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3   Title: Male success against con- and heterospecific competitors indicates a positive but modest role for

4   sexual selection as driver of speciation

5

6   RRH: Sexual selection and species isolation

7   Keywords: speciation; reproductive isolation; sexual selection; sperm competition; remating

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14   Data archiving: no data to archive.

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16

17   **Abstract**

18   For sexual selection within species to drive the evolution of reproductive isolation between lineages,

19   sexually selected and reproductive isolating traits must both share underlying mechanisms and operate

20   in the same direction. While some work has been done to evaluate mechanistic overlap, fewer studies

21   have evaluated whether intraspecific sexually-selected variation is associated with elevated

22   reproductive isolation between species. Here we evaluate this association by assessing the relationship

23   between male reproductive success against conspecifics versus heterospecific males at each of two

24   different mating stages. We find that male precopulatory performance (remating success following a

25   conspecific versus a heterospecific first mating) was not associated between conspecific and

26   heterospecific contexts, but postcopulatory success (sperm competition against conspecific versus

27   heterospecific males) was modestly positively correlated. We discuss two lines of evidence that suggest

28   this modest association is due to incomplete mechanistic overlap between postcopulatory competition

29   in conspecific and heterospecific mating contexts. This study provides an explicit test of a necessary

30   condition for sexual selection to drive speciation, and finds that while sexual selection is not individually

31   sufficient to explain the magnitude of reproductive isolation in this system, it could nonetheless

32   facilitate the evolution of isolation via postcopulatory sperm competition.

33

34

Introduction

35

36   Sexual selection is frequently proposed as a powerful driver of speciation (Ritchie 2007),

37   however for this to be the case, two conditions must be met. First, traits that are the target of sexual

38   selection must also be involved in reproductive isolation, so that the two processes share mechanisms

39   and underlying genes in common. Second, sexual selection and reproductive isolation must act in the

40   same direction. In particular, in order to drive speciation, sexual selection must favor trait changes

41   within species that act to amplify reproductive isolation between species. Some empirical studies have

42   generated evidence that sexual selection and species reproductive isolation act via shared traits,

43   mechanisms, and/or genes (Groot et al. 2013, Arbuthnott 2009, Castillo and Moyle 2014, and see

44   below), although whether they are sufficiently mechanistically coupled for sexual selection alone to

45   drive isolation remains equivocal (Panhuis et al. 2001, Ritchie 2007, Bolnick and Kirkpatrick 2012, Saffran

46   et al. 2013). Moreover, it is equally unclear that the direction of sexual selection and reproductive

47   isolation will consistently align; theory suggests that they might act at cross purposes under some

48   conditions (Servedio and Burger 2014), but there are few empirical studies that explicitly examine the

49   strength and direction of association between traits that mediate sexual success within and between

50   species.

51   A range of sexual traits could potentially be involved in both intraspecific reproductive

52   performance and reproductive isolation against heterospecifics. In particular, studies of precopulatory

53   traits (reviewed Ritchie 2007), especially male traits related to courtship, have produced some evidence

54   that male signal traits that are strongly preferred by conspecific females also strongly contribute to

55   interspecies mating isolation (e.g. *Laupala* crickets: Mendelson and Shaw 2005, Shaw and Parsons 2002).

56   Nonetheless, other less examined mating interactions might also play an influential role in both

57   intraspecific mating success and interspecific reproductive barriers. Remating in *Drosophila* offers a

58   context within which to investigate multiple such mating traits with consequences for both conspecific

59   and heterospecific interactions. Among *Drosophila*, remating is a common sexual strategy. In *Drosophila*

60   *melanogaster*, paternity tests from natural field collected females indicate that most have mated

61   between 2 to 6 times (Imhof et al. 1997, Harshman and Clark 1998), while remating estimates are >80%

62   in laboratory settings (reviewed Singh et. al. 2002). Accordingly, the majority of matings are expected to

63   be rematings, making male performance in remating contexts an important aspect of lifetime fitness. To

64   secure offspring with a female that has previously mated, a second male needs both to successfully

65   court this female and, once mated, to effectively displace the sperm of the first mated male (referred to

66   as 'offensive' sperm competition; Boorman and Parker 1976). In the initial (pre-copulatory) interaction,

67   the male must convince the female—via acoustic, visual, and chemical cues—to accept a second mating,

68   despite potentially detrimental effects to her and despite biochemical manipulation by the first male

69   that decreases her receptivity (Parker and Partridge 1998, Sirot et al. 2009). Because remating provides

70   females with a degree of control over paternity of offspring, especially when the first male is deemed

71   suboptimal (Fricke et al. 2006), female remating decisions likely involve an assessment of the quality of

72   the second male. In interactions with conspecifics alone, this assessment can be based on factors such

73   as sperm depletion (when a first mating was not recent; Clark et al. 199, Gromko and Markow 1993) but

74   also on optimizing mating with the highest quality males ('trading up' Byrne and Rice 2005). However,

75   it's unclear whether the same performance characters would be assessed, or assessed to the same

76   degree, by females whose first mating was with a heterospecific male—a mating context that is almost

77   always suboptimal. If females evaluate different qualities when choosing remating partners depending

78   on whether a first male was conspecific or heterospecific, sexual selection on precopulatory remating

79   traits need not act in the same direction as reproductive isolation.

80   Following a successful remating event, a second male must also outcompete the first male in

81   sperm competition. In *Drosophila*, it is well documented that the second (or 'P2') male frequently sires

82   more offspring than the first ('P1') male (a phenomenon referred to as 'P2 precedence'), whether the

83   first mating is conspecific or heterospecific (Price et al. 1999, Price 1997). Nonetheless, P2 success

84   against conspecifics can vary among males, depending upon the factors such as condition and sperm

85   count (Letsinger and Gromko 1985) as well as female genotype (Clark et al. 1999, Bjork et al. 2007). In

86   contrast to intraspecific sperm competition, second mated conspecifics almost always sire the majority

87   of offspring following a first heterospecific mating, a phenomenon known as conspecific sperm

88   precedence (Price 1997). This phenotypic observation suggests that sperm competition between

89   conspecifics and heterospecifics might not be based on identical mechanisms. Indeed, it has been shown

90   that *Drosophila* females manipulate and store sperm differently depending upon whether it is from

91   conspecific versus heterospecific males (Manier et al. 2013a). Nonetheless, intraspecific sperm

92   competition in *Drosophila melanogaster* is also well characterized at the genetic level (Begun et al. 2000,

93   Findlay et al 2008), and it has been shown that genes mediating intraspecific sperm competition

94   (Wolfner 1997, Neubaum and Wolfner 1999) also significantly affect the efficacy of conspecific sperm

95   precedence (Castillo and Moyle 2014; Civetta and Finn 2014, and see Discussion). Pre- and

96   postcopulatory remating success therefore offers a model for investigating mating traits in parallel for

97   their role in both intraspecific and interspecific reproductive interactions.

98   Here we evaluated the potential link between these male reproductive traits in conspecific and

99   heterospecific mating contexts, using a worldwide set of fifteen *Drosophila melanogaster* populations.

100   To focus specifically on male performance traits, we examined remating success and offensive sperm

101   competition in males from these different populations, all with a single female line and all against a

102   standard conspecific and heterospecific first male genotype. We first evaluated evidence for variation

103   among lines in pre- and postcopulatory male competitive traits against both conspecific and

104   heterospecific males. With these data, we examined whether male success against conspecific first

105   males was associated with success against heterospecific first males. By examining two different male

106   performance traits, we were able to assess if either of these phenotypes meets the requirement for

6

107   sexual selection to drive speciation—that these traits act in the same direction—and whether male

108   reproductive success differences against conspecifics are alone sufficient to explain patterns of

109   reproductive isolation. Finally, we also evaluated non-competitive male fecundity, to confirm that male

110   sexual success was specifically due to male traits mediating competitive interactions. Based on our

111   findings, as well as previous theoretical and empirical work, we identify several general conditions which

112   might favor a positive relationship between sexual selection and reproductive isolation, and therefore

113   enable a role for sexual selection in the evolution of strengthened reproductive barriers. Nonetheless,

114   we conclude that even under such conditions, sexual selection is unlikely to be sufficient to drive

115   reproductive isolation on its own.

116

117   **Methods**

118   *Fly stocks and maintenance*

119   All fly stocks were reared on standard cornmeal media prepared by the Bloomington Drosophila

120   Stock Center (BDSC) at Indiana University, and were kept at room temperature (~22C). Fourteen of our

121   Drosophila melanogaster experimental male lines were drawn from the founder lines of the Drosophila

122   Synthetic Population Resource (King et al. 2012; and provided by Stuart MacDonald) and were chosen as

123   they represent a diverse sample of worldwide populations with genome sequence data for downstream

124   studies (see Figure 1 for locations of origin). The fifteenth male line was the Austria w132 line, originally

125   collected by Christian Schlotterer and donated to us by Kristi Montooth (University of Nebraska –

126   Lincoln). This line was also used as the female genotype in all trials in this study. P1 male lines for

127   intraspecific and interspecific competition trials were Green Fluorescent Protein (GFP) labeled *D.*

128   *melanogaster* 32170 (obtained from the BDSC) and *D. simulans* 14021-0251.2663 (obtained from the

129   University of California San Diego *Drosophila* species stock center), respectively. The same female line,

130   and first male *D. melanogaster* and *D. simulans* GFP lines, were used in Castillo and Moyle's (2014)

131   analysis of the role of three known sperm competition loci in the expression of conspecific sperm

132   precedence.

133

134   *Male intra- and interspecific performance assays*

135   To assess differences in offensive competitive ability of males from our 15 target lines, our assay

136   evaluated siring success of male lines with tester (Austria w132) *D. melanogaster* females following a

137   first mating with either a GFP-marked male *D. melanogaster* (to assess intraspecific sexual competition)

138   or *D. simulans* (to assess interspecific sexual interactions). The intra- and interspecific assays were

139   designed so that the only difference between them was the identity of the first (P1) male, enabling us to

140   directly compare the relative competitive success of each target male line against a common

141   heterospecific and conspecific male tester genotype (Figure 1).

142   For first matings, virgin female Austria flies, GFP male *D. melanogaster* flies (for intraspecific

143   trials), and GFP male *D. simulans* flies (for interspecific trials), were isolated 1 day prior to first mating. 4-

144   6 virgin females and 4-6 males (either *D. melanogaster* or *D. simulans* P1 genotypes) were housed in a

145   single vial and allowed to mate for 3 days. Females were then removed singly to individual blue-dyed

146   food vials and allowed to oviposit for 24 hours, then checked for eggs to ensure at least one mating had

147   occurred with a GFP-labeled first male. Females that did not oviposit were recorded as 'unmated' and

148   removed from the remainder of the experiment (Figure 1). Females confirmed to have mated were then

149   moved to a new vial to be singly paired with a virgin male from one of the 15 target male lines. The

150   second pairing was maintained for 24 hours, after which the male was removed and the female moved

151   to a new vial for 24 hours. Each female was then moved to a new vial every 24 hours for two additional

152   days, for a total of 4 days of oviposition (3 of these after contact with second male). Progeny from all

153   four vials were allowed to mature into adulthood and, upon eclosion, all adult individuals were gently

154   anesthetized and viewed under a Leica M205FA stereo scope equipped with a UV light for visualizing

155   GFP. The presence or absence of GFP in the ocelli of the eye was used as a marker of paternity; progeny

156   with GFP ocelli must have been sired by the first mated (P1) GFP-labelled male, whereas progeny with

157   wildtype ocelli are counted as progeny of the target male populations. For each timepoint per trial—the

158   post-P1 blue vial and 3 post-remating vials—each progeny individual was scored for presence or

159   absence of GFP in the ocelli. In instances where no wildtype offspring were observed in the 3 post-

160   remating vials, we assumed that a successful remating event with the second male did not occur, and

161   these were scored as 'unremated' (Figure 1). Both intra- and interspecific trials were repeated until at

162   least 5 successful replicates (i.e. trials in which there was evidence that a second/remating event

163   occurred) for each focal (second) male line were obtained.

164

165   *Estimating variation in first mating frequency*

166   Because our experiment was designed so that post-conspecific and -heterospecific mating

167   assessments were entirely parallel, the first pairing involved multiple males and females (4-6 each) for

168   both conspecific (GFP *D. melanogaster* males) and heterospecific (GFP *D. simulans*) first matings. The

169   latter was used to ensure that heterospecific matings occurred at a reasonable frequency, however a

170   corollary effect is that females in conspecific first pairings might have experienced >1 copulations within

171   the 3 day co-housing period. Because we did not directly observe matings, we used a secondary assay to

172   estimate the number of conspecific males each female likely mated with during these first pairings, as

173   follows: 5 virgin Austria females were paired with 5 conspecific males that were a mix of GFP males and

174   males from a single focal line (either 2 GFP/3 focal, or 3 GFP/2 focal), and kept co-housed for 3 days as

175   above. A total of 54 paired trials were run (half of 3 GFP/2 focal males, and half of 2 GFP/3 focal males).

176   Within these 27 pairs of trials, focal males were drawn from four of our lines: Austria (10/27 pairs) and

177   California (7/27 pairs), and Israel (5/27) and Spain (5/27). Following mating, females were allowed to

178   oviposit for 24 hours in individual vials. Upon eclosion, progeny were assessed for presence versus

179   absence of GFP in the occeli; each trial was then scored as 'GFP', 'WT' or 'mixed' matings based on the

180   types of progeny found in the offspring. This procedure was not performed for heterospecific first

181   matings, as the mating rate was low enough that females were assumed to be singly mated.

182   Assay results—the number of females that produced only monotypic versus mixed offspring—

183   were used to estimate the likely number of copulations per female in these first mating trials, based on

184   the general expectation that fewer mixed versus monotypic offspring broods is consistent with a lower

185   frequency of females that have copulated more than once. We found that 16/54 trials produced 'mixed'

186   offspring, all of which must have been the product of at least two copulations; the remaining females

187   produced monotypic offspring (16/54 all GFP-labelled, and 22/54 all wild type). From these

188   observations, and some simplifying assumptions (see Supplementary text), we infer that the majority of

189   females mate either 1 or 2 times in first mating trial (for example, we estimate 48% are single matings),

190   whereas the likely frequency of three copulations is low (Supplementary text).

191

192   *Quantifying male competitive success*

193   We used success of second males in securing a remating as our data for offensive precopulatory

194   success, for each male line. In particular, we tallied the number of second mating trials where there was

195   at least one non-GFP offspring in subsequent vials—indicating a second mating with the target male—

196   versus the number of trials that had only GFP offspring (unremated). The number of successes and

197   failures were compared among male lines and between intra- and interspecific assays to determine if

198   either second male line identity or identity of first male (conspecific vs. heterospecific) affected mean

199   success in obtaining a remating (see analyses below).

200   We used the proportion of offspring sired after the second mating to estimate offensive

201   postcopulatory (sperm competitive) success. For all trials in which a second (focal male) was successful

202   at remating (i.e., for which there was at least one non-GFP labelled offspring in post-remating vials), the

203   proportion of all adult flies that were wild-type versus GFP (tallied across the 3 post-remating vials) was

204   calculated as an estimate of postcopulatory siring success, either in competition with conspecific (for

205   intraspecific trials) or heterospecific (interspecific trials) males.

206

207   *Non-competitive male fecundity assay*

208   To determine if our competitive male phenotypes were associated with non-competitive male

209   fecundity, we assessed fecundity for each of the 15 target male lines when singly mated with the tester

210   female line Austria w132. Virgin males and females were isolated and housed individually for 24 hours

211   to reach maturity, prior to being aspirated into individual vials that contained one Austria female and a

212   single male of a focal line. Each pair was then co-housed for 24 hours on standard media, after which

213   the male was removed and the female allowed an additional 24 hours to oviposit before being removed.

214   We assessed male fecundity at two stages—average number of eggs produced by an inseminated

215   female and total adult offspring produced—in each assay, allowing us to also verify that adult progeny

216   counts reflect successful fertilization events. Fecundity assays were performed for at least 5 replicates

217   for each male line. We found that egg count and adult offspring count were quantitatively

218   indistinguishable (i.e., with few exceptions, all eggs developed into adulthood), therefore non-

219   competitive fecundity measures used in all subsequent analyses were based on adult offspring count

220   (see Supplementary Text for egg data).

221

222   *Statistical analyses*

223   *Phenotypic variance for pre- and postcopulatory success*:

224   We used a χ^2 test of independence to evaluate whether our 15 focal male lines differed in their

225   mean precopulatory success at obtaining a second mating following a first mating with a tester

226   conspecific male line (intraspecific trials), or following a first mating with a tester heterospecific male

227 line (interspecific trials.) To evaluate whether male lines differed in postcopulatory offensive sperm

228 competitive ability, we used analyses of variance (ANOVA) to assess whether identity of focal male

229 genotype significantly affects the mean proportion of progeny sired by the second (P2) male. ANOVAs

230 were performed separately for sperm competitive success following conspecific and heterospecific first

231 matings, on a logit transformation of the proportion of offspring sired by the target male (i.e., not GFP

232 labelled). To assess whether focal male lines differed in non-competitive fecundity, we used an ANOVA

233 to assess whether focal male genotype significantly affected the mean number of adult offspring

234 produced within 24 hours of a single non-competitive mating. For completeness, we also evaluated

235 whether variation in either con- or heterospecific success at either mating stage was associated with

236 global geographical patterns, and found that neither continent of origin, nor temperate versus tropical

237 origin, significantly predicted competitive success in any of these cases (data not shown).

238

239 *Relationship between male performance after intraspecific versus interspecific matings, and between*

240 *pre- versus post-copulatory success:*

241   For both pre- and postcopulatory success traits we used Pearson's correlation coefficient to

242 assess the association between line means for reproductive phenotype following intraspecific versus

243 interspecific first matings. For the male precopulatory success data, we assessed the correlation

244 between proportion successful remating after conspecific trials versus after heterospecific trials, using

245 line mean values. For postcopulatory success, we tested for a correlation between line mean siring

246 success against conspecific versus heterospecific males. Within each class of interaction (intraspecific or

247 interspecific) we also evaluated the correlation between pre- and postcopulatory success phenotypes

248 using a Pearson's correlation coefficient. Because assessing the correlation between mean phenotypes

249 in these analyses does not take into account any within-line variance exhibited for postcopulatory

250 phenotypes in our dataset, we also performed several bootstrap analyses to assess the effect of within-

251   line (among-individual male) variance on the strength of these associations between male performance

252   phenotypes (see Supplementary text).

253

254   *Relationship between competitive and non-competitive male reproductive performance traits:*

255   To assess the relationship, if any, between non-competitive male performance and success in

256   offensive reproductive interactions, we evaluated the correlation between male non-competitive

257   fecundity and both pre- and postcopulatory success traits when competing against conspecifics. For

258   each we computed a Pearson's correlation coefficient between average fecundity of each male line

259   (after single matings) against the logit transformation of proportion data for either pre- or post-

260   copulatory success following a conspecific first mating. For completeness, we also examined the

261   relationship between non-competitive fecundity and male pre- and postcopulatory success following a

262   heterospecific first mating.

263

264   **Results**

265   *Significant variation among male lines for reproductive success traits*

266   We found significant phenotypic variance between focal male lines in their ability to secure a

267   mating following conspecific (χ^2 = 24.784; *P* = 0.037) and following heterospecific (χ^2 = 23.804; *P* = 0.048)

268   first matings (Figure 2). Following conspecific first matings, precopulatory success ranged from 0.217 to

269   0.857, with the majority of populations exhibiting a relatively high success rate (>0.6) but Colombia,

270   Spain, South Africa, Israel, and Malaysia performing more poorly. The proportion of successful remating

271   attempts following heterospecific males ranged from a perfect success rate (shared by 5 of the

272   populations) down to 0.5 for the Colombia male line (Table 1).

273   We also found significant phenotypic variation between focal male lines in their postcopulatory

274   success as the second (offensive) male genotype, against both conspecific first males (*F*(14, 45.937); *P* =

275   0.022) and against heterospecific first males (F(14, 25.989); P = 0.007) (Figure 3). Interestingly, for

276   postcopulatory success against conspecifics only, this was accompanied by a relatively high variance in

277   performance among males within each line (Figure 3). Because this variation cannot be explained by

278   male or female genotype effects, and the rearing environment of all flies was uniform, it might be due to

279   variation among individual females in whether they had copulated once or twice prior to our remating

280   assay (that is, within the initial 3-day pairing with conspecific tester males; see methods and

281   supplement). Consistent with this, we see much less among-male within-line variation following

282   heterospecific first matings, in which females are expected to only have mated once. Importantly,

283   despite within-line male performance variation, we still detect significant line mean differences in pre-

284   and postcopulatory success against the conspecific (GFP) tester male genotype.

285   In contrast to competitive male reproductive phenotypes at both pre- and postcopulatory

286   stages, we found no significant difference among male lines in non-competitive fecundity (F(14, 1.006);

287   P = 0.459).

288

289   *Positive but modest relationship between male sexual performance against conspecific males and*

290   *heterospecific males*

291   To assess whether sexual selection and reproductive isolation could act in the same direction,

292   we examined the relationship between success after conspecific versus heterospecific first matings for

293   both pre- and postcopulatory traits. We found no significant correlation between precopulatory success

294   after conspecific versus heterospecific first matings (r(13) = 0.450, P = 0.090)(Figure 4). In contrast,

295   mean male line values of postcopulatory success against conspecific first males versus against

296   heterospecific first males were significantly positively associated (r(13) = 0.552, P = 0.032)(Figure 5). This

297   suggests that male lines that are on average better sperm competitors against conspecific males are also

298   better able to displace heterospecific sperm.

299

Postcopulatory success is associated with precopulatory success after conspecific first matings

301   Because we assessed second male success in terms of both precopulatory and postcopulatory

302   competitive ability, we could also evaluate whether these different sexual performance traits are

303   associated with each other. For male performance after conspecific first matings, we found a significant

304   correlation between remating success and sperm competitive ability (r(13) = 0.562, *P* = 0.029). In

305   contrast, pre- and postcopulatory success following heterospecific matings were not associated (r(13) =

306   0.014, *P* = 0.959) (Figure 6).

307

Differences in remating and sperm competitive success are not determined by non-competitive male

fecundity

310   To better interpret the causes of variation in second male reproductive success, we evaluated

311   whether male line variation in either precopulatory or postcopulatory success could be explained by

312   differences in intrinsic, non-competitive male fecundity. We found no significant correlation between

313   intrinsic fecundity and precopulatory remating success following conspecific (r(13) = 0.046, *P* = 0.870) or

314   heterospecific (r(13) = 0.476, *P* = 0.073) first matings. We also found no correlation between non-

315   competitive fecundity and postcopulatory success against either conspecific (r(13) = -0.047, *P* = 0.868) or

316   heterospecific (r(13) = 0.188, *P* = 0.503) first males.

317

Discussion

319   Because of its effects on reproductive trait evolution, sexual selection is commonly invoked as a

320   driver of speciation. However questions still remain about the mechanistic connection between

321   conspecific and heterospecific sexual interactions, including whether sexual selection generally acts in

322   the same or different directions as reproductive isolation. Here we examined phenotypic variation in,

323   and the relationship between, pre- and postcopulatory male performance in *Drosophila melanogaster*

324   when competing against a conspecific or heterospecific first male. Although we found no significant

325   association between precopulatory success—securing a remating—after a conspecific versus a

326   heterospecific first mating (Figure 4), average postcopulatory competitive success was significantly

327   positively associated between the two contexts (Figure 5). Our findings speak both to whether sexual

328   selection could be expected to act in concert with, or in opposition to, reproductive isolation, and

329   whether selection within species is individually sufficient to drive reproductive isolation. They also raise

330   the question of why the male performance traits examined here might differ in their degree of coupling

331   between conspecific and heterospecific mating contexts, and suggest conditions under which these

332   positive associations are expected to be more or less likely. Overall, our results indicate that sexual

333   selection on some male reproductive traits can facilitate the evolution of isolating barriers, but may be

334   insufficient to drive isolation on its own.

335

336   *Intraspecific competitive success has a positive but modest contribution to reproductive isolation*

337   The specific connection between sexual selection and reproductive isolation determines

338   whether sexual dynamics within species could shape the evolution of reproductive isolation between

339   species, including facilitating or even constraining the emergence of isolating barriers. Our results

340   indicate that, for the male performance traits we examined here, sexual selection does not appear to

341   constrain reproductive isolation and could, for post-copulatory competitive traits, act to facilitate the

342   expression of stronger reproductive isolation. Previous analyses have found mixed evidence for this

343   association. While some theoretical work has suggested that sexual selection amplifies isolation under

344   specific conditions (Gavrilets and Waxman 2002, Gavrilets 2000, Kondrashov and Shpak 1998, Higashi et

345   al. 1999), other models indicate that sexual selection can oppose speciation, especially when strong

346   female preferences reduce the variation in male mating traits that is required for reproductive isolation

347   to evolve (Servedio 2012, Kirkpatrick and Nuismer 2004, Servedio and Burger 2014). Interestingly, most

348   of the latter predictions have been generated specifically in the context of divergence with gene flow

349   (i.e. in partial sympatry); it is unclear whether similar expectations hold broadly in allopatry, where there

350   is no direct or indirect selection for increased reproductive isolation but also where gene flow—

351   including the movement of strong female preference alleles between species (e.g. Servedio and Burger

352   2014)—does not oppose the build-up of isolation.

353   The few previous empirical assessments of relationships between sexually-selected traits and

354   reproductive isolation have also produced variable findings. These differing outcomes mostly appear to

355   depend on the specificity of female preferences for con- or heterospecific male traits in any given case.

356   At least two studies have inferred that sexual selection works against reproductive isolation because an

357   exaggerated male trait is preferred by both conspecific and heterospecific females in sympatry, thereby

358   increasing the propensity for gene flow between species (*Girardinichthys*, Macias Garcia et al 2012; *O.*

359   *pumilio*, Yang et al. 2016). In contrast, in both Laupala crickets (Mendelson and Shaw 2005, Shaw and

360   Parsons 2002) and Tungara frogs (Boul et al. 2007), male signal traits that are strongly preferred within

361   species are also used by females to discriminate against heterospecific males. While our analysis was not

362   primarily designed to assess the pattern of female preferences for con- and heterospecific male traits,

363   we found that the *D. melanogaster* female line we used generally chooses to remate with conspecific

364   second males after a heterospecific first mating (i.e. more often than foregoing a second mating; Figure

365   4), and strongly prefers the sperm of second conspecific males after heterospecific first matings (i.e.

366   siring success against *D. simulans* is well above 50% for all conspecific male lines; Figure 5), regardless of

367   the significant variation among male lines in their relative success in each phenotype. Together, our

368   observations and these prior studies suggest that strong female preferences for conspecific male traits

369   could help to facilitate positive associations between sexual selection and reproductive isolation. Note,

370   however, that female preferences need not always be required, especially in the specific context of

371   sexual conflict over fertilization; for example, the sperm of *Caenorhabditis* lineages experiencing strong

372   antagonistic selection on sperm competition traits have been shown to cause traumatic female sterility

373   in heterospecifics (Ting et al. 2014), indicating that sexual antagonism acting within species can also

374   produce isolation between species.

375   While our findings suggest that male sexual performance against conspecifics and

376   heterospecifics can act in the same direction, at least for competitive fertilization traits, they also

377   indicate a fairly modest association between sexually selected and reproductive isolating phenotypes.

378   This observation implies that sexually selected intraspecific phenotypes can contribute to isolating

379   barriers, but are likely insufficient to individually drive the reproductive isolation observed between

380   species. Other studies that have investigated this general relationship—both theoretically and

381   empirically—have made similar inferences (e.g. Bolnick and Kirkpatrick 2012). Using patterns of isolation

382   observed in empirical examples, Bolnick and Kirkpatrick (2012) modelled the strength of intraspecific

383   assortative mating that would be necessary to produce phenotypic differences sufficient to cause

384   reproductive isolation, and showed that the magnitude of sexual selection fails to sufficiently explain the

385   amount of reproductive isolation observed between lineages. Additional analytical theory and

386   simulations suggest that male traits must be under both natural (divergent) selection and sexual

387   selection in order to successfully contribute to isolation in sympatry (Kirkpatrick and Ravigne 2002;

388   Servedio and Burger 2014; Servedio and Boughman 2017); sexual selection alone is insufficient to drive

389   increases in isolation.

390   If sexual selection can be a positive but insufficient driver of isolation, it is important to examine

391   the conditions under which sexual selection can be more effective (or less) at generating reproductive

392   barriers. Interestingly, our data themselves suggest at least one other condition that could determine

393   the strength of this association: the degree to which there are shared underlying mechanisms

394   controlling competitive success against conspecifics versus heterospecifics. Below we discuss two lines

395   of evidence that suggest that varying degrees of mechanistic overlap between these mating contexts

396   likely explains both the modest association we observed for postcopulatory traits, and lack of

397   relationship for precopulatory traits.

398

399   *The degree of mechanistic overlap influences the strength of association between sexually selected traits*

400   *and reproductive barriers*

401   Both of the sexual performance phenotypes we examined here are likely a product of multiple

402   underlying mechanisms, that could vary in their degree of overlap between conspecific and

403   heterospecific mating contexts and therefore in the expected strength of association between these

404   contexts. Interestingly, evidence both from prior characterization of these pre- and postcopulatory

405   performance phenotypes, and from direct genetic studies, suggests that the degree of mechanistic

406   overlap could be greater for postcopulatory phenotypes. Prior analyses of the mechanics of

407   postcopulatory sperm success in *Drosophila* indicate that it primarily acts on only three classes of trait:

408   sperm traits (reviewed in Snook 2005), ejaculate traits (reviewed in Mautz et al. 2013) and female

409   reproductive responses (including sperm transfer, sperm displacement, sperm ejection, and sperm

410   selection for fertilization (Manier et al. 2013b, Manier et al. 2010, Miller and Pitnick 2002)). This

411   circumscribed suite of traits increases the chance that superior male performance against conspecific

412   and heterospecific males share some physiological mechanisms in common. Within *D. melanogaster*, for

413   example, in addition to size and speed of sperm—traits that affect displacement success (e.g., Lüpold et

414   al 2012)—intraspecific sperm success has been shown to vary in part due to female ejection timing

415   (Lüpold et al 2013). Interspecifically, in *D. simulans* it has been shown that postcopulatory fertilization

416   success of conspecific males over heterospecific males also depends on both male-mediated sperm

417   displacement success and female-controlled mechanisms of sperm ejection, among other traits (Manier

418   et al. 2013a). In addition, in *D. melanogaster* there is direct evidence that these postcopulatory

419   mechanisms also share genes in common (Castillo and Moyle 2014). In assays using the same female line

420   and the same first male genotypes as examined here, Castillo and Moyle (2014) showed that knockdown

421   or null lines for two genes (*Acp36DE*, *CG9997*) with known roles in sperm competition within *D.*

422   *melanogaster* were also significantly less competitive in offensive (second male) sperm competition with

423   *D. simulans* first males. A third evaluated locus—*Sex Peptide*—is essential for effective sperm

424   competition among conspecifics (Liu and Kubli 2013), but had no phenotypic effect on competitive

425   performance against heterospecific first males (Castillo and Moyle 2014). Together, both direct

426   phenotypic observations and genetic studies provide good evidence that there is some degree of

427   overlap, although incomplete, in the mechanisms that mediate postcopulatory sperm success when

428   competing against conspecific and heterospecific males.

429   In contrast, a much larger range of traits could mediate the precopulatory success of males in

430   persuading *Drosophila* females to remate (reviewed Markow et al. 2005), including a complex set of

431   non-independent behavioral cues (Welbergen et al. 1992) as well as several mating signals along at least

432   two sensory axes – acoustic (reviewed Hoikkala 2005) and chemosensory (reviewed Chung and Carrol

433   2015). Intraspecifically, *D. melanogaster* females are known to use both acoustic (e.g., pulse songs with

434   more pulse (Talyn and Dowse 2003)) and chemical signals (e.g., presence and abundance of male

435   pheromone 7-Tricosene; Billeter et al. 2009) to evaluate conspecific mating partners. How *D.*

436   *melanogaster* females discriminate against heterospecific *D. simulans* males is less well understood

437   (reviewed Sawamura and Tomaru 2002), however courtship song interpulse interval is known to affect

438   interspecific courtship success (Kyriacou and Hall 1986). Given this broad variety of potentially

439   important precopulatory traits*,* it is possible that mechanisms involved in male remating success after

440   heterospecific versus conspecific first matings are not even on the same sensory axis. Moreover, the

441   criteria for assessing both the benefit of remating and the suitability of second males, likely varies

442   depending on the species identity of the first male. Following a heterospecific first male, females will

443   almost certainly benefit from remating by compensating for an unambiguously low quality first mating

444   (the "rescue effect", Fricke et. al. 2006), whereas following a conspecific mating, the benefits of

445   remating are more strongly dependent on whether the second male is a higher quality male (enabling

446   the female to "trade up", Byrne and Rice 2005). In the first instance, the criteria for identifying suitable

447   second mates might be focused more on species-recognition traits, whereas the latter might assess

448   more nuanced differences in male quality or persistence (although it remains unclear if mate recognition

449   traits are generally distinct from species recognition traits; reviewed Servedio and Boughman 2017).

450   Interestingly, our own observations following conspecific first matings suggest that females might

451   indeed be making these remating decisions based on male quality; generally, male lines with better

452   precopulatory remating success are also better sperm competitors against conspecifics following this

453   mating (Figure 6). In contrast, the success of male lines in persuading females to remate following a

454   heterospecific first mating is uncorrelated with their ability to displace heterospecific sperm (Figure 6).

455   This discrepancy between these two associations is further evidence that overlap between mechanisms

456   that underlie these traits is incomplete, as we might otherwise expect to see a similar pattern of success

457   following a heterospecific mating as we do a conspecific if these mechanisms were identical. In either

458   case, there are clear reasons that the degree of mechanistic overlap might be expected to differ

459   systematically between traits involved in precopulatory and postcopulatory male success, when

460   compared between conspecific and heterospecific first male contexts.

461

462   *Conditions most likely to connect sexual selection and reproductive isolation*

463   As we've addressed above, our findings—in conjunction with previous theory and data—suggest

464   several primary conditions where sexual selection within species is expected to have its largest role in

465   reproductive isolation. In particular, when female preferences for intraspecific male traits is strong,

466   these preferences are expected to also contribute to discrimination against heterospecific males. In

467   addition, when a limited number of potential mechanisms underlie mating interactions, the likelihood

468   that these mechanisms will be involved in both intra- and interspecific contexts is increased. The

469   importance of pleiotropy between sexually selected traits and isolating barriers is a repeated and

470   general inference from numerous models of sexual selection and speciation (Ritchie 2007), and the

471   differences we observe between different mating traits (pre- versus post-copulatory) here might reflect

472   the overall propensity for these different classes of traits to participate pleiotropically in both sexual

473   selection and isolation.

474   Finally, apart from these conditions, there are other general factors that might influence the

475   extent to which some sexually selected traits might be less reliable or consistent contributors to

476   reproductive isolation. For example, while environmental conditions can play a role in postcopulatory

477   mating traits (for example, by changing the number or quality of sperm produced, depending upon

478   physiological condition; reviewed in Pitnick et al. 2009), there are reasons to expect that precopulatory

479   traits (such as visual, auditory, and other sensory cues) are more directly influenced by external

480   environmental factors. For example, elaborate courtship traits can make males more apparent to

481   predators, a potentially important natural selective agent; similarly, the type and intensity of male

482   signals can be shaped by the environment in which these signals must be transmitted (Boughman 2002).

483   The extent to which sexual selection and natural selection interact to influence the evolution of isolating

484   barriers is an area of substantial ongoing research (Boughman 2013, Safran et al. 2013), but clear

485   examples from the sensory drive literature (Boughman 2001, Seehausen et al. 2008) and elsewhere

486   (Hardwick et al. 2013) suggest that sexual selection on precopulatory mating traits might often take a

487   back seat to direct natural selection on these same traits, a conclusion also supported by theory (Van

488   Doorn et al. 2009, Servedio and Boughman 2017). The operation of additional (and potentially

489   constraining) forces of natural selection on precopulatory traits is therefore another factor that might

490   favor a stronger role for postcopulatory traits in mediating the connection between sexual selection and

491   reproductive isolation.

492   Regardless, it is evident that in order for sexual selection to drive speciation, phenotypes

493   associated with success in one context must also be successful in the other. Here we explicitly tested for

494   a relationship between sexually selected (intraspecific), and reproductive isolating (interspecific) traits

495   by comparing male success directly between the two contexts for two different male mating success

496   traits. While we found little support that sexual selection would be sufficient to drive reproductive

497   isolation on its own, postcopulatory competitive success appears to act in the same direction in both

498   contexts. In conjunction with previous evidence that competitive fertilization within and between

499   species also depends upon some shared genes (Castillo and Moyle 2014) and overlap in mechanisms of

500   sperm displacement and cryptic female choice (e.g., Manier et al. 2013a) it is clear that sexual selection

501   on this class of male competitive traits could contribute to elevated reproductive isolation between

502   species, in the form of competitive heterospecific sperm displacement. In the absence of many empirical

503   analyses of the direct association between traits important for success in both intra- and inter-specific

504   interactions, it remains unclear if this observation applies more generally beyond *Drosophila*

505   *melanogaster* remating traits. Nonetheless, our analysis shows that such direct tests are critical for

506   assessing sexual selection's relationship with reproductive isolation, including the relative role of sexual

507   selection could play as a driving force in speciation.

508

509   **Acknowledgements**

514

515   **Citations**

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689   assortative mate preferences in allopatry but not sympatry. *Evolution*, *70*(12), 2778–2788.
690   http://doi.org/10.1111/evo.13079

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693 **Table 1: Mean mating data for each male population. Precopulatory success is represented as**

694 **proportion of total trials where P2 males succeeded in securing a remating, and postcopulatory**

695 **success is shown as average proportion of total offspring that are sired by the P2 male. Non-**

696 **competitive fecundity is shown as average number of adult offspring produced in 24 hours following a**

697 **single mating with each male line. Standard error is included for postcopulatory success and non-**

698 **competitive fecundity data.**

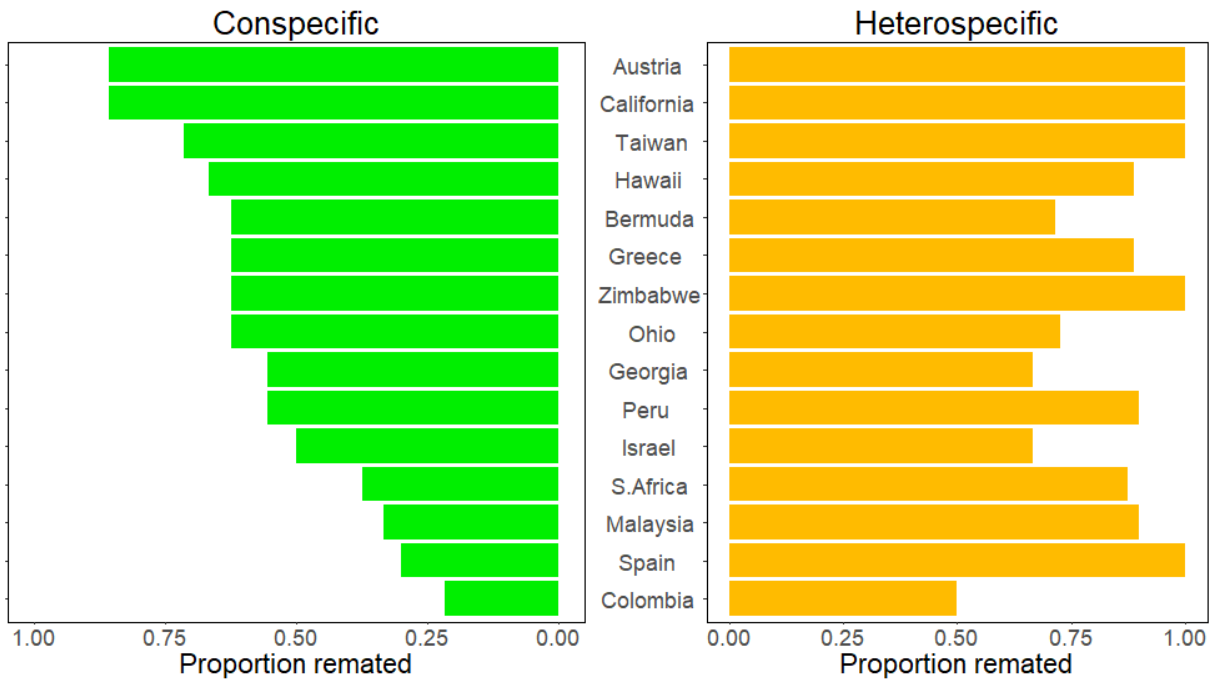|		Precopulatory success		Postcopulatory success		
P2 male identity		Heterospecific first male	Conspecific first male	Heterospecific first male	Conspecific first male	Non-competitive fecundity
A1	Ohio	0.727	0.625	0.942 ± 0.11	0.457 ± 0.03	23.8 ± 2.2
A2	Colombia	0.500	0.217	0.845 ± 0.13	0.436 ± 0.05	23.4 ± 1.7
A3	Spain	1.000	0.300	0.762 ± 0.05	0.095 ± 0.06	25.4 ± 1.0
A4	Zimbabwe	1.000	0.625	0.866 ± 0.06	0.354 ± 0.03	25.0 ± 1.2
A5	Greece	0.888	0.625	0.842 ± 0.09	0.222 ± 0.03	23.6 ± 0.7
A6	Georgia	0.667	0.556	0.867 ± 0.15	0.389 ± 0.06	22.6 ± 1.8
A7	Taiwan	1.000	0.714	0.930 ± 0.08	0.360 ± 0.02	26.0 ± 1.1
B1	Bermuda	0.714	0.625	0.893 ± 0.13	0.420 ± 0.03	19.8 ± 1.1
B2	S. Africa	0.875	0.375	0.885 ± 0.06	0.195 ± 0.03	20.2 ± 3.2
B3	Israel	0.667	0.500	0.935 ± 0.09	0.283 ± 0.03	21.8 ± 1.2
B4	California	1.000	0.857	0.935 ± 0.10	0.499 ± 0.02	23.8 ± 1.4
B5	Hawaii	0.889	0.667	0.955 ± 0.10	0.352 ± 0.01	25.2 ± 1.8
B6	Peru	0.900	0.556	0.959 ± 0.09	0.499 ± 0.01	25.2 ± 1.7
B7	Malaysia	0.900	0.333	0.933 ± 0.07	0.249 ± 0.02	24.8 ± 1.7
WT	Austria	1.000	0.857	0.893 ± 0.02	0.488 ± 0.02	22.8 ± 3.4

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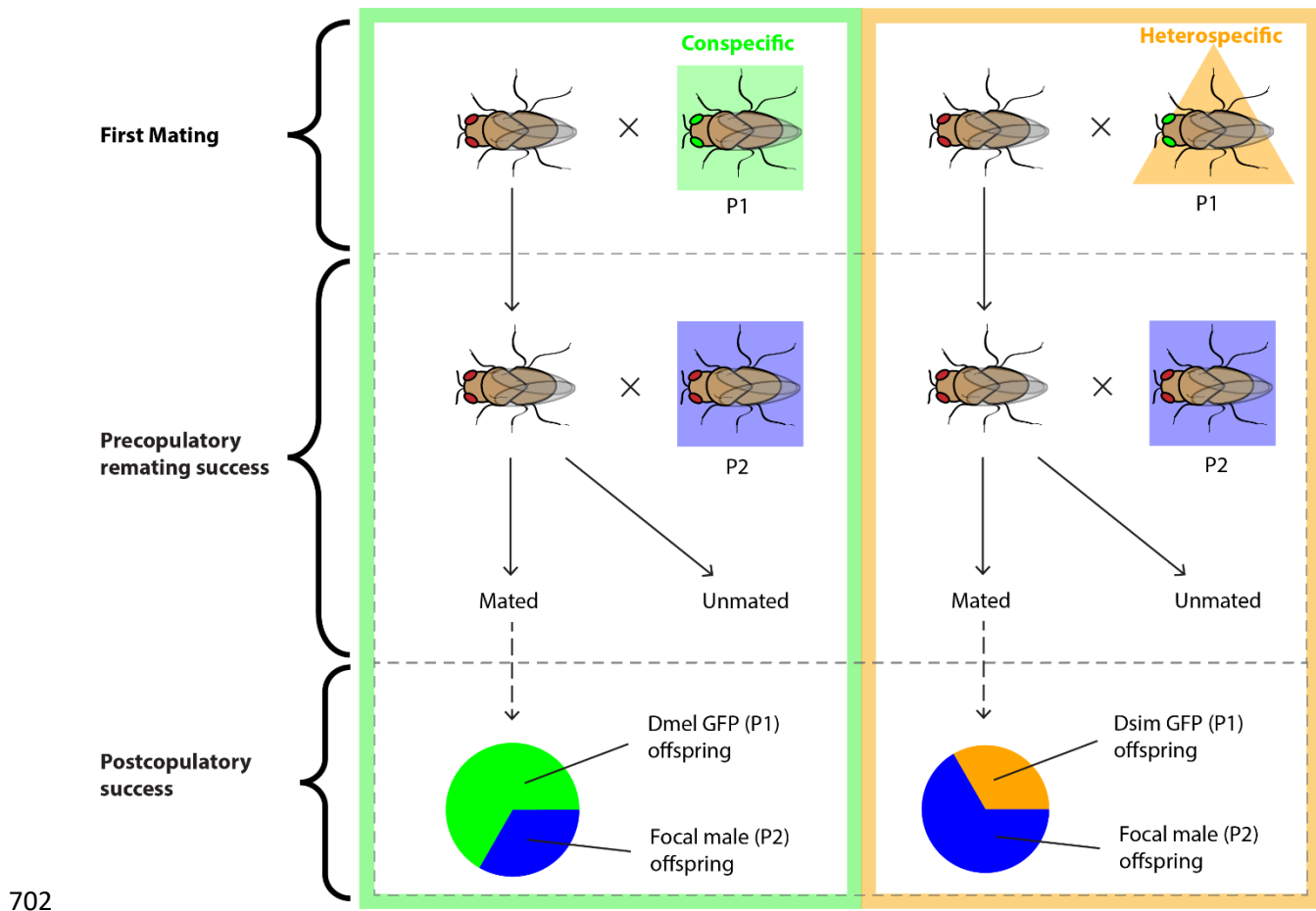
Figure 1: Schematic of experimental procedure. The experimental structure for assessing competitive success against conspecific (left) versus heterospecific (right) males is parallel, the only difference being the species identity of the first (P1) GFP-labelled male genotype. In each trial, a single female is first mated with a GFP-labelled first (P1) male before being paired with a second (P2) male drawn from one of the 15 target populations (Table 1). Adult offspring are scored for GFP ocelli after each second pairing, producing two datasets: precopulatory success (judged by presence/absence of wild-type (non-GFP) offspring) and, for pairings in which a second mating is confirmed, postcopulatory success (determined by proportion of offspring sired by P2 male). Both first male GFP genotypes and the female genotype – Austria w132 – are the same for all trials.

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Figure 2: Precopulatory remating success following conspecific (left) and heterospecific (right) first matings. Precopulatory success is the proportion of remating trials that resulted in at least one non-GFP offspring, indicating the proportion of trials in which P2 males secured a remating. Male lines significantly vary in their precopulatory success following both conspecific (χ^2 = 24.784; *P* = 0.037) and heterospecific (χ^2 = 23.804; *P* = 0.048) first matings.

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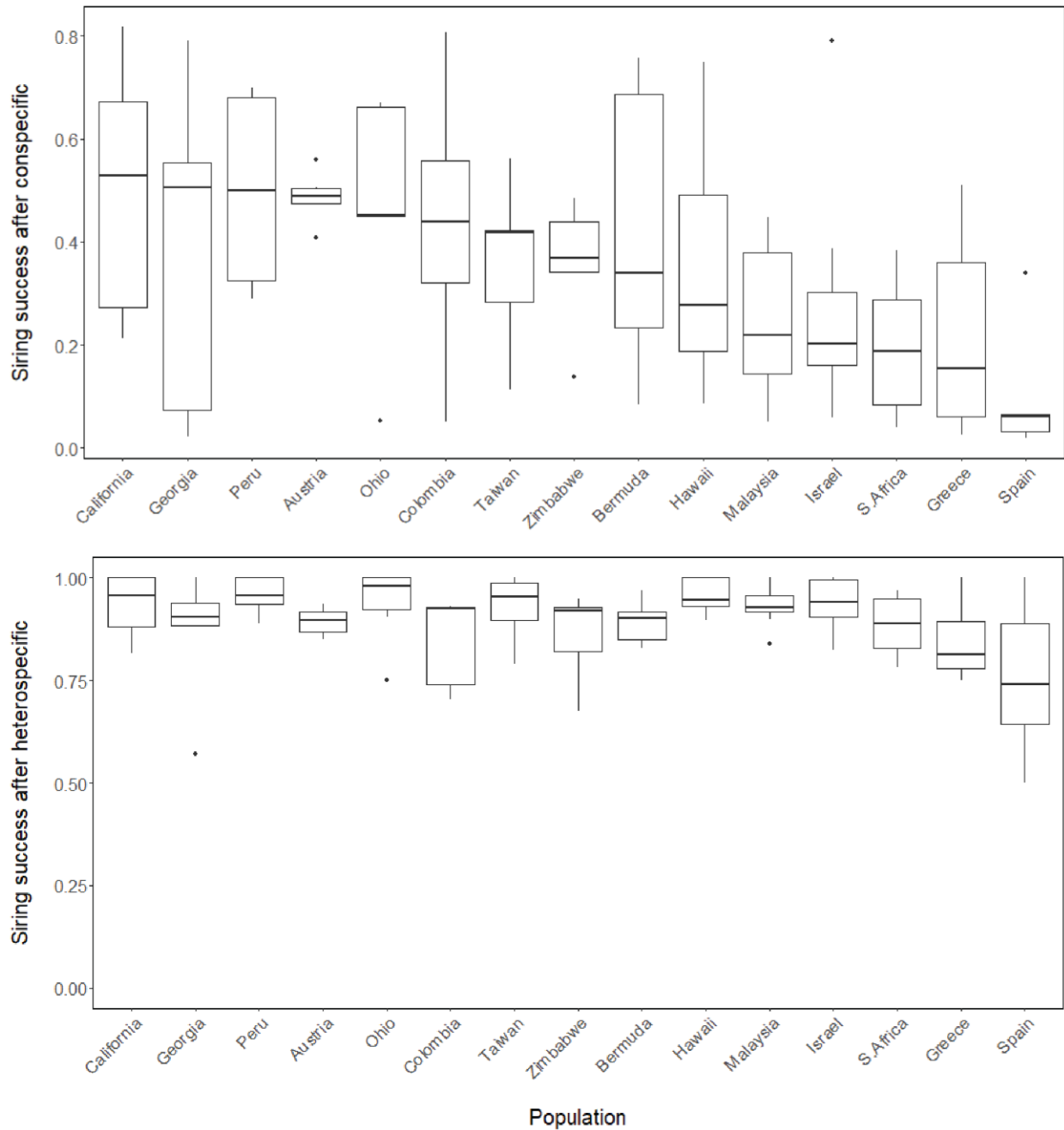
Figure 3: Postcopulatory siring success of second males from each focal line following intraspecific (top) and interspecfic (bottom) first matings. Boxes are mean proportion sired plus the 1st and 3rd quartiles; lines show standard errors. ANOVA on logit transformed proportions of offspring sired by the P2 male found significant male line effects for sperm competitive success after both conspecific (F(14, 25.989); P = 0.006877) and heterospecific (F(14, 45.937); P = 0.02192) first matings.

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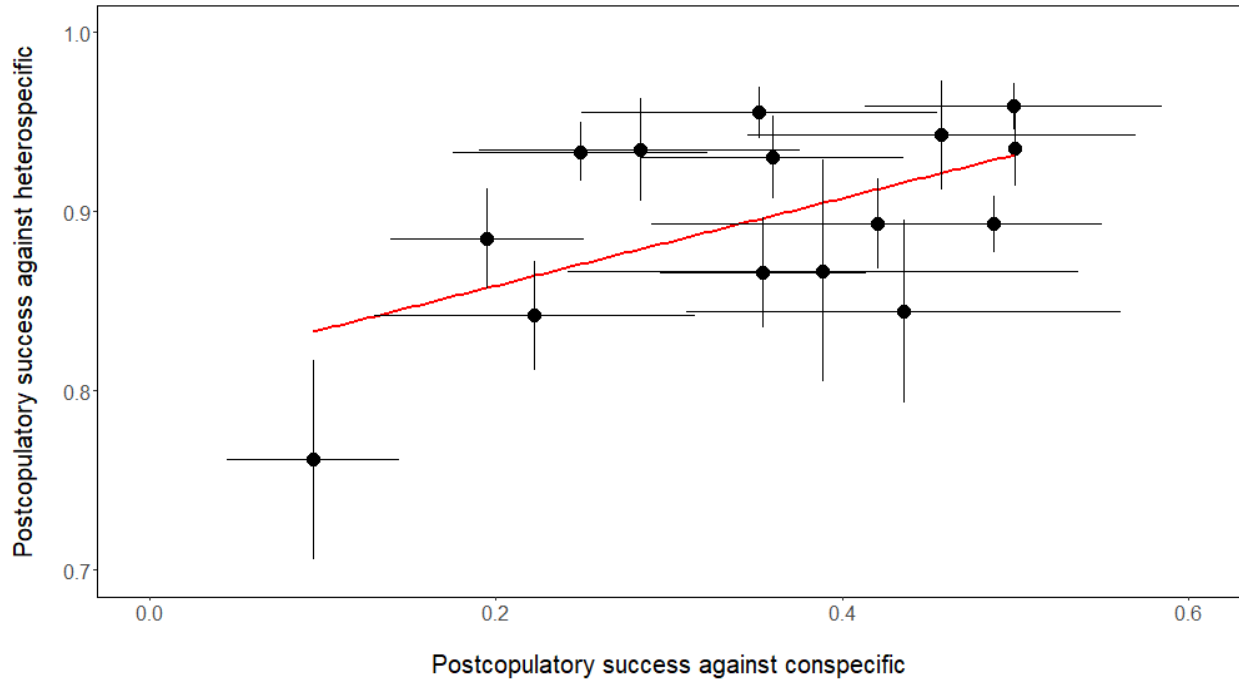

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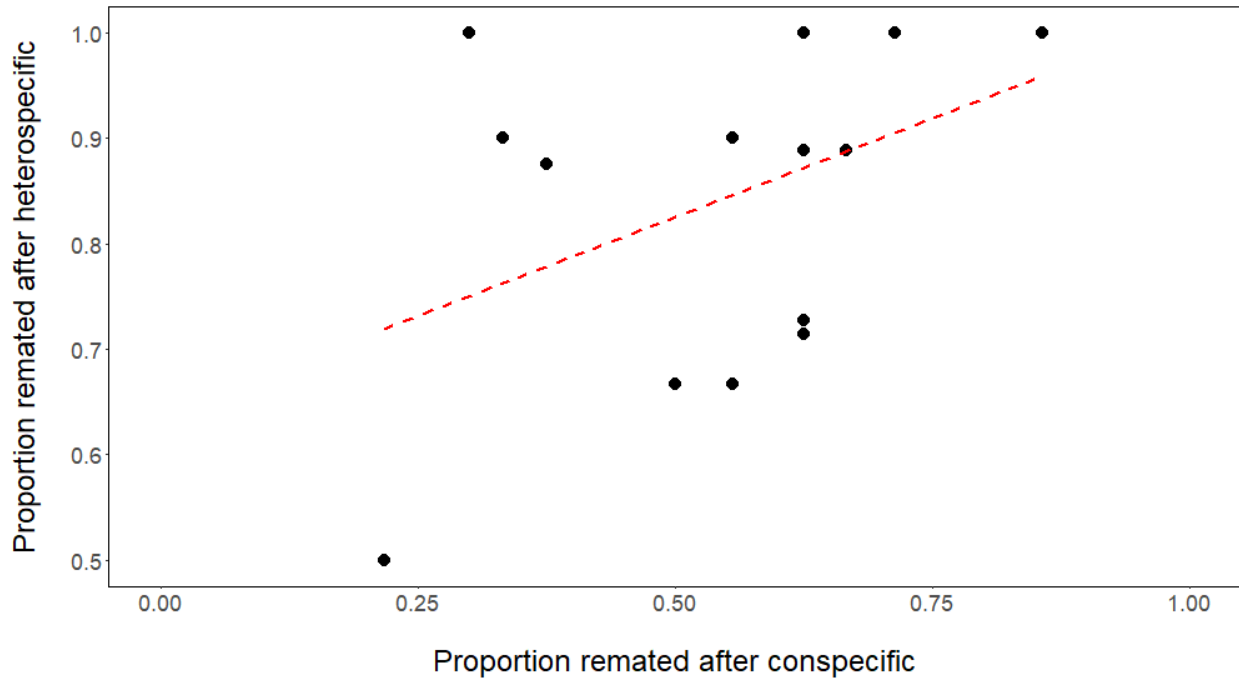
Figure 4: Mean precopulatory success of focal male lines after first matings with a conspecific (X-axis) versus heterospecific (Y-axis) first male is not significantly correlated (r(13) = 0.450, _P_ = 0.090). Each point represents the proportion of second mating trials that were successful for a focal male line.

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Figure 5: Mean postcopulatory competitive success of target male lines following first matings with conspecific (X-axis) or heterospecific (Y-axis) males is significantly correlated ($r(13) = 0.552$, $P = 0.032$). Points represent the average proportion of offspring sired by each male genotype, across all trials involving that genotype. The lines represent the standard errors for each population for the two phenotypes.

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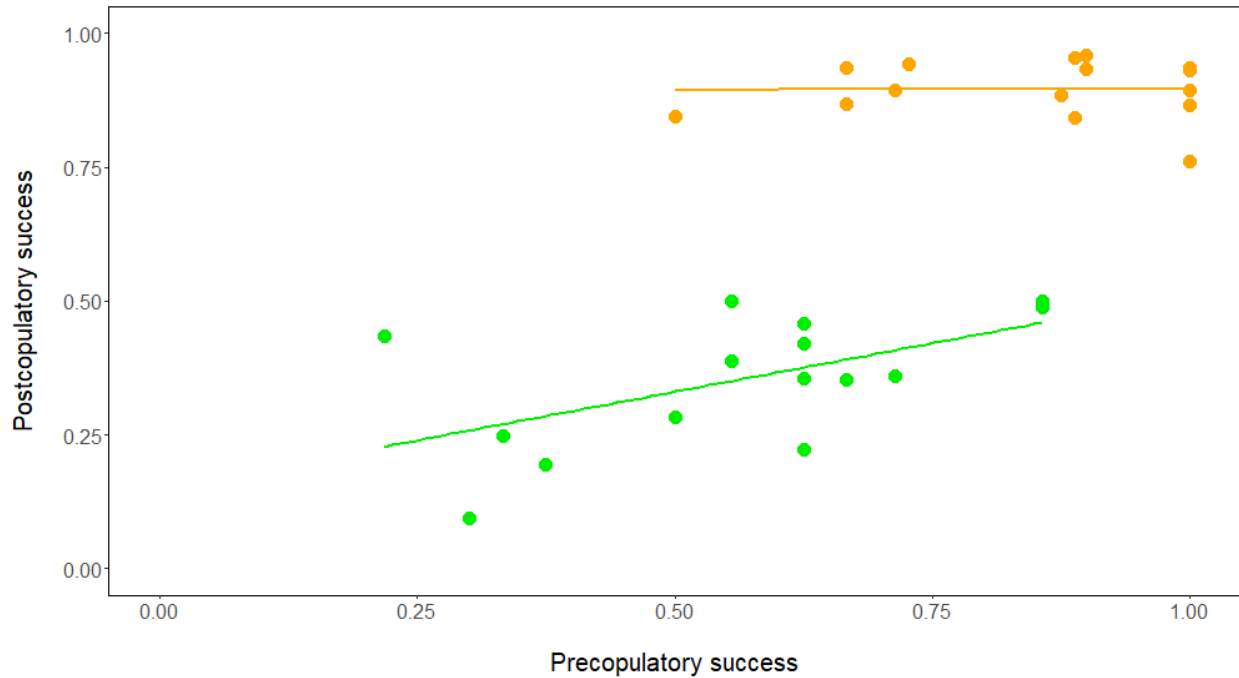
Figure 6: Associations between second male (P2) success at pre- and postcopulatory stages when competing against conspecific males (green) or heterospecific males (orange). For male performance against conspecifics, pre- and postcopulatory success is significantly correlated (r(13) = 0.562, _P_ = 0.029); pre- and postcopulatory male performance following a heterospecific first mating is unassociated (r(13) = 0.014, _P_ = 0.959).

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