Frequency-dependent hybridization contributes to habitat segregation in monkeyflowers

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Abstract

14

15   Spatial segregation of closely related species is usually attributed to differences in stress

16   tolerance and competitive ability. For both animals and plants, reproductive interactions between

17   close relatives can impose a fitness cost that is more detrimental to the rarer species. Frequency-

18   dependent mating interactions may thus prevent the establishment of immigrants within

19   heterospecific populations, maintaining spatial segregation of species. Despite strong spatial

20   segregation in natural populations, two sympatric California monkeyflowers (*Mimulus nudatus*

21   and *M. guttatus*) survive and reproduce in the other's habitat when transplanted reciprocally. We

22   hypothesized that a frequency-dependent mating disadvantage maintains spatial segregation of

23   these monkeyflowers during natural immigration. To evaluate this hypothesis, we performed two

24   field experiments. First, we experimentally added immigrants in varying numbers to sites

25   dominated by heterospecifics. Second, we reciprocally transplanted arrays of varying resident

26   and immigrant frequency. Immigrant seed viability decreased with conspecific rarity for *M.*

27   *guttatus*, but not *M. nudatus*. We observed immigrant minority disadvantage for both species, but

28   driven by different factors– frequency-dependent hybridization for *M. guttatus*, and competition

29   for resources and/or pollinators for *M. nudatus*. Overall, our results suggest a potential major role

30   for reproductive interference in spatial segregation that should be evaluated along with stress

31   tolerance and competitive ability.

Introduction

32

33   Patterns of habitat segregation are common in closely related species and have been long

34   mechanistically attributed to trade-offs between stress tolerance and competitive ability (Baker

35   1909, Connell 1961, Grace and Wetzel 1981, Yost et al. 2012, Peterson et al. 2013, Chen and

36   Schemske 2015, Ferris and Willis 2018). Competition is well established to be an important

37   mechanism driving zonation among closely related species with a high potential for niche

38   overlap (Burns and Strauss 2011). However, mating between closely related species may also

39   contribute to their spatial segregation (Anacker and Strauss 2014). The fitness costs of

40   interspecific mating (reproductive interference) can drive patterns of segregation or coexistence

41   through demographic displacement (Groning and Hochkirch 2008) or the evolution of increased

42   pre-zygotic isolation in sympatry (Servedio and Noor 2003). The ecological and evolutionary

43   outcomes of costly interspecific mating – spatial segregation (Groning and Hochkirch 2008) or

44   character displacement (Brown and Wilson 1956) – depend on the relative rate of evolutionary

45   change versus the rate of demographic decline in sympatry (Kyogoku and Wheatcroft 2020).

46   While the initial establishment of habitat segregation could result from exclusion in sympatry or

47   evolve directly in response to reproductive interference (Kyogoku and Kokko 2020), the long-

48   term maintenance of zonation patterns requires mechanisms that result in the demographic

49   decline of rare immigrants.

50   Plant species that co-occur and overlap in flowering often compete for pollinators and

51   often suffer consequent fitness reductions (Mitchell et al. 2009). Competition for pollination can

52   reduce fitness through reductions in visitation or through interspecific pollen transfer (Waser

53   1978 a, b). Both mechanisms of competition for pollination can affect fitness in a frequency-

54   dependent manner. Rarer species may receive fewer visits and are expected to receive less

55   conspecific pollen and more heterospecific pollen than common species. Models of reproductive

56   interference thus predict fitness declines with increasing rarity (Levin and Anderson 1970, Kuno

57   1992). Rare-species disadvantage caused by reproductive interference is major factor limiting

58   persistence of polyploid species that arise in populations of their diploid progenitors (minority

59   cytotype exclusion, Levin 1975). Thus, considerable focus has been placed on these dynamics in

60   co-occurring diploid and polyploid species (Husband 2000, Baack 2005, Buggs and Pannell

61   2006). However, the conditions experienced by rare polyploids are similar to that of parapatric

62   species that meet in secondary contact (Lewis 1961, Ribeiro and Spielman 1986, Thum 2007),

63   immigrants of species that are spatially segregated in regional sympatry (Singer 1990, Friberg et

64   al 2013, Christie and Strauss 2020), or nonnative species interacting with native congeners

65   (Takakura et al. 2009, Takakura and Fujii 2010, Takakura 2013, Takakura and Fujii 2015).

66   An extreme cost to reproductive interactions is the production of inviable seeds from

67   interspecific pollen transfer. This hybrid seed inviability is a common reproductive isolating

68   barrier in plants and particularly among species in the *Mimulus guttatus* species complex

69   (Coughlan et al. 2020, Garner et al. 2016, Oneal et al. 2016, Sandstedt et al. 2021, Vickery

70   1978). Inviable hybrid seeds produced between species in the *M. guttatus* species complex have

71   a characteristic flat or shriveled shape, which is caused by arrested endosperm development.

72   These inviable seeds can be distinguished by eye from round viable seeds with fully formed

73   endosperm produced by conspecific pollinations.

74   Populations of the widespread *M. guttatus* have repeatedly evolved tolerance to harsh

75   serpentine soils (Selby and Willis 2018) and co-occur with the geographically restricted

76   serpentine soil endemic *M. nudatus* in the Northern coast range of California. *M. guttatus*

77   typically grows in streams, seeps, or meadows, whereas *M. nudatus* typically grows in washes,

78 on rock outcroppings, or drier rocky areas adjacent to the streams inhabited by *M. guttatus*. *M.*

79 *nudatus* typically grows in barer habitats with fewer co-occurring con- and heterospecifics than

80 *M. guttatus*, a common pattern in serpentine soil endemics compared to species that occur both

81 on and off serpentine soils (Sianta and Kay 2019). Since habitats occupied by *M. guttatus* and *M.*

82 *nudatus* are often in close proximity (within meters) and immigrant individuals are regularly

83 found, dispersal limitation does not explain spatial segregation in these species. When

84 transplanted reciprocally at equal frequencies, *M. guttatus* has higher survival and produces more

85 seed than *M. nudatus* in both habitats, demonstrating that stress tolerance or competition are not

86 sufficient to explain spatial segregation patterns either (Toll and Willis 2018). In these reciprocal

87 transplants, both species produced many inviable F1 hybrid seeds as immigrants, demonstrating

88 the occurrence reproductive interference (Toll and Willis 2018). While a combination of factors

89 limits the persistence of *M. nudatus* immigrants in habitats occupied by *M. guttatus*, including a

90 lack of tolerance to flooding in *M. nudatus* (Toll and Willis 2018) and inbreeding depression

91 (Toll et al. 2021), these factors do not sufficiently explain the absence of *M. guttatus* in the drier

92 habitats occupied by *M. nudatus*. We therefore hypothesize that frequency-dependent

93 reproductive interference may maintain this spatial segregation pattern by imposing a strong

94 fitness cost on natural immigrants, which usually occur alone or in very small numbers.

95 Using observational and experimental data collected across two field seasons, we asked

96 three questions in this study. First, does hybridization reduce fecundity when *M. guttatus* and *M.*

97 *nudatus* naturally occur in close proximity? If hybridization contributes to the maintenance of

98 spatial segregation, we expect that seed viability will increase with increasing conspecific

99 frequency. Second, when species immigrate to sites occupied by heterospecifics, do components

100 of fitness in immigrants or residents (flowers per plant, seeds per flower, and seed viability)

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101   increase with their relative frequency? If resource competition contributes to the maintenance of

102   habitat segregation, we expect that flowers per plant will increase with increasing conspecific

103   frequency. If a lack of conspecific mates or pollinator competition contributes to the maintenance

104   of habitat segregation, we expect that seeds per flower will increase with increasing conspecific

105   frequency. Finally, if hybridization contributes to the maintenance of habitat segregation, we

106   expect that seed viability will increase with increasing conspecific frequency. Third, does

107   lifetime fecundity (measured as viable seeds per plant) in immigrants or residents increase with

108   increasing conspecific frequency in experimental sympatry? If minority disadvantage contributes

109   to the maintenance habitat segregation, we would expect that viable seeds per immigrant or

110   resident plant to increase with their increasing frequency, changing the relative performance of

111   immigrants relative to residents.

112

113   # **Materials and methods**

114   *Reproductive interference in natural populations: an observational transect*

115   To characterize the existence and possible magnitude of reproductive interference in co-

116   occurring populations of *M. guttatus* and *M. nudatus*, we surveyed plants along a transect

117   spanning an exposed serpentine outcrop that was inhabited by a pure stand of *M. nudatus*, a

118   transition zone where both species were present at lower density, terminating in a serpentine seep

119   with a pure stand of *M. guttatus* (38°51.528′ N, 122°24.614′ W). The entire length of the

120   transect, spanning the two pure stands through a small zone of overlap, was six meters. We

121   counted all the fruit produced by each species within eight 42 x 66 cm gridded quadrats centered

122   on the transect line, with the long side parallel to the transect line and evenly spaced along the

123   length of the transect. We counted all fruit present within the quadrat at 7cm intervals along the

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124   long side of the quadrat grid. At approximately 21cm intervals along the long side of the quadrat,

125   we collected one plant if only one species was present (n=13 *M. nudatus* and n=9 *M. guttatus*) or

126   two plants if both species were present (n=10 *M. nudatus* and n=10 *M. guttatus*) at the mid-point

127   of the short side of the quadrat. We counted fruit numbers instead of species abundances because

128   flower number, not plant number, is what pollinators in dense overlapping stands (Gardner and

129   MacNair 2000). We counted all seeds produced by the plants collected at 21 cm intervals and

130   categorized them as round viable (conspecific) or flat inviable F1 hybrid seeds based on

131   morphology (Oneal et al. 2016). We calculated seed viability by dividing the number of round

132   viable conspecific seeds by the total number of seeds produced by each plant. We estimated

133   Pearson correlation coefficients for the relationship between the proportion of conspecific fruit

134   and seed viability per sampled plant in R v 4.0.3 (R Core Team 2020).

135

136   *Experimental Transplants*

137   To test whether frequency-dependence contributes to the maintenance of habitat

138   segregation, we performed two reciprocal transplants over two years. In the first field season

139   (2018), we transplanted immigrants in varying abundances into natural populations of resident

140   heterospecifics. This allowed us to simulate a situation where immigrants arrive at low

141   abundances to sites already established by heterospecifics. However, since we did not transplant

142   the naturally occurring resident species with the immigrants, we lacked an appropriate

143   comparison for immigrant performance relative to residents. In the second field season (2019),

144   we transplanted immigrants and residents at varying frequencies reciprocally, allowing us to

145   compare the overall fecundities of experimental immigrants and residents.

146

147   *M. guttatus immigration experiment*

148   In February 2018, we transplanted variable numbers of immigrants into naturally dense

149   patches of native seedlings (>100 per half meter). While we performed this experiment

150   reciprocally, all but nine immigrant *M. nudatus* transplants died before flowering. We lacked

151   sufficient seedlings to replace them, and they were not analyzed further. Therefore, this

152   experiment only allowed us to examine the immigration dynamics of *M. guttatus* into *M. nudatus*

153   habitat (the direction less-well explained by our previous studies).

154   We collected seeds from a local population of *M. guttatus* at the University of California

155   McLaughlin reserve (38°51.528′ N, 122°24.614′ W) in 2013. We grew seeds from field-collected

156   maternal families in a greenhouse and crossed a single plant from each family to a single plant

157   from a different family to produce outbred maternal families in 2013 (described in more detail in

158   Toll et al. 2021). To minimize effects of inbreeding on fecundity (see Toll et al 2021), we

159   germinated 6 outbred maternal families of *M. guttatus* to use as focal plants. These plants were

160   initially grown in the shade house at the UC McLaughlin reserve in 2018. We did not have

161   sufficient seed to use these same outbred lines as neighbors, so we pooled equal numbers of seed

162   from twenty field-collected maternal families of *M. guttatus* to germinate in the shade house to

163   use as neighbors. We planted 72 blocks of focal *M. guttatus* plants in random order by maternal

164   family, then randomly assigned each family to one of the 6 levels of the initial conspecific

165   neighbor treatments (0, 8, 24, 48, 80, and 120 neighbors, Figure 1A). We transplanted a range of

166   seedlings that were ecologically relevant if a *M. guttatus* fruit dispersed into *M. nudatus* habitat,

167   given that each fruit produces an average of 200 seeds (Toll and Willis 2018). *M. guttatus*

168   seedlings were planted within naturally dense patches of *M. nudatus* (>100 native *M. nudatus*

169   within a half meter of each focal *M. guttatus* plant, Figure 1B). We planted *M. guttatus* neighbors

170   within a half-meter of each focal plant in a regularly spaced array and we planted these blocks

171   over three meters away from one another.

172   We replaced *M. guttatus* focal transplants as they died of transplant shock, resulting in 62

173   focal plants (of the original 72 plus 28 replacements) that survived to flower by the end of the

174   season. Neighbor transplant mortality rates were also high; 78% of *M. guttatus* transplants did

175   not survive to flowering, resulting in a range of flowering neighbors from 0 to 103, with a

176   median of 1 neighbor surviving to flowering. In subsequent analyses of the effect of conspecific

177   neighbors on components of fitness, we only included neighbors that successfully survived to

178   flower, as only they could potentially reduce reproductive interference. Mortality rates from this

179   transplant experiment are consistent with previous estimates at this site: 73%, 33%, and 82% of

180   *M. guttatus* seedlings died at this site in 2015, 2016, and 2017 respectively (site "QV" in Toll

181   and Willis 2018). Thus, we are confident that our transplants represent an ecologically realistic

182   range of potential surviving immigrant *M. guttatus* seedlings into *M. nudatus* habitat. We

183   counted all seeds produced by the 62 focal plants that produced fruit and categorized them as

184   viable round conspecific-pollinated or inviable flat heterospecific-pollinated (hybrid) seeds based

185   on morphology.

186   *Statistical analysis.* To examine how the number of conspecific neighbors affected focal

187   plant fitness components (flowers per plant, total seed per flower, and seed viability per focal *M.*

188   *guttatus* transplant) we used generalized linear mixed models in the R package *glmmTMB*

189   (Brooks et al. 2017). We fit models with each fitness component as the dependent variable, the

190   number of surviving conspecific neighbors as a fixed effect, and maternal family (n=6) of the

191   focal plant as a random effect. Models of total seeds per flower had an additional nested random

192   effect term for individual plant (n=62) since each focal plant produced multiple flowers.

9

193   We fit each count model first with a Poisson error distribution and identified the best

194   fitting error distribution by evaluating model diagnostics with the R package DHARMa (Hartig

195   2019). The effect of conspecific neighbors on flowers per focal transplant was estimated using a

196   negative binomial model because Poisson models were significantly overdispersed. The effect of

197   conspecific neighbors on seeds per flower was estimated using zero-inflated negative binomial

198   models with a constant intercept because Poisson and negative binomial models were

199   significantly overdispersed and zero-inflated. The effect of conspecific neighbors on seed

200   viability was estimated using a beta-binomial model because the binomial model was

201   significantly overdispersed.

202   We assessed the significance of the fixed effect (the number conspecific neighbors) with

203   the best fitting model for each fitness component using Wald Type II Chi-squared tests (R

204   package *car,* Fox and Weisberg 2019). We predicted marginal effects and bootstrapped (n=500

205   iterations) 95% confidence intervals with the R package *ggeffects* (Lüdecke 2018). We plotted

206   raw data and predictions with *ggplot2* (Wickham 2016) and combined plots with *patchwork*

207   (Pederson 2020).

208

209   *Frequency-manipulation reciprocal transplant*

210   To test whether frequency-dependence contributes to the maintenance of habitat

211   segregation, we reciprocally transplanted *M. guttatus* and *M. nudatus* at different frequencies but

212   with the same number of individuals in experimental patches. These mixed species patches were

213   transplanted into a seep dominated by *M. guttatus* and a wash dominated by *M. nudatus*. The

214   plants used in this experiment were all collected directly from natural populations at each

215   transplant site in the year (2019) of the experiment. In April 2019, we dug up 380 seedlings per

216 species from each site and repotted them in yellow 49-mL (2.54 cm diameter) Cone-tainers^{TM}

217 filled with potting soil (Stuewe and Sons, Inc., Tangent, OR, USA). We maintained seedlings in

218 a shadehouse at the UC McLaughlin Reserve for a week prior to redistributing seedlings in

219 Cone-tainers^{TM} at both sites. We sunk Cone-tainers^{TM} into the ground, so the top was flush with

220 the soil surface at each site in 19 plant hexagonal arrays (Figure 1C). Arrays were planted within

221 natural populations of each species, but we cleared native *Mimulus* plants within 0.25 m of each

222 array. We designed this experiment following the frequency manipulation arrays from Nagy

223 (1997): 0% immigrants (0 immigrants, 19 residents); 5% immigrants (1 immigrant, 18 residents);

224 ~25% immigrants (4 immigrants, 15 residents); ~50% immigrants (9 immigrants, 10 residents)

225 (Figure 1D). Higher levels of immigrants than natives would have been unrealistic in this system.

226 For clarity, we abbreviate frequency treatments as the ratio of *M. guttatus* (G) and *M. nudatus*

227 (N) transplants. We assigned plants to four frequency treatments in the seep site (19G, 18G:1N,

228 15G:4N, and 10G:9N) which is naturally occupied by *M. guttatus*, and four frequency treatments

229 in the wash site (18N, 18N:1G, 15N:4G, and 10N:9G) which is naturally occupied by *M.*

230 *nudatus*. We planted 5 replicates per frequency treatment per site, for a total of 20 blocks per site

231 and 40 blocks overall. Blocks were planted at least three meters away from each other. We added

232 supplemental water to blocks for a month after transplanting and replaced seedlings that died

233 prior to flowering to ensure that the same number of plants flowered in each frequency treatment.

234 We left containers in the field until all plants terminated flowering and ripened their fruit. We

235 collected fruit from each transplant in the field prior to digging up containers. We counted and

236 categorized all seeds produced by experimental plants and estimated the hybridization rate using

237 the same methods as the observational transect. We estimated lifetime fecundity (viable seeds

238 per plant) from three fitness components: the total number of flowers produced per plant, the

239   total number of seeds produced per flower, and seed viability (=viable seeds/total seeds

240   produced).

241   *Statistical analysis.* To examine how the immigrant frequency treatments affected plant

242   fitness components (flowers per plant, total seed per flower, and seed viability) and lifetime

243   fecundity (viable seeds per plant), we used generalized linear mixed models in the R package

244   *glmmTMB* (Brooks et al. 2017). We tested whether fitness components differed among frequency

245   treatments for each species in each site separately because no immigrants were transplanted in

246   the 0% immigrant frequency treatment (Figure 1D). We fit models with each fitness component

247   as the dependent variable, immigrant frequency as a fixed effect and block as a random effect

248   (block n=15 for immigrants, n=20 for residents). We also tested whether lifetime fecundity

249   differed between species, but only within the treatments where both immigrants and residents

250   were present (5%, 25%, and 50% immigrant frequency treatments) in each site separately. We fit

251   models with lifetime fecundity as the dependent variable, immigrant frequency, species and their

252   interaction as fixed effects and block as a random effect (block n=15).

253   Poisson models of count fitness components and lifetime fecundity were significantly

254   overdispersed, and models of seeds per flower and viable seeds per plant were significantly zero-

255   inflated. Thus, the effect of immigrant frequency on flowers per plant was estimated using a

256   negative binomial distribution, while total seeds per flower and viable seeds per plant were

257   estimated using a zero-inflated negative binomial distribution with a constant zero-inflation

258   intercept applied to all observations. The effect of immigrant frequency on seed viability was

259   analyzed using a binomial model for immigrants, and a beta-binomial model for residents

260   because binomial models for residents were significantly overdispersed.

261   We assessed the significance of the fixed effect (immigrant frequency treatment) with the

262   best fitting model for each fitness component using Wald Type II Chi-squared tests and assessed

263   the significance of the fixed effects (immigrant frequency treatment, species, and their

264   interaction) for lifetime fecundity using Wald Type III Chi-squared tests (R package *car,* Fox

265   and Weisberg 2019). We tested whether fitness components differed among treatments within

266   species, and whether species differed in lifetime fecundity within each frequency treatment with

267   Tukey post-hoc contrasts (R package *emmeans*, Lenth 2020). We estimated marginal means and

268   bootstrapped (n=500 iterations) 95% confidence intervals with the R package *ggeffects* (Lüdecke

269   2018). We plotted marginal means with *ggplot2* (Wickham 2016) and combined plots with

270   *patchwork* (Pederson 2020).

271

272   **Results**

273   *Reproductive interference in natural populations: an observational transect*

274   Individuals collected along a transect produced a high proportion of viable seeds in zones

275   where only conspecifics were present (average 0.92 for *M. guttatus*; 0.84 for *M. nudatus*), even

276   though heterospecifics were in very close proximity (always within 3m). In contrast, individuals

277   collected in the 3m overlap zone of the two species produced far lower proportions of viable

278   seeds (average 0.53 in *M. guttatus*; 0.66 in *M. nudatus*, Figure 2). Seed viability was positively

279   correlated with the local frequency of conspecific fruit for *M. guttatus* plant sampled along the

280   transect (Pearson correlation coefficient= 0.66; 95% CI= 0.29, 0.86; t = 3.61, df=17, *p* = 0.002),

281   meaning that greater numbers of adjacent conspecific flowers and fruits were associated with

282   greater proportions of viable seeds. However, seed viability was not significantly correlated with

283   the local frequency of conspecific fruit *M. nudatus* plants sampled along the transect (Pearson

284   correlation coefficient= 0.18; 95% CI= -0.28, 0.57; t = 0.79; df = 19; p = 0.44). These data from

285   a natural transition zone suggest that reproductive interference is more likely to be frequency-

286   dependent in *M. guttatus* immigrants than *M. nudatus* immigrants.

287

288   *Experimental Transplants*

289   Seed viability was positively associated with immigrant frequency for immigrant *M.*

290   *guttatus* transplants, but not immigrant *M. nudatus* transplants, consistent with the findings from

291   the observational transect. Resident transplants of both species also suffered from decreased seed

292   viability as immigrant transplants increased in frequency. Unexpectedly, fitness components

293   declined for immigrant *M. nudatus* transplants when they were most common, resulting in

294   immigrant disadvantage in the highest frequency treatment. Immigrant transplants had lower

295   lifetime fecundity, measured as viable seeds per plant, than resident transplants when immigrants

296   were rarest. For immigrant *M. guttatus*, this rarity disadvantage could not be explained by fitness

297   component differences prior to hybridization. In contrast, rarity disadvantage for immigrant *M.*

298   *nudatus* was observed for fitness components prior to hybridization.

299

300   *M. guttatus immigration experiment*

301   As expected from the observational transect, *M. guttatus* immigrant transplants

302   hybridized less and produced a greater proportion of viable conspecific seeds when surrounded

303   by more flowering conspecific neighbor transplants (Figure 3C), as would occur if an intact fruit

304   or multiple seeds colonized a habitat patch occupied by heterospecifics. Seed viability increased

305   with increasing numbers of conspecific neighbors (Wald type II Chi-square test: $\chi2$ = 7.49, df =

306   1, p = 0.006; coefficient estimate = 0.023, SE = 0.009, z-value = -2.74, p = 0.006). The model-

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307   predicted seed viability for rare immigrants increased from 0.24 for immigrants without

308   conspecific neighbors, to 0.78 in the plant with 103 neighbors (Figure 3C).

309   Flowers per plant and total seeds per flower for focal immigrant *M. guttatus* transplants

310   were not significantly associated with the number of flowering conspecific neighbor transplants

311   (Figure 3A, 3B; Wald type II Chi-square tests: flowers per plant $\chi2 = 0.11$, df = 1, $p = 0.73$;

312   seeds per flower $\chi2 = 0.52$, df = 1, $p = 0.47$).

313

314   *Frequency-manipulation reciprocal transplant*

315   *Fitness components in wash*. For immigrant *M. guttatus* transplants in the wash, seed

316   viability was significantly associated with the frequency treatment (Fig 4E, Wald type II Chi-

317   square test: $\chi^2 = 10.602$, df = 2, $p = 0.005$). In contrast, flowers per plant (Fig 4A, Wald type II

318   Chi-square test: $\chi^2 = 1.75$, df = 2, $p = 0.42$) and seeds per flower (Fig 4C, Wald type II Chi-

319   square test: $\chi^2 = 0.44$, df = 2, $p = 0.80$) were not associated with the frequency treatment. Seed

320   viability in immigrant *M. guttatus* transplants increased from 0.14 to 0.48 between the 5%

321   (1G:18N) and 50% (9G:10N) immigrant frequency treatments (Tukey Post-hoc contrast: $p =$

322   0.006, Table S1).

323   For resident *M. nudatus* transplants in the wash, seed viability was significantly

324   associated with the frequency treatment (Fig 4F, Wald type II Chi-square test: $\chi^2 = 11.40$, df = 3,

325    $p = 0.01$), but flowers per plant (Fig 4B, Wald type II Chi-square test: $\chi^2 = 3.20$, df = 3, $p =0.36$)

326   and seeds per flower were not (Fig 4D, Wald type II Chi-square test: $\chi^2 = 6.47$, df = 3, $p = 0.09$).

327   Seed viability in resident *M. nudatus* transplants decreased from 0.83 to 0.68 between the 0%

328   (1G:18N) and 25% (9G:10N) immigrant frequency treatments (Tukey Post-hoc contrast: $p =$

329   0.005, Table S2).

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331   *Fitness components in seep.* For immigrant *M. nudatus* transplants in the seep, flowers

332   per plant (Fig 5A, Wald type II Chi-square test: $\chi^2 =11.74$, df = 2, $p = 0.003$) and seeds per

333   flower (Fig 5C, Wald type II Chi-square test: $\chi^2 = 12.21$, df = 2, $p = 0.002$) were significantly

334   associated with frequency treatment, but seed viability was not (Fig 5E, Wald type II Chi-square

335   test: $\chi^2 = 1.52$, df = 2, $p = 0.47$). Flowers per plant in immigrant *M. nudatus* transplants

336   decreased from ~6 to ~2 flowers per plant between the 25% (4N:15G) and 50% (9N:10G)

337   immigrant frequency treatments (Tukey post-hoc contrast: $p = 0.005$, Table S3). This decline

338   resulted in a lower proportion of *M. nudatus* flowers than expected in the highest immigrant

339   frequency treatment (Proportion immigrant *M. nudatus* flowers by immigrant frequency

340   treatment: 5% treatment (1N:18G) = 0.06, 25% (4N:15G) treatment = 0.33, 50% (9N:10G) =

341   0.29). Seeds per flower in immigrant *M. nudatus* transplants increased from 27 to 64 seeds per

342   flower between the 5% (1N:18G) and 25% (4N:15G) immigrant frequency treatments and

343   decreased from 64 to 29 between the 25% (4N:15G) and 50% (9N:10G) immigrant frequency

344   treatments (Tukey post-hoc contrasts: $p < 0.05$, Table S3).

345   For resident *M. guttatus* transplants in the seep, seed viability was significantly associated

346   with frequency treatment (Fig 5F, Wald type II Chi-square test: $\chi^2 = 21.56$, df = 3, $p < 0.001$),

347   but flowers per plant (Fig 5B, Wald type II Chi-square test: $\chi^2 = 3.14$, df = 3, $p = 0.37$) and seeds

348   per flower were not (Fig 5D, Wald type II Chi-square test: $\chi^2 = 5.80$, df = 3, $p = 0.122$). Seed

349   viability decreased from 0.84 to 0.60 between the 0% (0N:19G) and 50% (9N:10G) immigrant

350   frequency treatments (Tukey post-hoc contrast: $p < 0.001$, Table S4) and decreased from 0.81 to

351   0.60 between the 5% (1N:18G) and 50% (9N:10G) immigrant frequency treatments (Tukey post-

352   hoc contrast: $p = 0.001$, Table S4)

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354   *Lifetime fecundity in wash.* Viable seeds per plant in the wash was significantly

355   associated with immigrant frequency treatment, species, and an immigrant frequency by species

356   interaction (Fig 6A, Wald type III Chi-square test: frequency $\chi^2 = 6.83$, df $= 2$, $p = 0.03$; species

357    $\chi^2 = 12.14$, df $= 1$, $p < 0.001$; frequency x species $\chi^2 = 14.12$, df $= 2$, $p < 0.001$). Immigrant *M.*

358   *guttatus* transplants produced one-sixth as many viable seeds per plant as resident *M. nudatus*

359   transplants in the 5% (1N:18G) immigrant frequency treatment (60 vs 347; Tukey post-hoc

360   contrast $p = 0.008$, Table S5).

361

362   *Lifetime fecundity in seep.* Viable seeds per plant in the seep was significantly associated

363   with species and an immigrant frequency treatment by species interaction (Fig 6B, Wald type III

364   Chi-square test: frequency $\chi^2 = 2.14$, df $= 2$, p $= 0.34$; species $\chi^2 = 18.85$, df $= 1$, $p < 0.001$;

365   frequency x species $\chi^2 = 15.21$, df $= 2$, $p < 0.001$). Immigrant *M. nudatus* transplants produced

366   one-twelfth as many viable seeds per plant as resident *M. guttatus* transplants in the 5%

367   (1N:18G) immigrant frequency treatment (15 vs 187, Tukey post-hoc contrast $p < 0.001$) and

368   one-sixth as many in the 50% (9N:10G) treatment (19 vs 111, Tukey post-hoc contrast $p = 0.001$,

369   Table S6).

370

371   # Discussion

372   In this study, we found that frequency-dependent hybridization likely contributes to

373   habitat segregation between two closely related monkeyflower species. Each species suffered

374   from hybridization when in close contact with heterospecifics in both field experiments as well

375   as in natural populations. Immigrants of both species had lower lifetime fecundity than residents

17

376   when rare. However, this minority disadvantage was caused by different factors for each species.

377   Partitioning of the fitness components demonstrated that rarity disadvantage for *M. guttatus*

378   immigrants was most consistent with reproductive interference, whereas aboveground resource

379   and/or pollinator competition together with reproductive interference all likely contributed to

380   rarity disadvantage for *M. nudatus* immigrants. *M. nudatus* immigrants also suffered fitness

381   reductions consistent with intra-specific competition when they were at their highest frequency.

382   This negative frequency dependence is predicted to facilitate immigrant invasion and break down

383   spatial segregation (Grainger et al. 2019). However, since it was identified only for *M. nudatus*,

384   it is unlikely to lead to breakdown. Our previous studies in this system identified flooding and

385   inbreeding depression as major factors limiting the long-term establishment of *M. nudatus* in

386   habitats occupied by *M. guttatus* (Toll and Willis 2018, Toll et al. 2021). The strong rarity

387   disadvantage for *M. guttatus* immigrants is further evidence that multiple asymmetric processes

388   contribute to the maintenance of strong spatial segregation between these closely related species.

389   In foundational studies on intertidal algae and barnacles, stress tolerance and competition

390   differed between species, determining zonation patterns (Baker 1909, Connell 1961). The factors

391   limiting each species are mechanistically different in our study, but the asymmetric nature of the

392   limitation is fundamentally similar to the mechanisms of zonation found in those classic studies.

393   For example, we found that a strong barrier limiting *M. nudatus* persistence in wetter sites,

394   occupied by *M. guttatus*, was its inability to survive seasonal flooding (Toll and Willis 2018).

395   Persistent flooding occurs during the winter rainy season in the seeps and meadows inhabited by

396   *M. guttatus*. In contrast, flooding is uncommon in quickly draining washes and rocky outcrops

397   inhabited by *M. nudatus*. An additional asymmetrical barrier limiting immigrant persistence in

398   this system is inbreeding depression (Toll et al. 2021). Since immigrants have fewer

399   opportunities to mate with conspecifics, they likely produce most viable seeds through selfing.

400   While recurrent selfing reduces fitness for both species, the reduction is greater for *M. nudatus*

401   especially in foreign habitats. Selfing reduces immigrant *M. nudatus'* fitness to zero in fewer

402   generations than it does for immigrant *M. guttatus* (Toll et al. 2021). Combined, these prior

403   studies provided strong mechanistic rationale for why *M. nudatus* does not invade *M. guttatus*

404   streams. However, why *M. guttatus* does not invade *M. nudatus* washes was still largely

405   unexplained, and the experiments presented in this study suggest frequency-dependent

406   hybridization is a contributing, and probably dominant, factor.

407

408   *Frequency dependence of hybridization*

409   This study identifies frequency-dependent hybridization as a strong barrier limiting the

410   persistence of *M. guttatus* in habitats occupied by *M. nudatus* (Figure 4E). This result is similar

411   to that of reciprocal transplant experiments of *Limnanthus* species and *Gilia* subspecies, where

412   frequency-dependent reductions in fecundity for one species (or subspecies) but not the other

413   was attributed to asymmetric impacts of heterospecific pollen transfer on seed set (Nagy 1997,

414   Runquist 2012, Runquist and Stanton 2012). In contrast, Christie and Strauss (2020) observed

415   symmetric declines in seed viability of reciprocally transplanted *Streptanthus* species, even

416   though experimental crosses reduced seed set asymmetrically (Christie and Strauss 2019). Since

417   heterospecific pollen transfer between *M. nudatus* and *M. guttatus* reduces viable seed set

418   symmetrically (Oneal et al. 2016), asymmetry of the frequency-dependence of seed viability is

419   surprising. However, this asymmetry can be explained when we consider how fecundity

420   components prior to hybridization changed across immigrant frequency treatments for *M.*

421   *nudatus* immigrants. *M. nudatus* immigrants produced the fewest flowers per plant in the highest

422   immigrant frequency treatment, resulting in a lower proportion of *M. nudatus* gametes than

423   suggested by the plant frequency treatment (~50% plants vs ~29% flowers). *M. nudatus* has

424   fewer ovules and pollen grains per flower than *M. guttatus* (Oneal et al. 2016, Ritland and

425   Ritland 1989). This difference in pollen production further amplified the difference in effective

426   gamete frequency than would be expected solely by the manipulation of the frequency of *M.*

427   *nudatus*. Thus, while other studies were able to predict asymmetries in frequency-dependence

428   based on lab crossing data (Runquist and Stanton 2012, Nagy 1997), the asymmetry we observe

429   is driven by ecological interactions (the reduction in flower number with increasing conspecific

430   frequency) and evolved life history differences (pollen production).

431   While naturally occurring *M. nudatus* also hybridizes with *M. guttatus,* seed viability was

432   also not associated with conspecific fruit frequency in the observational transect (Figure 2). Our

433   result differs from that of Gardner and MacNair (2000), who found a significant negative

434   correlation between fruit frequency and seed viability in *M. nudatus* co-occuring with *M.*

435   *guttatus*. Our studies occurred in the same region but in qualitatively different sites. Gardner and

436   MacNair (2000) only sampled *M. nudatus* plants with heterospecifics in close proximity,

437   equivalent to the middle portion of our transect where both species co-occurred but did not

438   extend the transect into pure populations. Compared to *M. guttatus*, seed viability in the

439   transition zone for *M. nudatus* was more variable (Figure 2), but since we did not record

440   phenology or pollinator behavior over time, the factors contributing to this pattern remain open

441   questions.

442   Our observations from the transect study suggests that fitness costs of hybridization are

443   highly localized and decay within meters from the nearest heterospecific (Figure 2), which

444   makes sense given the narrow boundaries that we see in most populations. In the reciprocal

445 transplant experiment, resident transplants of both species suffered decreased seed viability when

446 immigrant transplants were at high frequencies (Figure 4F, 5F), despite the high abundance of

447 native conspecifics for resident transplants at each site. These results suggest that low pollinator

448 constancy leads to high rates of interspecific pollen transfer between species at the local scale,

449 and that most pollination is occurring at a very small scale. These localized and frequency-

450 dependent fitness costs to residents are consistent with the observed stability of spatial

451 segregation patterns. Immigration rates would have to be extremely high, and immigrants would

452 have to be widespread to negatively affect resident populations.

453

454 *Competition may contribute asymmetrically to habitat segregation*

455 The *M. guttatus/M. nudatus* study system presented a unique opportunity to separate

456 effects of hybridization on fitness from effects of competition and abiotic factors. While the

457 combined effects of reproductive and ecological niche overlap could amplify positive frequency-

458 dependence and hasten exclusion (Schreiber et al. 2019), we instead observed declines in flowers

459 per plant and seeds per flower for *M. nudatus* immigrants in their highest frequency treatment.

460 The decline at high densities suggests that some form of competition was more important than

461 release from reproductive interference for *M. nudatus* immigrants at high immigrant frequencies.

462 As the transplants occurred in plastic conetainers, pre-empting below-ground competition,

463 above-ground competition for space, light, and/or pollinator visits may have suppressed flower

464 and seed production in these potted immigrants (Figure 1D). Due to the experimental design of

465 our frequency arrays (Figure 1C), we were unable to estimate the strength of intra- vs

466 interspecific competition (Inouye 2001).

21

467   Immigrant *M. nudatus* transplants produced the greatest number of seeds per flower in

468   the intermediate immigrant frequency treatment (Figure 5C). We do not have a clear

469   interpretation for this phenomenon. However, we hypothesize that pollinator behavior might

470   have driven to this U-shaped pattern in fecundity. Benadi and Pauw (2018) observed that South

471   African Fynbos species received the highest visitation rates at intermediate flower abundances,

472   relative to rare and common species. Future studies on pollinator visitation rates are needed to

473   test this hypothesis.

474   In greenhouse studies, Brigham (2003) failed to find deleterious effects of interspecific

475   competition to either *M. guttatus* or *M.nudatus*. In the field, Brigham (2001) found that effects of

476   intra-specific competition for *M. nudatus* varied by year, site and the timing of germination.

477   Neighbor removals increased, decreased, or had no effect on *M. nudatus* survival (Brigham

478   2001). In our study, we did not observe reductions in *M. guttatus* fecundity prior to hybridization

479   (i.e., in flower or fruit set). These findings accord well with expected competitive ability of *M.*

480   *guttatus*, which occur in streams that are full of both conspecifics as well as a high density of

481   many other plant species. The rocky serpentine washes inhabited by *M. nudatus* are mostly

482   sparsely vegetated, with only occasional areas of high density of monkeyflowers and very few

483   other small forbs. This is consistent with the broader pattern of serpentine endemics occurring in

484   barer (low competition) habitats compared to closely related species (Sianta and Kay 2019).

485   Therefore, it is perhaps not surprising that in our arrays, which intentionally created a density

486   somewhat intermediate and appropriate for both habitats, *M. nudatus* would suffer from

487   competition.

488

489   *Reciprocal transplant experiments and the asymmetry of barriers*

22

490   Reciprocal transplant experiments provide strong evidence of local adaptation, whether

491   between populations, ecotypes, or closely related species (Clausen et at. 1940, Kawecki and

492   Ebert 2004, Hereford 2009). In annual plants, the response variable in these studies is usually

493   seed set. Disentangling the relative effects of interspecific mating from environmental

494   differences between habitats can be achieved by varying the frequencies of each species

495   reciprocally in native and foreign sites. This allows comparisons across frequencies of fitness

496   components that are not affected by interspecific mating interactions (e.g., survival, flower

497   number) to those that could be (e.g., seed abortion, hybrid seed production, Nagy 1997, Runquist

498   and Stanton 2013, Christie and Strauss 2020).

499   In reciprocal transplants, the fitness of local residents and foreign immigrants are

500   compared at native and foreign sites and changes in the rank of fitness, where local residents

501   outperform foreign immigrants at each site, is taken as evidence of adaptation to local conditions

502   (Kawecki and Ebert 2004). Beyond differential habitat-based selection, transplanting individuals

503   of closely related species reciprocally reduces the distance between heterospecific individuals.

504   This in turn increases the chances of reproductive interference. While reciprocal transplant

505   experiments are often used to explain habitat segregation between closely related species (e.g.,

506   Yost et al. 2012, Peterson et al. 2013, Chen and Schemske 2015, Ferris and Willis 2018,

507   DiVittorio et al. 2020), reproductive interactions are rarely considered as important drivers of

508   these patterns (but see Toll and Willis 2018, Christie and Strauss 2020). Since heterospecific

509   pollen transfer can reduce seed set in a pattern indistinguishable from divergent adaptation,

510   deleterious effects of interspecific mating on fitness need to be accounted for in assessments of

511   local adaptation.

512   As demonstrated here, both divergent habitat adaptation (i.e., differences in flooding

513   tolerance) and reproductive interference contribute the spatial separation of the two

514   monkeyflower species. The underlying mechanisms of resident species advantage in reciprocal

515   transplants commonly operate asymmetrically (Popovic and Lowry 2020), and thus a pattern

516   consistent with divergent adaptation may arise as an emergent property of several asymmetric

517   barriers to foreign habitat persistence. Therefore, interpretations of a single fitness metrics (i.e.,

518   flower production, seed set) across species are difficult to interpret without knowledge of

519   frequency- and density-dependent processes and the underlying mechanisms. In a previous

520   study, *M. guttatus* and *M. nudatus* were transplanted reciprocally at equal frequencies (Toll and

521   Willis 2018) and thus, could not test for of minority disadvantage as we did here.

522

Conclusion

524   Patterns of spatial segregation are ubiquitous across animal and plant taxa and diverse

525   environments (Wisheu 1998). In closely related species, negative reproductive interactions can

526   contribute to these patterns if they occur in a frequency-dependent manner and are stronger than

527   intraspecific competition (Schreiber et al 2019, Weber and Strauss 2016). Using both

528   experimental and observational approaches, we found that hybridization operated in a frequency-

529   dependent manner for one species of monkeyflower when invading habitats dominated by its

530   congener but was not frequency-dependent when the other species invaded. Despite this

531   asymmetry, hybridization contributed to rare immigrant disadvantage for both species. Overall,

532   this result, combined with other results from this system (Toll and Willis 2018, Toll et al. 2021),

533   suggests that spatial segregation is an emergent property of many asymmetric barriers operating

534   in complementary and contrasting ways. Therefore, it seems likely that evidence of divergent

535   adaptation from reciprocal transplant experiments of closely related species have the potential to

536   be driven by reproductive interference, in addition to the more commonly invoked differences in

537   stress tolerance and competitive ability. Parsing out the specific effects of each mechanism

538   across many systems will be necessary for a fuller understanding of how closely related species

539   segregate into different habitats.

540

Acknowledgments

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Figure Legends

Figure 1. (A) Schematic of the 2018 *M. guttatus* immigration experiment showing positions of focal immigrant *M. guttatus* plants (filled circle) and conspecific neighbors (open circles) within each block. (B) Photograph of an *M. guttatus* transplant with no conspecific neighbors, surrounded by native *M. nudatus*. (C) Schematic of the 2019 frequency-manipulation reciprocal transplant experiment showing the showing positions of immigrant (X) and resident (•) plants within each immigrant frequency treatment. Figure adapted from Nagy (1997). Five replicates per treatment were planted at each site, resulting in 20 blocks containing residents and 15 blocks containing immigrants at each site. (D) Photograph of an example 50% immigrant (9N:10G) plot in the seep site. The diameter of each pot was 2.54 cm and the largest diameter of each array was 12.7cm. Native *Mimulus* plants within 0.25m of each array were removed prior to transplanting.

Figure 2. Transect of co-occurring *M. nudatus* and *M. guttatus*. Background colors depict the frequency of *M. nudatus* fruit (blue) vs *M. guttatus* fruit (red). Points indicate the proportion of inviable F1 hybrid seeds produced by individual *M. nudatus* (triangles) and *M. guttatus* (circles) plants collected every 21cm along the transect.

Figure 3. Components of fitness from the 2018 *M. guttatus* immigration experiment. Fitness components: flowers per plant (A), total seeds per flower (C, D), and seed viability. Each red circle represents a single immigrant focal *M. guttatus* transplant in a wash dominated by *M. nudatus*. Conspecific neighbor abundance was significantly associated with focal plant hybridization rate.

Figure 4. Predicted marginal means and 95% confidence intervals for components of fitness in the wash transplant site from the 2019 reciprocal transplant experiment. Models: Fitness component = immigrant frequency + (1|Block). Fitness components: flowers per plant (A, B), total seeds per flower (C, D), and seed viability (E, F). Immigrant *M. guttatus* = red circles (A, C, E), Resident *M. nudatus* = blue triangles (B, D, F). Letters indicate groupings from Tukey post-hoc contrasts.

Figure 5. Predicted marginal means and 95% confidence intervals for components of fitness in the seep transplant site from the 2019 reciprocal transplant experiment. Models: Fitness component= immigrant frequency + (1|Block). Fitness components: flowers per plant (A, B), total seeds per flower (C, D), and seed viability (E, F). Immigrant *M. nudatus* = blue triangles (A, C, E), resident *M. guttatus* = red circles (B, D, F). Letters indicate groupings from Tukey post-hoc contrasts.

Figure 6. Predicted marginal means and 95% confidence intervals for lifetime fecundity in a wash (A) and seep (B) in the 2019 reciprocal transplant experiment. *M. nudatus* = blue triangles, *M. guttatus* = red circles. Marginal means were predicted from models using data from plots where both species were present at each site. Models: Lifetime fecundity = Species + Immigrant frequency + Species x Immigrant Frequency + (1|Block). Letters indicate significant groupings from Tukey post-hoc tests.
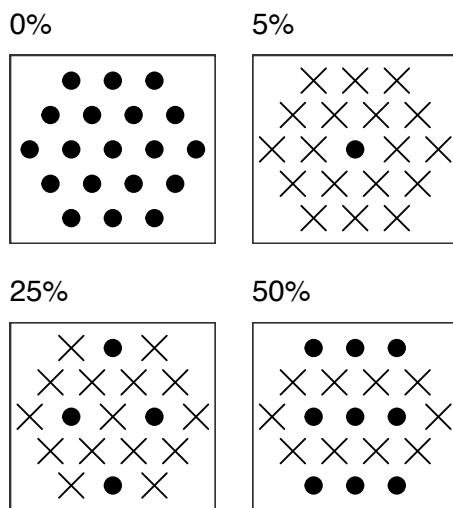
A

M. guttatus immigration experiment
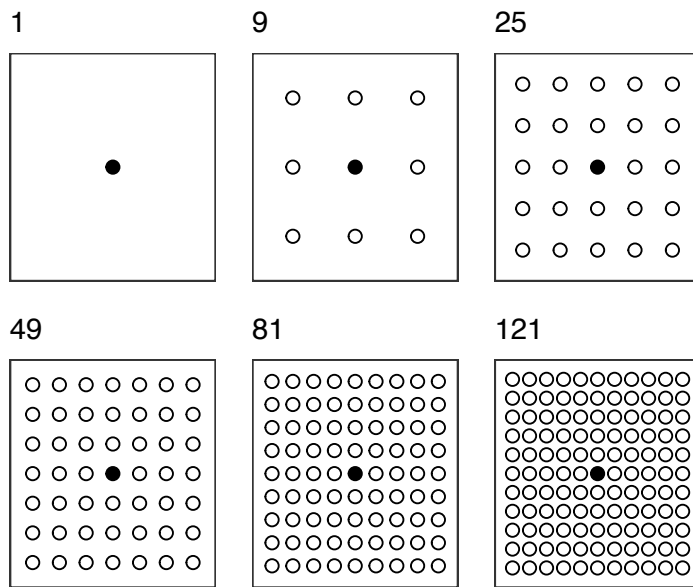
B
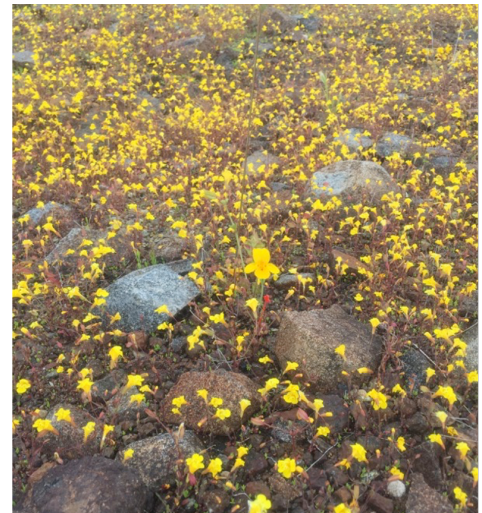
C

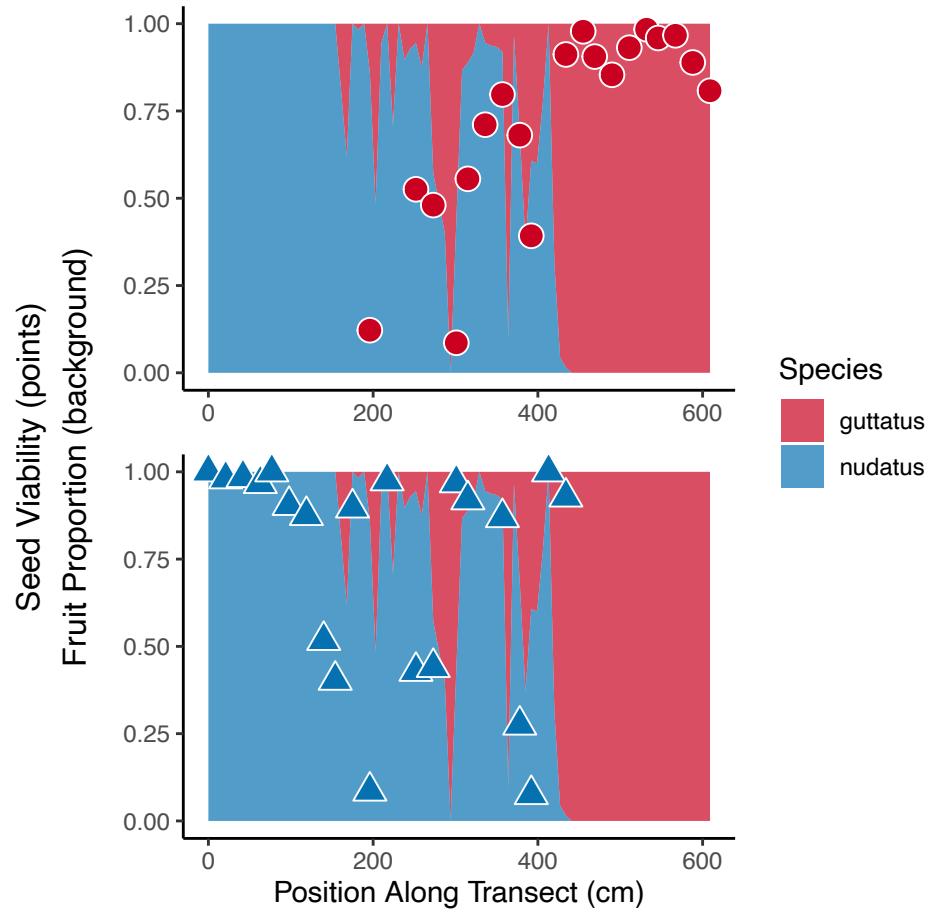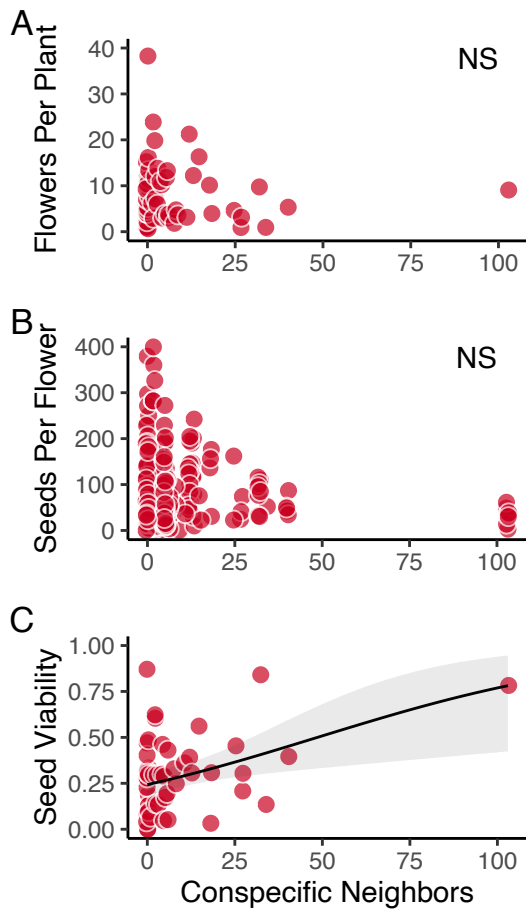Frequency–manipulation reciprocal transplant

D

Figure 2.

Figure 3.

Figure 4.

Figure 5.
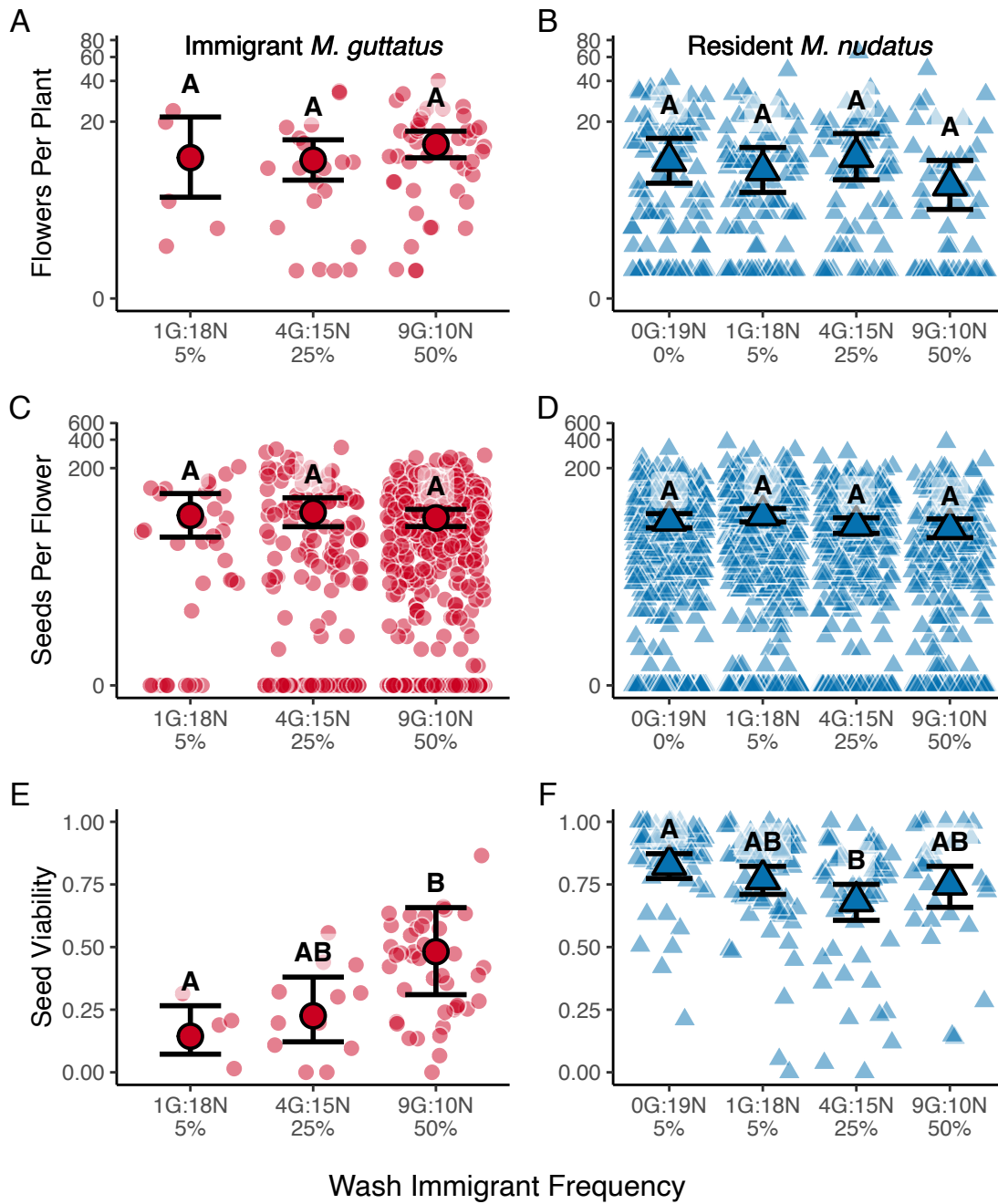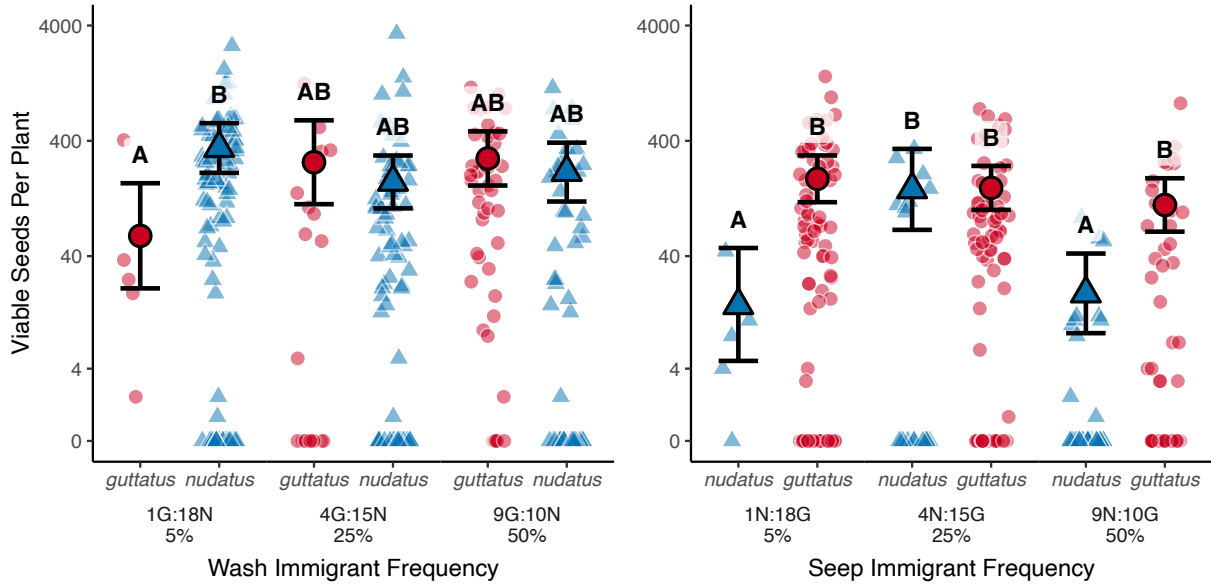
Figure 6.