsic reproductive isolation between two sisters
813   species of *Drosophila. Evolution* **64**: 903–920.

814 Matute, D. R. 2014. The magnitude of behavioral isolation is affected by characteristics of
815   the mating community. *Ecology and Evolution*, **4**: 2945-2956.

816 Matute, D.R. 2015. Noisy neighbors can hamper the evolution of reproductive isolation by
817   reinforcing selection. *Am. Nat.* **185**: 253-269.

818 Miller, G. T. and Pitnick S. 2002. Sperm-female co-evolution in *Drosophila. Science* **298**:
819   1230-1233.

820 Mueller, J. L., Ram K. R., McGraw L.A., Qazi, M.B., Siggia, E.D., Clark, A.D., Aquadro,
821   C.F., and Wolfner, M.F. 2005. Cross-species comparison of *Drosophila* male accessory
822   gland protein genes. *Genetics* **171**: 131-143.

823 Murphy, H.A. and Zeyl, C.W. 2015. A potential case of reinforcement in a facultatively
824   sexual unicellular eukaryote. *Am. Nat.* In press.

825 Nielsen, R., Korneliussen, T., Albrechtsen, A., Li, Y., and Wang J. 2012. SNP Calling,
826   Genotype Calling, and Sample Allele Frequency Estimation from New-Generation
827   Sequencing Data. *PLoS ONE* **7**: e37558

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

829 Noor, M.A.F. 1999. Reinforcement and other consequences of sympatry. *Heredity* **83**: 503–
830   508.

831 Nosil, P., Crespi, B.J., and Sandoval, C.P. 2003. Reproductive isolation driven by the
832   combined effects of ecological adaptation and reinforcement. *Proc. R. Soc. Lond. B.*
833   **270**: 1911–1918.

834 Nosil, P., and Hohenlohe, P. A. 2012. Dimensionality of sexual isolation during
835   reinforcement and ecological speciation in *Timema cristinae* stick insects. *Evol. Ecol.*
836   *Res.* **14**: 467-485.

837 Nosil P. 2005. The role of selection and gene flow in the evolution of sexual isolation in
838   *Timema* walking-sticks and other Orthopteroids. *J. Orthoptera Res.***14**: 247–253.

839 Palumbi, S. R. 2008. Speciation and the evolution of gamete recognition genes: pattern and
840   process. *Heredity* **102**: 66-76.

841 Panhuis, T. M., Butlin, R., Zuk, M., and Tregenza, T. 2001. Sexual selection and speciation.
842   *Trends Ecol. Evol.* **16**: 364-371.

843 Pfennig, K.S. 1998. The evolution of mate choice and the potential for conflict between
844   species and mate-quality recognition. *Proc. R. Soc. Lond. B.* **265**: 1743-1748.

845 Pfennig, K.S. 2000. Female spadefoot toads compromise on mate quality to ensure
846   conspecific matings. *Behav. Ecol.* **11**: 220-227.

847 Pfennig, K.S., and Pfennig, D.W. 2005. Character displacement as the "best of a bad
848   situation": fitness trade-offs resulting from selection to minimize resource and mate
849   competition. *Evolution* **59**: 2200-2208.

850 Pfennig, K.S., and Pfennig, D.W. 2009. Character displacement: ecological and reproductive
851   responses to a common evolutionary problem. *Q. Rev. Biol.* **84**: 253-276.

852 Pfennig, D. W. and Pfennig, K. S. 2012. Evolution's Wedge: Competition and the Origins of
853   Diversity. University of California Press, Berkeley, CA.

854 Pfennig, K. S., and Rice, A. M. 2014. Reinforcement generates reproductive isolation
855   between neighbouring conspecific populations of spadefoot toads. *Proc. R. Soc. B.* **281**:
856   20140949.

857 Pinheiro, J., and Bates D. 2000. Mixed-effects models in S and S-PLUS. Springer, New York,
858   NY, USA.

859 Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., and the R Development Core Team. 2013.
860   nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-113. URL:
861   http://cran.r-project.org/web/packages/nlme/index.html

862 Pitnick S., Markow T.A., and Riedy M.F. 1991. Transfer of ejaculate and incorporation of
863   male-derived substances by females in the *nannoptera* species group (Diptera:
864   Drosophilidae). *Evolution* **45**: 774-780.

865 Pitnick, S., Spicer, G.S., and Markow, T.A. 1997. Phylogenetic examination of male
866   ejaculatory donations in *Drosophila*. *Evolution* **51**: 833-845.

867 Pitnick, S., T. A. Markow, and G. S. Spicer. 1999. Evolution of multiple kinds of female

868   sperm-storage organs in *Drosophila*. *Evolution* **53**: 1804-1822.

869   Pitnick, S., and G. T. Miller. 2000. Correlated response in reproductive and life history traits

870   to selection on testis length in *Drosophila hydei*. *Heredity* **84**: 416-426.

871   Powell, J.R. 1997. Progress and Prospects in Evolutionary Biology: The *Drosophila* Model.

872   Oxford University Press. New York.

873   Price, T.D. 2007. Speciation in Birds Roberts & Co. Publishers, CO, USA.

874   Prince, G.J., and Parsons, P.A. 1977. Adaptive behaviour of *Drosophila* adults in relation to

875   temperature and humidity. *Aust. J. Zool.* **25**: 285-290.

876   Ram, K. R., and Wolfner, M. F. 2007. Seminal influences: *Drosophila* Acps and the

877   molecular interplay between males and females during reproduction. *Integr. Comp. Biol.*

878   **47**: 427-445.

879   Ranz, J. M., Maurin, D., Chan, Y. S., Von Grotthuss, M., Hillier, L. W., Roote, J., Ahsburner,

880   M., Bergman, C. M. (2007). Principles of genome evolution in the *Drosophila*

881   *melanogaster* species group. *PLoS Biol.* **5**: e152.

882   Rice, W. R., and Hostert, E. E. 1993. Laboratory experiments on speciation: what have we

883   learned in 40 years? *Evolution* **47**: 1637-1653.

884   Rice, W. R. 1996. Sexually antagonistic male adaptation triggered by experimental arrest of

885   female evolution. *Nature* **381**: 232-234.

886   Rice, A.M., and Pfennig, D.W. 2008. Analysis of range expansion in two species undergoing

887   character displacement: Why might invaders generally "win" during character

888   displacement? *J. Evol. Biol.* **21**: 696-704.

889   Ritchie, M. G. 2007. Sexual selection and speciation. *Annu. Rev. Ecol. Evol. Syst*., **38**: 79-

890   102.

891   Rundle, H.D., and Schluter, D. 1998. Reinforcement of stickleback mate preferences:

892   sympatry breeds contempt. *Evolution* **52**: 200–208.

893   Rundle, H.D., Chenoweth, S.F., and Blows, M.W. 2009. The diversification of mate

894   preferences by natural and sexual selection. *J. Evol. Biol.* **22**:1608–1615.

895   Ryan, M.J., and Rand, A.S. 1993. Species recognition and sexual selection as a unitary

896   problem in animal communication. *Evolution* **47**: 647-657.

897 Safran, R. J., Scordato, E. S., Symes, L. B., Rodríguez, R. L., and Mendelson, T. C. 2013.
898   Contributions of natural and sexual selection to the evolution of premating reproductive
899   isolation: a research agenda. *Trends Ecol. Evol*. **28**: 643–650

900 Sanderson, N. 1989. Can gene flow prevent reinforcement? *Evolution* **43**: 1223–1235.

901 Schully, S. D., and Hellberg, M. E. 2006. Positive selection on nucleotide substitutions and
902   indels in accessory gland proteins of the *Drosophila pseudoobscura* subgroup. *J. Mol.*
903   *Evol*. **62**: 793-802.

904 Servedio, M.R. and Kirkpatrick, M. 1997. The effects of gene flow on reinforcement.
905   *Evolution* **51**: 1764–1772.

906 Servedio, M.R. 2000. Reinforcement and the genetics of nonrandom mating. *Evolution* **54**:
907   21–29.

908 Servedio, M.R., and Noor, M.A. 2003. The role of reinforcement in speciation: theory and
909   data. *Annu. Rev. Ecol. Syst.* **34**: 339–364.

910 Shaw, K. L., and T. C. Mendelson. 2013. The targets of selection during reinforcement. *J.*
911   *Evol. Biol.* 26:286–287.

912 Singmann, H. 2013. afex: Analysis of Factorial Experiments. R Package Version, 0-6. URL:
913   http://www.psychologie.uni-freiburg.de/Members/singmann/R/afex

914 Springer, S.A., and Crespi, B.J. 2007. Adaptive gamete-recognition divergence in a
915   hybridizing *Mytilus* population. *Evolution* **61**: 772–783.

916 Swanson, W. J., Clark, A. G., Waldrip-Dail, H. M., Wolfner, M. F., and Aquadro, C. F. 2001.
917   Evolutionary EST analysis identifies rapidly evolving male reproductive proteins in
918   *Drosophila. Proc. Natl. Acad. Sci. USA*. **98**: 7375-7379.

919 Swanson, W.J., Wong, A., Wolfner, M.F., and Aquadro, C.F. 2004 Evolutionary expressed
920   sequence tag analysis of *Drosophila* female reproductive tracts identifies genes
921   subjected to positive selection. *Genetics* **168**: 1457–1465.

922 Timofeef-Ressovsky, H., and Timofeef-Ressovsky, N.W. 1940. Population-genetische
923   Versuche an *Drosophila* II. Aktionbereiche von *Drosophila funebris* und *Drosophila*
924   *melanogaster. Zeitschrift für induktive Abstammungs-und Vererbungslehre.* **79**: 35–44.

925 Tonzetich, J. 2004. Orcein staining and the identification of polytene chromosomes. *Methods*

926 *Mol. Biol.* **247**: 249-56.

927 Turner, E., Jacobson, D.J., and Taylor, D.J. 2010. Reinforced postmating reproductive
928 isolation barriers in *Neurospora*, an Ascomycete microfungus. *J. Evol. Biol.* **23**: 1642–
929 1656.

930 Walker, T.J. 1974. Character displacement and acoustic insects. *Am. Zool.* **14**: 1137–1150.

931 Wigby, S., and Chapman, T. 2005. Sex Peptide causes mating costs in female *Drosophila*
932 *melanogaster*. *Curr. Biol*. **15**: 316-321.

933 Wigby, S., Sirot, L. K., Linklater J. R., Buehner N., Calboli F.C.F., Bretman A., Wolfner M.
934 F., and Chapman T. 2009. Seminal fluid protein allocation and male reproductive
935 success. *Curr. Biol.* **19**: 751-757.

936 Wullschleger, E.B., Wiehn, J., and Jokela, J. 2002. Reproductive character displacement
937 between the closely related freshwater snails *Lymnaea peregra* and *L. ovata*. *Evol. Ecol.*
938 *Res.* **4**: 247–257.

939 Yapici, N., Kim, Y.J., Ribeiro, C., and Dickson, B.J. 2008. A receptor that mediates the post-
940 mating switch in *Drosophila* reproductive behaviour. *Nature* **451**: 33-37.

941 Zachar, N., and Neiman, M. 2013. Profound effects of population density on fitness-related
942 traits in an invasive freshwater snail. *PLoS One*, **8**: e80067.

943 Zeileis, A., and Hothorn, T. 2002. Diagnostic checking in regression relationships. R News **2**:
944 7-10. URL http://CRAN.R-project.org/doc/Rnews

945 Zouros, E., and d'Entremont, C. J. 1980. Sexual isolation among populations of *Drosophila*
946 *mojavensis*: response to pressure from a related species. *Evolution*, **34**: 421-430.