1   **Title**: Fitness and mating consequences of variation in male allocation in a wind pollinated plant

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9   **Keywords:** gain curves, protandry, mate diversity, monoecy, sex allocation, wind pollination

10   **Abstract:** In hermaphrodites, the allocation of resources to each sex function can influence

11   fitness through reproductive success and mating success. In wind pollinated plants, sex allocation

12   theory predicts that male fitness increases linearly with investment of resources into male

13   function. However, there have been few empirical tests of this prediction. We experimentally

14   manipulated allocation to male function in *Ambrosia artemisiifolia* (common ragweed) in a field

15   experiment and measured mating success using genetic assays. We investigated the effects of

16   various morphological traits and flowering phenology on male fitness, and on male and female

17   mate diversity. Our results provide evidence for a linear relation between allocation to male

18   function and fitness. We find earlier onset of male flowering time increases reproductive success,

19   while later onset flowering time increases the probability of mating with diverse individuals.

20   This research is among the first empirical studies testing the prediction of linear male fitness

21   returns in wind pollinated plants. Our results provide insight into the large investment into male

22   function by wind pollinated plants and temporal variation in sex allocation.

Introduction

Hermaphroditic organisms attain fitness through both female and male sex functions and they can maximize reproductive success through a wide variety of different strategies. Most flowering plants are hermaphroditic and because of vector-mediated gamete transfer, plant mating can be highly promiscuous with individuals mating with numerous sexual partners through either sex function. The ecological and genetic factors that determine reproductive success have been well documented for female function because of the relative ease of assessing fitness through maternal contribution. In contrast, success through male function has been more difficult to quantify because it relies on using molecular markers to identify paternity. With current molecular techniques, we are beginning to gain an understanding of male mating success, including who has mated with whom and how often (e.g. Tomaszewski et al. 2018; Christopher et al. 2019; Santos del Blanco et al. 2019).

Sex allocation theory considers how the fitness acquired through each sex function varies with the investment of limited resources into female or male reproductive structures (Charnov 1982; Charlesworth 1991; Brunet 1992; Emms 1993). In general, hermaphroditic organisms should invest in both sex functions to the point where the marginal fitness returns are equal, at which point investment should turn to the sex function with the more linear, or less decelerating, sex function (Charlesworth 1991; Brunet 1992). The shape of the fitness gain curve is determined by a variety of intrinsic and extrinsic factors, including dispersal dynamics, biotic interactions, sibling competition, and mating patterns (Harder and Thomson 1989; Harder and Barrett 1995; Zhang 2006). While there has been extensive theoretical treatments of sex allocation theory,

45   empirical support for their predictions, particularly regarding fitness through male function,

46   remains limited.

47

48   In sessile plants that rely on external vectors for transferring and receiving pollen, various

49   features of the pollination environment can affect the shapes of fitness gain curves. Many animal

50   pollinated plants likely have diminishing returns on investment in male function due to a variety

51   of processes, including pollinators becoming saturated with pollen after visiting a plant (Lloyd

52   1984), pollinator grooming removing pollen (Harder 1990), and deposition of related pollen

53   causing local mate competition (Charnov 1982). In contrast, in wind pollinated plants the male

54   gain curve is expected to be more linear (Burd and Allen 1988; Klinkhamer et al. 1997; Sakai

55   and Sakai 2003). There are several explanations for this. First, it is unlikely that the air can

56   become saturated with pollen, at least within the scope of biologically plausible pollen

57   production. Second, once pollen is liberated from a plant, pollen loss from the airstream should

58   be stochastic and independent of an individual plant (Niklas 1985). And finally, conspecific

59   pollen captured by stigmas is likely proportional to its concentration in the air (although there

60   might be subtle differences depending on pollen size (Paw U and Hotton 1989; Friedman and

61   Harder 2005). Similar arguments have been made for the male gain curve in sessile spermcasting

62   marine organisms (reviewed in Schärer 2009). Together these factors suggest that for a given

63   individual, producing more pollen should result in siring more offspring (ie. linear relation

64   between allocation to pollen production and fitness).

65

66   Sex allocation theory also explains shifts in relative resource allocation to female or male

67   function with plant size (Klinkhamer et al. 1997; Zhang 2006). Larger and taller plants often

4

68   have more resources to invest in gamete production leading to greater fecundity. This can

69   produce a "budget" effect of plant size on sex allocation. Generally, access to more resources is

70   associated with greater relative allocation to female function (Lloyd 1984; Korpelainen 1998;

71   Chen et al. 2017), because of the greater cost associated with producing seeds and fruit. In

72   addition to the budget effect, size can also have a direct effect on fitness, where larger plants

73   have greater fitness than small plants for a given investment in reproduction. For example, in

74   wind pollinated plants, the physical placement of male flowers at higher positions or at the tips

75   of long branches, results in more effective pollen dispersal and greater siring success (Young and

76   Schmitt 1995; Tonnabel et al. 2019a). The influence of budget and direct effects of plant size on

77   sex function may be aligned, but the underlying mechanism might be through selection on

78   fecundity or through access to more mates.

79

80   Male mating with more than one female partner is almost ubiquitous in the flowering plants

81   although whether this arises as a by-product of selection on siring success in general, or on

82   outcross mate diversity in particular is not clear (Pannell and Labouche 2013). Mating with

83   multiple partners can provide an advantage— regardless of any increase in total fecundity—by

84   increasing the genetic diversity of offspring (Barrett and Harder 2017). Higher mate diversity

85   almost certainly increases the variance in offspring genotypes and may decrease the variance in

86   final offspring number (for example, by minimizing the risk of unsuccessful pairings) which

87   raises the probability of successfully leaving offspring (Gillespie 1974; 1977). Producing a set of

88   genetically diverse offspring increases the probability of generating a "winning" phenotype

89   (Williams 1975; Maynard Smith 1976). Increasing the variance in offspring genotypes is

90   especially beneficial in heterogeneous environments if it produces genotypes that succeed in

91   different conditions, akin to bet-hedging strategies (Antonovics and Ellstrand 1984; Simons

92   2011). Finally, greater mate diversity may reduce sibling competition between related sibs

93   (Karron and Marshall 1990). From the maternal perspective, a common measure of mate

94   diversity is to determine the genetic contribution of different fathers to the seeds on a plant

95   (correlated paternity, r_p: Ritland 2002). This measure can be extended to consider the entire

96   mating portfolio of a plant (Barrett and Harder 2017) through both male and female function,

97   although a full quantification of mate diversity is rare (but see Tomaszewski et al. 2018;

98   Christopher et al. 2019).

99

100   Mating opportunities between plants are influenced by the timing of female and male function

101   (Lloyd 1980). The vast majority of studies on the evolution of flowering time have focused on

102   animal pollinated plants, and fitness through female function (Christopher et al. 2020), but of

103   course selection through the two sex functions need not be in harmony (Delph and Ashman

104   2006). Selection on male flowering time may be driven by mating opportunity or through genetic

105   covariation with other traits (Austen and Weis 2016a). Furthermore, many hermaphroditic and

106   monecious plants have some temporal separation in the onset of female and male function

107   (dichogamy; Bertin and Newman 1993) that leads to a shift in the mating environment (the

108   relative abundance of female and male phase flowers) through time. For example, in protandrous

109   plants, the floral sex ratio shifts from male- to female-dominated during a population's flowering

110   season (Brunet and Charlesworth 1995; Brookes and Jesson 2010). This means that in general,

111   early male-phase flowers encounter pollen competition for few ovules compared with later male-

112   phase flowers that have access to more ovules (Nakamura et al. 1989; Stanton 1994). This effect

113   is weakened if protandry in incomplete and flowering duration is long with substantial overlap in

114   sex function.

115

116   Our primary goal in this study is to use a manipulative field experiment to evaluate the effect of

117   allocation to male function on siring success and mate diversity in a wind pollinated herbaceous

118   plant. We also set out to determine if there were additional benefits accrued through plant size

119   (height, width, and plant biomass) on siring success; and to determine the effect of flowering

120   time on mating opportunities and reproductive success through male function. We used the

121   monoecious herb, *Ambrosia artemisiifolia* (common ragweed), a weedy annual plant that is

122   known to produce a prodigious amount of allergenic pollen. It has been well established in *A.*

123   *artemisiifolia* that sex allocation is both size and resource dependent, where taller plants and

124   those with high light availability invest more in male function (McKone and Tonkyn 1986;

125   Ackerly and Jasieński 1990; Traveset, 1992; Paquin and Aarssen 2004; Friedman and Barrett

126   2011a; Nakahara et al. 2018). To achieve our aims we experimentally manipulated allocation to

127   male function to limit confounding sex allocation with overall condition or plant size and to

128   produce individuals with a range of different male allocation patterns (Emms 1993, Schärer

129   2009). After allowing plants to naturally wind pollinate, we used genetic markers to estimate the

130   progeny sired by each plant. This study represents the first attempt to estimate the male gain

131   curve in a wind-pollinated plant after experimentally controlling for male allocation (similar to a

132   series of studies in free-spawning animals; Yund and McCartney 1994, McCartney 1997, Yund

133   1998, Johnson and Yund 2009) and to quantify mate diversity through paternal function.

134

135   **Methods**

7

136   *Study Species*

137   *Ambrosia artemisiifolia* L. (common ragweed) is an early successional weed that inhabits a

138   broad range of habitats including roadsides, cultivated fields, and disturbed lands (Bazzaz 1974).

139   The species is monoecious, producing male inflorescences at the tips of branches and

140   inconspicuous female flowers in leaf axils (Payne 1963). Ragweed can produce between 200 and

141   6000 seeds depending on the plant's condition (Fumanal et al. 2007), although fecundity is

142   substantially lower when plants are disturbed or transplanted. Plants are usually weakly

143   protandrous at the plant level (i.e. male flowers open before female flowers) and there is

144   plasticity in the degree and order of dichogamy (Friedman and Barrett 2011a). Ragweed is self-

145   incompatible (Friedman and Barrett 2008), so that all seed are outcrossed. In addition, it is an

146   annual plant reproducing entirely through seed (Bassett and Crompton 1975), which allows for

147   more easily quantifying fitness.

148

149   *Experimental Design*

150   Between June 1-5 2017, we collected *A. artemisiifolia* seedlings at the 4-leaf stage from 18

151   natural populations around Syracuse, NY, USA. The populations were selected arbitrarily,

152   ensuring they were approximately 4 km apart (range: 4-9 km). We collected plants from multiple

153   sites to increase the genetic variation in microsatellite loci, which we would later use for

154   paternity assignment. We dug up seedlings and immediately transplanted them into 2.5 cm pots

155   filled with Sunshine Mix4 (Sun Gro Horticulture). We maintained the plants in their pots for 8

156   days, then transplanted them into randomly predetermined locations in three blocks in a field at

157   Syracuse University, NY, USA (43°00'47"N 76°07'07"W). The three blocks were spaced

158   approximately 15m from each other, and each block was covered with groundcloth to inhibit

8

159   weed growth. Within each block, we planted 64 plants in a square grid, with individuals 50cm

160   away from each other. We planted equal numbers of all subpopulations into each block (n=4 per

161   subpopulation per block) and recorded the specific location of each plant. We monitored the

162   plants' condition and replaced any dead plants up to two weeks after transplanting in the field

163   (n=12 transplants).

164

165   We minimized pollen contamination by naturally occurring ragweed by mowing the field around

166   the three blocks every two weeks and manually removing any ragweed surrounding the field site

167   at least twice a week. The larger area around the experimental garden was maintained by

168   Syracuse University and received considerable mowing and horticultural maintenance, which

169   reduced the overall presence of ragweed plants.

170

171   To limit confounding sex allocation with overall condition or plant size (Emms 1993, Schärer

172   2009), we artificially manipulated male allocation by randomly assigning each plant to one of

173   four male allocation categories that differed in their maximum number of branches bearing male

174   inflorescences per plant. We started the manipulations between August 1-5 (which coincided

175   with when the very first plants began flowering) after allowing plants to grow for about seven

176   weeks. We cut male inflorescences from each plant to match its pre-assigned allocation category,

177   and maintained the number of inflorescences by removing a subset of newly emerged male

178   inflorescences twice a week. The four categories had a maximum of 4, 8, 16, or 32 branches

179   bearing male inflorescences per plant. Although the number of branches increased exponentially,

180   subsequent branches have fewer and shorter inflorescences, so this approximated a linear

181   increase in male flowers (Figure S1: category 4 has greater variance because we could only

182   remove excess branches from the randomly assigned individuals).

183

184   We measured plant height from ground level to the base of the inflorescence spike, and measured

185   width at the widest point of the plant including branches. We recorded these size measurements

186   three weeks after transplanting (17 July 2017) and repeated the measurements on 7 August and 7

187   September. We also recorded the date the first female and male flower opened on each plant.

188   Subsequent to the initial difference in onset of female and male flowers, plants continue

189   producing new flowers of both sex functions throughout the blooming period (see Figure 4 in

190   Friedman and Barrett 2011a). Once plants started senescing (between October 2 and 15, 2017),

191   we harvested male inflorescences from each plant and separately collected above-ground plant

192   material. We dried the male inflorescences and above-ground plant material for 3 days at 70°C,

193   then recorded the biomass as proxies of male allocation and plant size, respectively.

194

195   We allowed plants to pollinate naturally within the field. We randomly selected a subset of plants

196   (n=24 per plot, total=72) from which we collected at least 60 seeds twice: once when the plants

197   first matured seeds (between September 12 and 26, 2017), and once before the plants were

198   harvested (between October 1 and 12, 2017). We refer to these plants as 'maternal' plants but

199   recall that the species is monoecious, so these focal 24 plants are also 'paternal' plants. We

200   ensured that the maternal plants were evenly represented among the four male allocation

201   categories. We avoided collecting seeds from plants at the edges of plots as they were not

202   surrounded by experimental plants in all directions.

203

204   *Parent and progeny genotyping*

205   We collected fresh leaf tissue from all experimental plants in the field on August 23-25 for

206   subsequent genotyping. We stored leaf tissue at -80°C until DNA extractions could be

207   performed. On December 21, 2017, we stratified 60 seeds (from an equal mix of early and late

208   collection dates) from each of the 72 focal maternal plants in the dark at 4°C for 16 weeks, and

209   then planted them in 96-well plug trays filled with Sunshine Mix4 mixed with 25% sand. We

210   planted more seeds than we intended to genotype to ensure we had 24 seedlings per maternal

211   plant. The average germination probability of the seeds was 68.5 percent. We maintained plants

212   in the greenhouse under 16-hr photoperiod and 21°C/18°C day/night temperatures. After six

213   weeks, once the majority of plants were at the 4-leaf stage or larger, we collected fresh leaf tissue

214   from 24 progeny per maternal plant, and stored the plates at -80°C until DNA extractions could

215   be performed.

216

217   We extracted DNA from frozen leaf tissue of the 1728 progeny and 192 field plants using a

218   modified CTAB protocol. We then used seven polymorphic microsatellite markers that were

219   previously developed for *A. artemisiifolia* (Genton et al. 2005; Chun et al. 2009) to identify male

220   parentage. We performed polymerase chain reaction using Bio-Rad thermocyclers (see Table S1)

221   and labelled PCR products were analyzed at the Institute of Biotechnology at Cornell University

222   on an ABI 3730xl DNA Analyzer using GeneScan LIZ500 size standard (Applied Biosystems,

223   Foster City USA). We manually scored the genotypes using GeneMarker v.2.2.0 (Soft Genetics,

224   State College USA). We used CERVUS version 3.0.7 (Field Genetics Ltd, London UK), to

225   perform paternity analyses using stringent parameters with a minimum of 3 typed loci and

226   accounting for a 1% genotyping error rate and assigned paternity using a strict (95%) confidence

227   criterion.

228

229   *Data analysis*

230   We assessed reproductive success in three ways. First, we quantified the total number of seeds

231   sired by each plant and refer to this as *male reproductive success*. Second, we quantified the

232   number of unique plants on which a given individual sired seed and we refer to this as *male mate*

233   *diversity*. Third, we quantified the number of seeds sired by different pollen donor plants on a

234   given focal plant and we refer to this as *female mate diversity*. Note that we did not quantify total

235   seed production by plants (female reproductive success). Our primary aim was to evaluate the

236   association between male allocation and the measures of male reproductive success (total

237   number of seeds sired and number of mates), including their relation with each other, and effects

238   of size and flowering time.

239

240   All analyses were performed using R version 4.1.1 (R Core Team 2021). We used generalized

241   linear mixed models (GLMMs) using the glmmTMB package (Brooks et al. 2017) and marginal

242   means were estimated using the package emmeans package (Lenth 2021) We used the DHARMa

243   package for diagnostic tests of residuals (Hartig 2017). We had three sets of models, where the

244   response variable was either male reproductive success, male mate diversity, or female mate

245   diversity. In all models, we included the fixed effects of male allocation, female flowering day,

246   male flowering day, plant width, plant height, biomass, and whether the plant was located on the

247   edge or interior. The latter effect was excluded in models of female mate diversity since all

248   plants were located in the interior; in this model we additionally included germination

12

249 probability of seed. Two random effects were included in all models: block and source

250 population. We identified significant terms in the model using AIC scores and log-likelihood

251 ratios of model fit, and present the simplest model of best fit (except when the term reflected

252 experimental design, like edge effect). We tested for significant differences among categories

253 using Dunn-Sidak adjustment for multiple testing. Recall that we quantified male allocation in

254 two ways—the categorical manipulation the plant received, and the continuous measure of male

255 inflorescence weight. Thus, we ran separate models including either measurement as a fixed

256 effect. The two sets of models produced very similar results, which is not surprising because the

257 two measures of male allocation are strongly statistically associated (Figure S1).

258

259 Models of male reproductive success used a negative binomial distribution with the canonical

260 link function. Additionally, to specifically evaluate the shape of the male gain curve, we fit a

261 power function ($y=ax^b$; Charnov 1979; Johnson and Yund 2009) to the continuous male

262 allocation data. The exponent of the power function (b) describes the shape of the curve. If the

263 exponent does not differ significantly from 1 then reproductive success approximates a linear

264 function of male allocation; if it is significantly <1 or >1, reproductive success is either a

265 saturating or accelerating function of male allocation, respectively. Models of mate diversity

266 required accounting for the number of seeds sired. Thus, we used a binomial model (unique

267 mates/total seeds sired) with prior weights, which best captured the underlying biological process

268 and accommodated the frequent occurrence of the upper bound. Models of female diversity used

269 a poisson distribution with an offset parameter of the number of seeds genotyped and the

270 canonical link function.

271

13

272   We investigated the fitness accrued through male function by mating with additional mates by

273   estimating the slope of a least-square regression between standardized male mating success and

274   standardized male reproductive success ('Bateman gradient': Arnold and Wade 1984). The

275   model included covariates for flowering time, male allocation, edge location, and random effects

276   accounting for block and source population. We compared the likelihood of linear and quadratic

277   terms in the regression using AIC scores and log-likelihood ratios of model fit.

278

279   We considered the spatial dispersal of successful pollen transfer by calculating the Euclidean

280   distance between a pollen donor and the plant on which it sired offspring. Note that because of

281   our experimental design there was a 15m gap between any two blocks. We modeled mating

282   distance using generalized linear mixed models (GLMMs) using the glmmTMB package, with a

283   gamma distribution. To investigate the effect of morphological and phenological variables on

284   dispersal distance, we included male inflorescence weight, plant height, plant width, biomass,

285   female flowering time, male flowering time and edge as fixed effects, and candidate father

286   identity as a repeated random effect. To investigate the relation between mean dispersal distance

287   and reproductive success we modeled the effects of male siring success and male mate diversity

288   as fixed effects. To visualize variation in dispersal kernels, for every pollen donor plant that sired

289   more than 5 seeds, we fit a Weibull distribution to its dispersal kernel, and extracted the shape

290   parameter, κ, and the scale parameter, λ for each pollen donor plant. We also used the software

291   NMπ (Chybicki 2018) to calculate the parameters of the pollen dispersal model. Specifically, we

292   estimated average dispersal distance (δ_p), the shape of the exponential-power dispersal kernel

293   (b_p), the intensity of directionality of dispersal (κ_p), and the prevailing direction of pollen

294   dispersal (α_p).

14

295 **Results**

296 *Identification of mating outcomes*

297 A total of 1257 (72.7%) offspring were assigned paternity, the remaining 471 (27.3%)

298 individuals were excluded because either confidence was low, or no paternity matches were

299 found using the parental genotypes (ie. the seed were sired by plants outside the experimental

300 fields). Of the 192 experimental plants, 176 plants (91.7%) sired seed. Of the 16 plants that were

301 not represented in the paternity of seed, three plants were removed from the analysis because

302 they were typed at less than three loci, and the remaining 13 plants were not identified as the

303 father for any genotyped seed.

304

305 *Pollen dispersal and siring distance*

306 The incidence of mating strongly declined with distance, with a heavy-tailed distribution. Plants

307 sired most of their seeds locally, where siring peaked within half a meter of pollen source and

308 declined rapidly over the 25 m distance of the field (distance between mates: mean = 2.81 m, SD

309 = 5.03 m.; Figure 1A, solid line). Known intermate distances were vastly smaller than the

310 distances between all pairs of plants (ie. all possible mates; t=52.91, P<0.001) and also smaller

311 than the distances of all within-block pairs of plants (t=4.39, P<0.001) —reflecting the

312 predominance of mating amongst near neighbours. Of the seed we successfully genotyped,

313 90.7% were sired by fathers within the same experimental block (1127 of 1242), and only 9.3%

314 were due to longer distance siring events between blocks. None of the morphological or

315 phenological variables we investigated (male inflorescence weight (or allocation category), plant

316 biomass, width, height, the onset of female or male flowering) had any effect on siring distance,

317 and there was also no relation between siring distance and either the number of seeds sired or

15

318   mate diversity. The only variable that was significantly associated with dispersal distance was

319   whether plants were located in the interior or the edge (adjusted for other effects in the model,

320   mean dispersal distance: edge: 2.92 m, 95% CI: 2.38-3.59; interior: 2.24 m, 95% CI: 1.89-2.66;

321   t=1.94, P<0.05).

322

323   There was substantial variation among plants in their fitted dispersal distributions. To visualize

324   the variation in the distribution of inter-mate distances, we plot the fitted Weibull distribution for

325   a random subset of 10 plants (Figure 1B) and also show the trajectories of pollen dispersal for

326   the same subset of 10 plants (Figure 1C). The Weibull distribution is described by the shape

327   parameter, κ, and the scale parameter, λ. Among individual candidate fathers, estimated shape κ

328   had range=0.62-4.74 (mean=1.51) and the estimated scale λ had range=0.72-6.39 (mean=2.30).

329   We next used NMπ (Chybicki 2018) to calculate properties of the pollen dispersal kernel.

330   Similar to the results using other methods, the estimated average dispersal distance (δ_p) was 1.2m

331   with high variance (S.E.=1.3m), the shape of the exponential-power dispersal kernel (b_p) was

332   0.18 (S.E.=0.14), the intensity of directionality of dispersal (κ_p) was 0.48 (S.E.=0.31), and the

333   prevailing direction of pollen dispersal (α_p) was 2.68 with high variance (S.E.=8.93).

334

335   *Male reproductive success*

336   Individual plants sired a mean=6.60 (SD=6.53, Range=0-39) seed, of the successfully genotyped

337   offspring. The effect of our experimental manipulations resulted in plants with greater male

338   allocation siring significantly more seed than plants with less male allocation (Table 1A; Figure

339   2A). Adjusted for other effects in the model, plants in the lowest allocation category sired an

340   average of 4.44 seeds (95% CI: 3.36-5.87) while plants in the highest allocation category sired an

16

341   average of 8.05 seeds (95% CI: 6.28-10.31). When we consider allocation as a continuous

342   variable modeled as male dry inflorescence weight, we found that the number of offspring sired

343   increases significantly with male inflorescence weight (Table 1B; Figure 2B). The exponent of

344   the power function (b=0.85, SE=0.30) saturates slightly, but did not significantly differ from one

345   (t_{168}= -0.45, P=0.65), indicating a significant linear relation between male allocation and siring

346   success ($F_{1,168}$=7.68; P<0.001).

347

348   We found a strong effect of the timing of onset of male flowering on male reproductive success

349   where earlier flowering plants sired significantly more seed (Table 1). Moreover, we found a

350   significant interaction between allocation and male flowering time, whereby only plants in

351   categories 1, 2, and 3 experienced greater reproductive success if they started male flowering

352   earlier (Table 1A; Figure 2C). Plants in the highest allocation category had similar reproductive

353   success regardless of when they flowered (Table 1A; Figure 2C).

354

355   In contrast to our expectation that larger plants would sire more offspring, we discovered that

356   neither plant height nor biomass had a significant effect on the number of seeds sired. Instead,

357   we found that wider plants had greater male reproductive success (Table 1; $\beta \pm$ SE =0.08 $\pm$

358   0.005; Figure S3). Plants located on the edge of the arrays sired significantly fewer seeds than

359   plants located in the interior (edge: mean: 4.97 seeds, 95% CI: 4.06-6.08; interior: mean: 6.52

360   seeds, 95% CI: 5.45-7.82; Table 1).

361

362   *Mate diversity*

17

363   There was a significant positive linear Bateman gradient indicating a strong relation between

364   mating and reproductive success (Figure 3; $\beta \pm$ SE $=0.71 \pm 0.04$, $\chi^2 = 235.41$, $P <0.0001$). We

365   also found a significant quadratic term ($\gamma \pm$ SE $=0.12 \pm 0.03$, $\chi^2 = 24.75$, $P <0.0001$). These

366   relations were independent of male allocation.

367

368   We calculated male mate diversity by quantifying the number of mates to which a pollen-donor

369   plant successfully dispersed pollen and sired seed. Of the genotyped seed, plants donated pollen

370   to and mated with a mean=4.91 (SD=2.55, Range=1-14) different individuals. In raw numbers,

371   males with larger male allocation had greater mate diversity (mean cat1: 3.71; cat2: 4.08; cat3:

372   5.61; cat4: 5.98), however this was likely a consequence of siring more seeds overall. In models

373   that accounted for the total number of seeds sired, we found no significant effect of male

374   allocation on mate diversity (Table 2).

375

376   We previously reported that early flowering plants sired more total seeds (Figure 2C), when we

377   investigate mate diversity it appears that many of those seeds were more likely to be full-sibs

378   (i.e. sired on the same plant). Individuals that flowered later had a probability of greater mate

379   diversity (Table 2; Figure 4A), that is, they donated pollen to and sired seeds on more unique

380   mates. Amongst the morphological characteristics we measured (plant width, plant height, and

381   biomass) none of them had a significant effect on mate diversity, after controlling for the number

382   of seeds sired.

383

384   We quantified female mate diversity as the number of different pollen donor plants that sired

385   seed on a given plant. We genotyped a subset of seed per plant (mean=17.25, SD=2.43,

18

386   Range=11-22). Of these genotyped seed, plants mated with a mean=11.06 (SD=2.64, Range=5-

387   16) different pollen donors. Plants that initiated female flowering later produced seeds that were

388   sired by a greater diversity of pollen donors, than those with an earlier onset to female flowering

389   (Table 2, Figure 4B). There was no association between female mate diversity and the allocation

390   class of the individual, nor any of the measured morphological traits (height, width, biomass),

391   nor germination probability.

392

393   **Discussion**

394   Our results are consistent with the hypothesis that wind pollinated plants experience linear

395   fitness gains with increased male allocation. Experimentally manipulating the number of male

396   flowers elicited predicted changes in the number of offspring sired—plants with more male

397   flowers sired significantly more offspring than plants with fewer male flowers. We found

398   minimal direct effects of plant height on reproductive success, although plants with greater width

399   (longer branches) had higher male fitness. We found significant, and surprising, influences of the

400   timing of flowering, where an earlier onset to male flowering was beneficial for reproductive

401   success, especially for plants with fewer male flowers. In contrast, individuals with later male

402   flowering had relatively higher male mate diversity, and similarly later onset to female flowering

403   was associated with seeds sired by a greater diversity of mates. Finally, our quantification of

404   seed siring distance identified a strongly skewed and heavy-tailed distribution of successful

405   pollen, with most seeds sired on nearest neighbours. Below, we consider the implications of

406   these effects of sex allocation, intermate distance, and flowering time and their possible

407   consequences on the evolution of reproductive strategies in wind pollinated plants.

408

19

409   *Incidental and unintended experimental consequences*

410   Here we address the possible implications of several features of our experiment. First, we

411   artificially created classes of plants with different male allocation to avoid confounding our

412   intended variable of interest with overall condition or budget effects of plant size. This treatment

413   necessarily involved removing male flowers, and for obvious reasons we could not artificially

414   add male flowers. One implication of this is that we cannot test whether further exaggeration of

415   male allocation will result in a saturation of reproductive success. We followed previous workers

416   and fit a power function to our male gain curve data (Johnson and Yund 2009), and an implicit

417   assumption of this is that males could theoretically produce enough pollen to monopolize

418   fertilizations, which is presumably biologically impossible under at least some range of

419   conditions. Second, because of the inflorescence architecture of *A. artemisiifolia*, when we

420   removed male inflorescences we necessarily interfered with the height and width of plants,

421   because male inflorescences are borne on terminal racemes. There was a modest, but significant,

422   reduction in height and width for the lowest allocation treatment, but none of the other treatments

423   differed significantly from each other (Figure S2). Our analyses accounted for this statistically by

424   initially including all effects as covariates, but nonetheless we may have reduced power to

425   identify direct effects of size on reproductive success. Third, we used a square lattice design

426   which meant that plants are not equidistant to their first-order neighbours (i.e. horizontal and

427   vertical neighbours are 0.5 m, but neighbours along the diagonal are 0.7 m). This is not a

428   problem, per se, but should be considered when interpreting intermate distance.

429   *Male reproductive success and pollen dispersal*

430   Our results are consistent with predictions from sex allocation theory that male fitness increases

431   linearly with male allocation. To our knowledge, the only other explicit test of the male gain

20

432   curve in wind pollinated plants comes from a natural stand of white spruce trees, where trees that

433   produced more male cones sired a greater proportion of seeds (Schoen and Stewart 1986). There

434   is some evidence that in the wind-pollinated herb *Mercurialis annua* plants with greater pollen

435   production have greater reproductive success at high density (Tonnabel et al. 2019a). In this

436   species, males with morphological traits that facilitated farther pollen dispersal achieved greater

437   fitness, and only marginally improved fitness with greater pollen production. A previous study in

438   *A. artemisiifolia* demonstrated that male reproductive success increased with height and male

439   reproductive investment (Nakahara et al. 2018), but whether this was due to budget or direct

440   effects of plant size was unclear. Other studies have reported that male reproductive success

441   increases linearly with male allocation in spermcasting marine invertebrates (Yund and

442   McCartney 1994; McCartney 1997), or shown that the male gain curve becomes more linear

443   under certain ecological conditions like intense local sperm competition or mating over larger

444   distances (Yund 1998; Johnson and Yund 2009). Our finding of a linear gain curve means that

445   increased allocation to pollen production should be favored under some ecological conditions.

446   Previous work demonstrated substantial genetic variation for plasticity in male allocation in

447   ragweed (Friedman and Barrett 2011a), so that allocation may respond adaptively to ecological

448   conditions.

449

450   Because we experimentally manipulated plant size, we removed budget effects (i.e. the effect

451   where plants in better condition or larger plants invest more in reproduction because they have a

452   larger resource budget), any remaining effects of plant size on male fitness would more likely be

453   due to direct effects of size (Klinkhamer et al. 1997). Experimentally controlling for male

454   investment, we found no association between plant height and siring success. Similarly, there

455   was no significant effect of plant height on male reproductive success in *M. annua* (Tonnabel et

456   al. 2019). But our result is discordant with a different study in *A. artemisiifolia* that found a weak

457   effect of plant height on siring success, although the effect depended on model assumptions

458   about neighbourhood size (Nakahara *et al.* 2018). All of these studies were conducted in

459   common garden arrays where the benefit of height might be diminished compared to natural

460   settings, because of the reduction in intervening vegetation. Nonetheless. for herbaceous plants

461   occurring in open environments, siring success may not be strongly influenced by plant height,

462   but by other aspects of size that affects the ability to better disperse pollen.

463

464   We identified a benefit of plant width on male reproductive success. Although we did not

465   initially predict this, the architecture of the plant suggests that this effect is due to the

466   aerodynamics of pollen release in wind pollinated plants (Niklas 1985). All objects are

467   surrounded by a layer of still air, and the size of the boundary layer is determined by the size of

468   the solid structure disrupting air flow. An important adaptation for wind pollinated plants is

469   positioning male flowers away from vegetative structures to get them out of the boundary layer

470   and enhance pollen liberation from anthers (Timerman and Barrett 2021). In many species this

471   involves extending the anthers on long filaments that vibrate in the wind (Timerman and Barrett

472   2018). However, in *A. artemisiifolia* dehiscent anthers extend only just below the downward-

473   pointing floret (Payne 1963), and so the position of the staminate head on branches that extend

474   beyond foliage may better expose them to the wind to increase vibration and facilitate the release

475   of pollen into the airstream (Friedman and Harder 2005). Similarly, in *M. annua* selection favors

476   males with wider diameters in combination with longer branches and greater biomass (Tonnabel

477   et al. 2019a). Thus, there is increasing evidence that branching architecture may provide direct

478   beneficial effects for male fitness in wind-pollinated plants.

479

480   Wind-dispersed pollen typically has a leptokurtic distribution from point sources (Bateman 1947;

481   Gleaves 1973; Levin and Kerster 1974), so that the seed set of recipients should decrease rapidly

482   with distance from the pollen donor. Our results are consistent with this. Most plants sire seeds

483   on their nearest neighbours, and the distance between mates shows a strongly fat-tailed

484   distribution. Other studies have found similar results, for example pollination success in *Taxus*

485   declined with plant spacing (Allison 1990); in dioecious *Thalictrum* species female plants at

486   greater distance from males had reduced seed set (Steven and Waller 2007); and in *Festuca*

487   *pratensis*, most pollen was deposited within 75m of donors (Rognli *et al*. 2000). In our

488   experiment, none of the morphological variables we measured had any significant effect on the

489   distance between mates. Similarly, Nakahara et al. (2018) found no effect of plant height on the

490   the maximum distance between mates. These findings are in contradiction to the theoretical

491   expectation that taller wind pollinated plants will have farther pollen dispersal (Burd and Allen

492   1988), and indicate that plant height may have only limited consequences for pollen dispersal in

493   herbaceous plants in open fields. Indeed, successful matings at the farthest distances in our

494   experiment (between blocks) appear to be stochastic. Nonetheless, for plants that sired fewer

495   seeds overall, these long-distance siring events represent a greater proportion of their total seeds

496   sired. While most mating occurs very locally in wind pollinated plants, rare longer-distance

497   mating events may profoundly impact genetic structure and patterns of genetic variation

498   (Loveless and Hamrick 1984).

499

500   *Mating portfolios and the benefits of mate diversity*

501   We found high variance in both male reproductive success and male mating success, as expected

502   under Bateman's principle and sexual selection (Tonnabel et al. 2019b). To investigate the

503   fitness accrued by mating with more partners, we estimated the Bateman gradient for male

504   function, and identified significant positive linear and quadratic (accelerating) terms. This

505   relation was not influenced by allocation category or male inflorescence weight, suggesting

506   consistent benefits for all individuals. Similarly, while individuals with more male flowers had

507   greater mate diversity in absolute terms, this was entirely driven by their concomitant increase in

508   reproductive success. This finding supports the proposition that multiple mating in plants is a by-

509   product of selection on male function to increase siring success (Pannell and Labouche 2013).

510   Mating with multiple female partners (high mate diversity) provides a quantitative advantage

511   through male function, and our data corroborate that mating opportunities constrain male

512   reproductive success.

513

514   There is scant evidence that mate diversity, per se, is under selection or beneficial (Barrett and

515   Harder 2017). However, several lines of evidence suggest that genetic diversity may be

516   advantageous through a process of 'genetic bet-hedging'. When stochasticity is incorporated into

517   measures of natural selection, then fitness depends on both the mean and the variance in

518   offspring number (Gillespie 1974) and increasing the variance in offspring number of a genotype

519   will decrease its fitness (Gillespie 1977). To the extent that mating between any two individuals

520   leads to low fitness (e.g. due to genetic compatibility), then having a greater diversity of mates

521   could reduce the variance in offspring number and increase mean fitness. A second related

522   argument is that genetic diversity within families is beneficial for offspring success in the face of

523   temporal or spatial heterogeneity. When environments are heterogeneous, offspring diversity

524   raises the chances that some offspring succeed, thus decreasing the variance in success and

525   increasing geometric mean fitness (Slatkin 1974; Simons 2011). In animals, various lines of

526   evidence suggest that genetic bet hedging is unlikely to be solely responsible for maintaining

527   polyandry, unless the costs of multiple mating are very low (Yasui 1998; Jennions and Petrie

528   2000). Indeed, in plants the costs of multiple mating are likely low, especially for wind

529   pollinated plants that are not investing in showy flowers, raising the likelihood that genetic bet-

530   hedging provides a selective advantage.

531

532   Mate diversity can also be considered from the perspective of the interactions among siblings –

533   both during seed development and subsequent dispersal and establishment. First, genetic

534   variation among developing embryos provides an opportunity for maternal resources to be

535   distributed to the highest quality embryos and potential abortion of incompatible or low-quality

536   embryos (Zeh and Zeh 1996; Haig and Westoby 1988). However, sibling competition within the

537   developing fruit can be detrimental to both maternal and paternal parents, and several plant

538   reproductive strategies may have evolved to reduce mate diversity within an ovary (Kress 1981;

539   Bawa 2016). For example, generalist, indiscriminate pollinators are most likely to deliver

540   unrelated pollen grains onto stigmas, and there is a well-established association between uni-

541   ovulate flowers and abiotic and generalist pollinators (Charlesworth 1993; Friedman and Barrett

542   2011b). Like most wind pollinated plants. *A. artemisiifolia* has flowers with single ovules, so

543   differential maternal investment would occur among developing fruits on a plant. Second, greater

544   mate diversity increases the number of half-sib families rather than full-sib families, so in species

545   with restricted seed dispersal establishing seedlings are less related. Genetic diversity among

546   seedlings reduces sib-competition thereby increasing maternal (and paternal) fitness (Cheplick

547   1992). Furthermore, to the extent that pollen dispersal is limited, genetic diversity of offspring

548   will reduce biparental inbreeding (Uyenoyama 1986) and any accompanying inbreeding

549   depression (Charlesworth and Willis 2009). Future work is necessary to partition the relative

550   influence of these factors on selection for mate diversity.

551   *The benefits of temporal separation in flowering*

552   The temporal separation of flowering between sexes (dichogamy) generates uneven sex ratios

553   across a flowering season (Brunet and Charlesworth 1995; Sargent and Roitberg 2000), which is

554   paradoxical because frequency-dependent selection should act to equalize the availability of

555   pollen and ovules at every point in time. The benefits of dichogamy (and protandry in particular)

556   include avoiding interference between male and female sex organs (Lloyd and Webb 1986;

557   Bertin 1993) and preventing selfing (Darwin 1876). Neither of these mechanisms are likely

558   responsible for protandry in *A. artemisiifolia* because the species is monoecious and self-

559   incompatible. In a simulation model of the asynchrony in timing of pollen and ovule

560   presentation, Medan and Bartoloni (1998) demonstrated that selection favours protandrous

561   genotypes when there is substantial overlap in male and female function; as they have the

562   greatest access to mates (both pollen and ovules), although flowering too early can be wasteful.

563

564   Several results support this balance of influences on protandry. We found that earlier onset to

565   male function increased male reproductive success (especially for plants that had lower overall

566   male allocation), but later flowering increased the probability of mating with diverse partners.

567   Although we cannot identify the mechanisms here, we speculate that the potential for male

568   mating success depends on competition for access to ovules from other individuals—more

26

569   ovules will be available later in the season, but there will also be greater pollen competition.

570   Plants benefit from early flowering by monopolizing siring opportunities on the few individuals

571   that have female flowers, but shifting male flowering to later when more plants are blooming

572   benefits relative mate diversity. Similarly, slightly delaying female function resulted in seed sired

573   by a more diverse pollen pool. Other mechanisms may be at play, for example if plants with

574   earlier flowering donated their pollen to more fecund mates, had greater pollen competitive

575   ability, or if their mates had declining fruit set (Brunet 1996, Weis and Kossler 2004, Austen and

576   Weis 2016b). The pattern in our results suggests that the staggered onset to male and female

577   flowering benefits both sex functions (in "harmony" between the sexes: Delph and Ashman

578   2006), while fecundity selection and sexual selection through male function are acting in

579   opposing directions on the start of male flowering.

580

581   Sex ratio selection and availability of ovules will eventually constrain the continued evolution of

582   dichogamy, demonstrated in a theoretical model by Sargent et al. (2006). An extended blooming

583   season alleviates some of the costs of skewed sex ratios—the earliest blooming plants sacrifice

584   some male mating opportunities that are lost due to an absence of available ovules, but the

585   wasted pollen represents a small fraction of overall investment. This scenario is exemplified by

586   *A. artemisiifolia*, where flower production increases, and under some conditions accelerates,

587   through time (Friedman and Barrett 2011a: see Figure 4), mitigating the costs of wasted mating

588   opportunities by early blooming flowers. Further, plants flower for 6 weeks or more, and the

589   average degree of dichogamy in our experiment was 4.2 days (range= -9 to 17 days; 9% of plants

590   were protogynous), so plants express both sex functions for the majority of their flowering.

591

592   The duration of overlap between male and female function, or their temporal separation, may be

593   an adaptive response to environmental stochasticity. Under conditions where resource

594   availability is unpredictable and resource acquisition varies during the flowering season, the best

595   allocation strategy should be in favor of the sex function with the higher return on investment

596   (Zhang 2006). Indeed, *Ambrosia artemisiifolia* experiences substantial plasticity in sex allocation

597   and in the degree and order of dichogamy (Paquin and Aarssen 2004; Friedman and Barrett

598   2011a). Here we have demonstrated that the male gain curve is linear or nearly so, and if we

599   assume that the female gain curve is mostly linear (Nakahara et al. 2018), then we would expect

600   a sharp transition between sex functions (Zhang et al. 2006). The gradual transition that we

601   observe may be explained because male flowers are photosynthetic and contribute to available

602   resources (Bazzaz and Carlson 1979), and when reproductive resources are not a pool, but an

603   income, then the constraints on staminate flowering are altered (Burd and Head 1992).

604   Nonetheless, although the male gain curve is linear, there are likely diminishing marginal returns

605   of male investment through time due to the saturation of ovules.

606   *Conclusion*

607   Experimental tests of the shape of male gain curves and factors that affect it are scarce compared

608   to the amount of existing theoretical work. Our study is one of the few studies that empirically

609   test theoretical expectations of male gain curve in wind pollinated plants. In agreement with

610   predictions, we found a linear increase in fitness returns for increasing investment in male

611   function. Fitness through male function is likely limited by the availability of mates, and

612   increasing male investment results in proportional increases in the number of mating partners. In

613   wind-pollinated *A. artemisiifolia* there is likely strong selection on producing more pollen, with

614   the direct benefit increasing siring success and indirectly leading to more mating partners. While

28

615   early onset male flowering (particularly for lower male allocation plants) benefits male

616   reproductive success, later onset results in greater probability of mating with diverse partners

617   through both male and female function. Together this suggest an adaptive role for protandry to

618   adjust the pool of competing pollen for available ovules, and a balance between fecundity

619   selection and sexual selection through male function. Plants with lower male allocation might

620   experience particular benefits by flowering earlier and avoiding the competitive arena during full

621   population blooming, although a more explicit test of this prediction is necessary to rule out

622   alternative explanations.

623   **Author Contributions**: JF conceived the study, AA and JF designed the study, AA conducted

624   the field work and genetic assays, AA and JF analyzed the data and JF drafted the manuscript

625   with input from AA.

626

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Table 1. Models of effects on male reproductive success in *Ambrosia artemiisifolia*. Results for A) model with factorial measure of male allocation (manipulation category), and B) model with continuous measure of male allocation (male inflorescence weight), using negative binomial GLMMs. Random effects (not shown) included the effects of source population and block. Male flowering time is abbreviated MFT, and Male inflorescence weight is abbreviated MIW. Bold *P*-values indicate significant effects.

	Term Condition	**df**	χ^2	***P*-value**
A)	Allocation category	3	9.03	**<0.05**
	Male flowering time	1	9.34	**<0.001**
	Plant width	1	4.44	**<0.05**
	Edge	1	5.52	**<0.05**
	Allocation x MFT	3	9.46	**<0.05**
B)	Male inflorescence weight	1	6.67	**<0.01**
	Male flowering time	1	13.04	**<0.001**
	Plant width	1	5.99	**<0.05**
	Edge	1	5.62	**<0.05**
	MIW x MFT	1	7.23	**<0.01**

Table 2. Models of effects on proportional mate diversity in *Ambrosia artemiisifolia*. Results for (A) male mate diversity model with factorial measure of male allocation, (B) male mate diversity model with continuous measure of male allocation (male inflorescence weight), and (C) female mate diversity. Models in A) and B) used a binomial distribution weighted by the number of seeds sired per individual, model C) used a poisson distribution and an offset parameter of the number of seeds genotyped per individual. Random effects (not shown) in all models included the effects of source population and block. Bold *P*-values indicate significant effects.

	Term Condition	**df**	**x^2**	***P*-value**
A)	Allocation category	3	0.78	0.85
	Male flowering time	1	9.37	**<0.01**
	Edge	1	0.03	0.86
B)	Male inflorescence weight	1	0.01	0.91
	Male flowering time	1	8.96	**<0.01**
	Edge	1	0.09	0.76
C)	Allocation category	1	1.08	0.78
	Female flowering time	1	8.93	**<0.01**
	Germination probability	1	1.59	0.21

Figure 1. (A) The frequency distributions of interplant and intermate distances of *Ambrosia artemiisfolia* plants in experimental arrays. The solid line represents the probability density distribution of distance between mates in our progeny assays, while the dotted and dashed lines represent the probability density distributions of the distance between all plants in the experiment (dotted line) and distances between plants within a block (dashed line). (B) Modelled Weibull distribution of the dispersal kernel for a random subset of 10 experimental plants. (C) Distribution of 192 plants in experimental arrays (black dots) with the curves showing the trajectory of pollen to reach the known mates for the same random subset of 10 experimental plants. Note that the physical distance between the three blocks has been compressed for ease of figure presentation.

Figure 2. Influences on the number of seeds sired (male reproductive success) in *Ambrosia artemiisfolia* plants, including (A) treatment effects of four experimentally manipulated categories of male allocation, (B) male inflorescence weight, and (C) interaction between male allocation category and the julian day of the start of male flowering. Panel B shows the relation described by the power function regression, $y=ax^b$, with $b=0.85$. All results represent model-adjusted and back-transformed values from ln estimates. See Table 1 for statistical details.

Figure 3. Male-specific Bateman gradient in *Ambrosia artemisiifolia* in experimental array conditions. The equation of best fit line is: $y= 0.71x + 0.12x^2-0.1$, see text for statistical details.

Figure 4. Effect of the Julian day of the start of (A) male flowering time and (B) female flowering time on male and female mate diversity (number of unique mating partners through female function), respectively, in *Ambrosia artemiisfolia* plants. Results represent model-adjusted and back-transformed values from ln estimates. See Table 2 for statistical details.
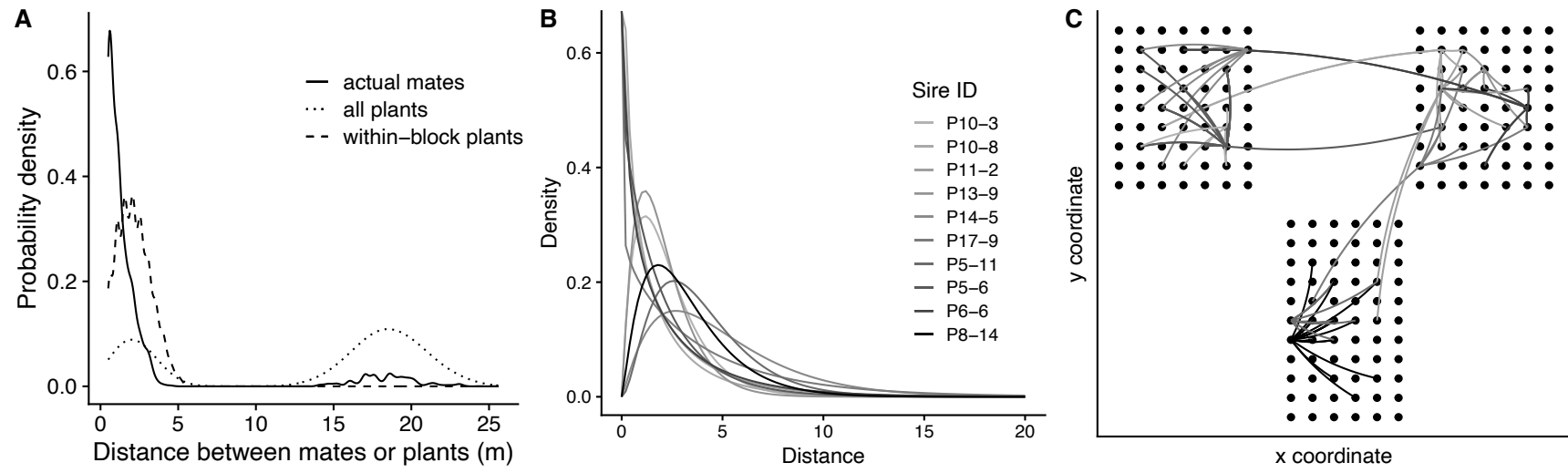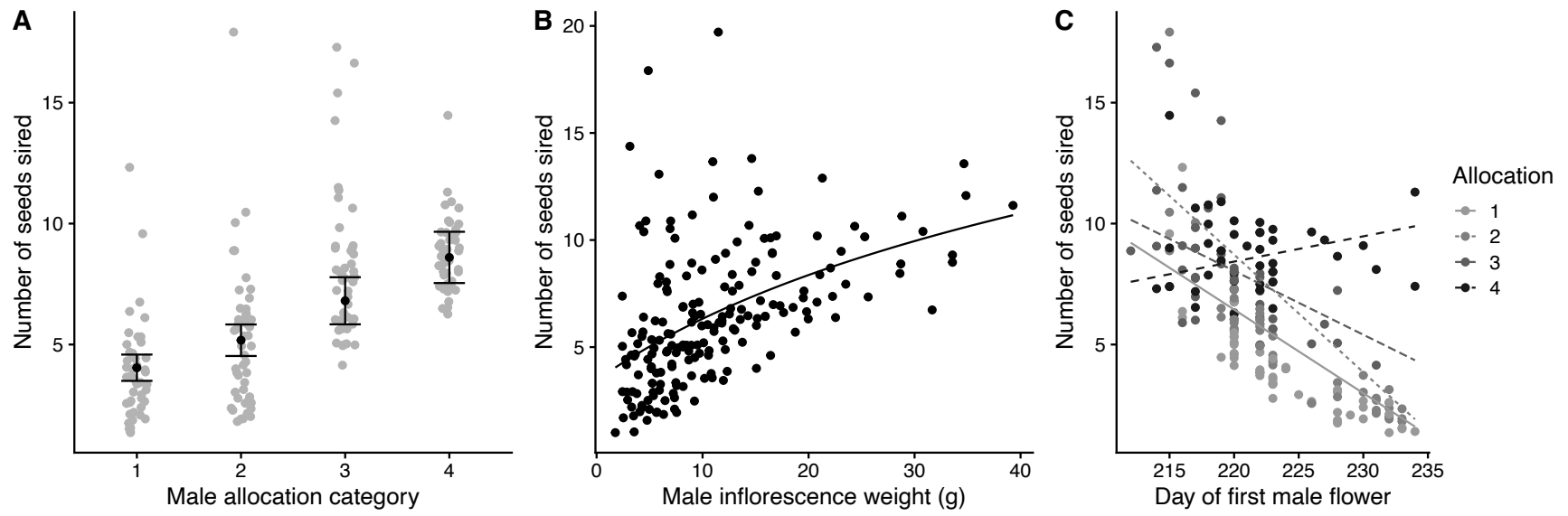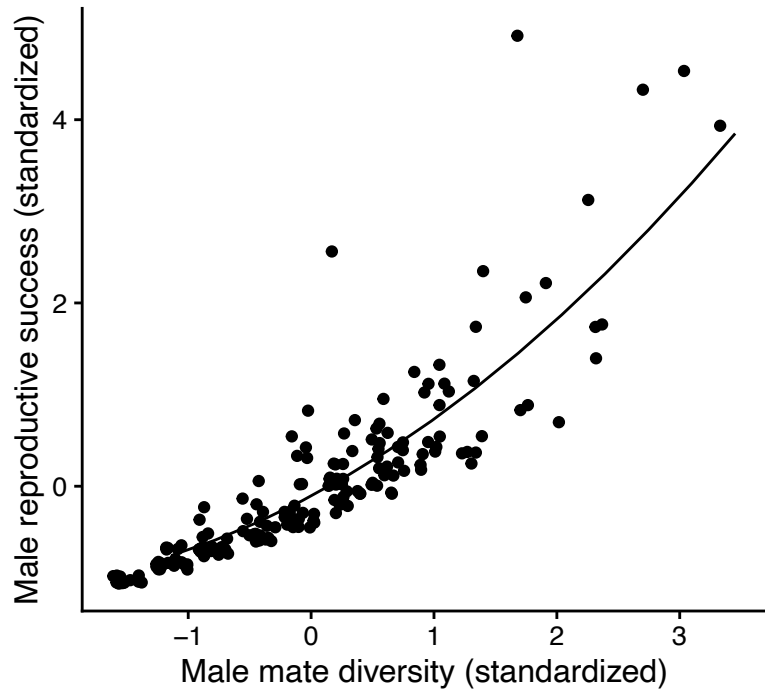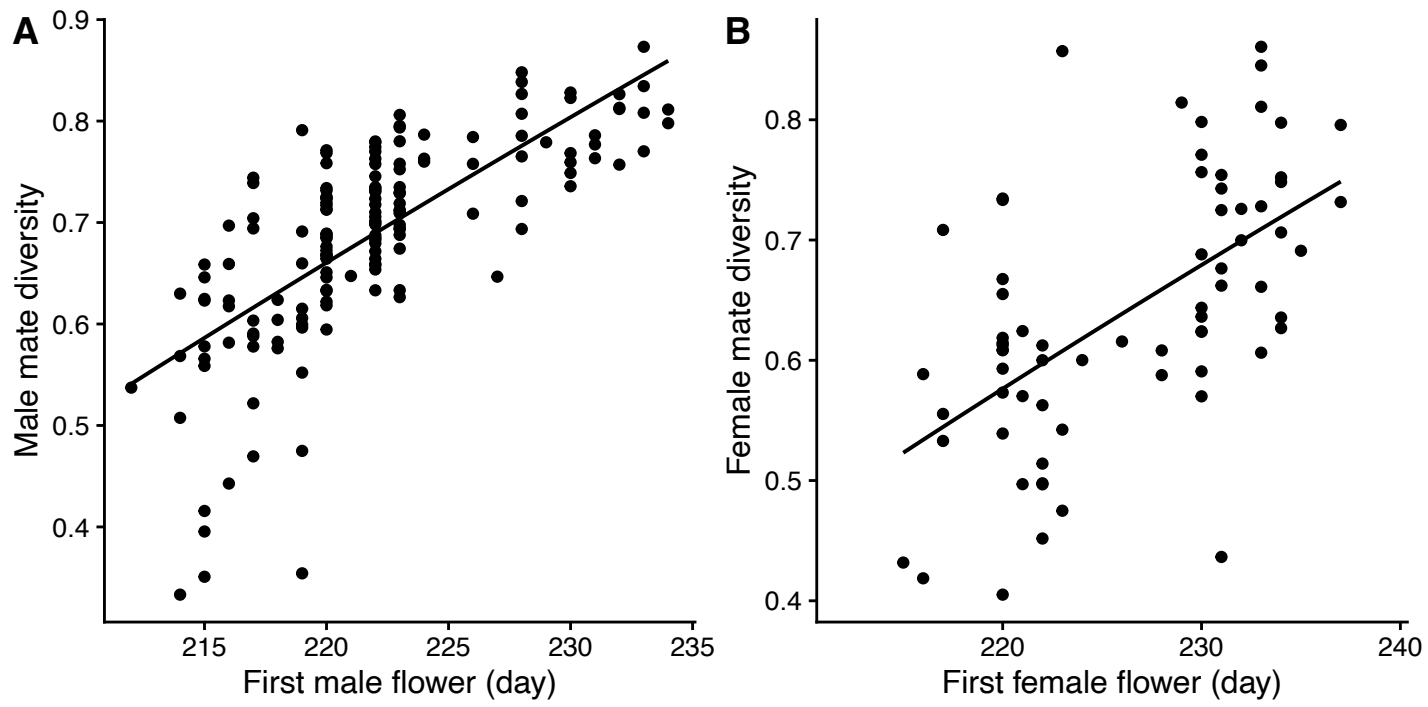
Figure 1.

Figure 2.

Figure 3.

Figure 4.

Supplementary Materials

Figure S1. Effects of the categorical experimental treatment of male flower manipulation on mean (±S.E.) male inflorescence weight in experimental *Ambrosia artemisiifolia* plants. Raw data are plotted, and means associated with different lowercase letters differed significantly.

Figure S2. Effects of the categorical experimental treatment of male flower manipulation on mean (±S.E.) A) plant height, and B) plant width, in experimental *Ambrosia artemisiifolia* plants. Raw data are plotted, and means associated with different lowercase letters differed significantly.

Figure S3. Influence of plant width (cm) on the number of seeds sired (male reproductive success) in *Ambrosia artemiisfolia* plants. Results represent model-adjusted and back-transformed values from ln estimates. See Table 1 for statistical details.
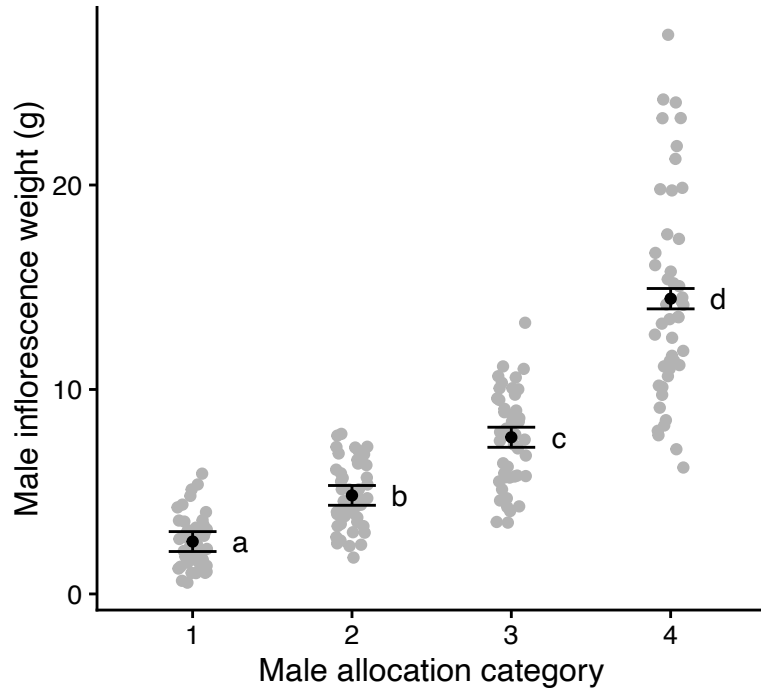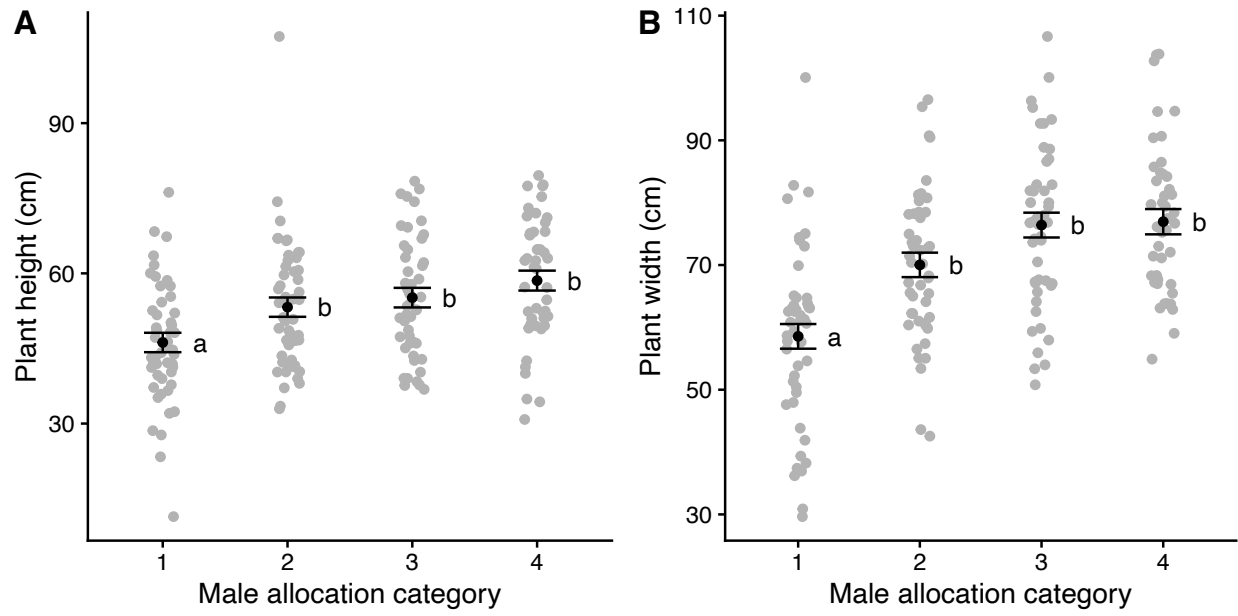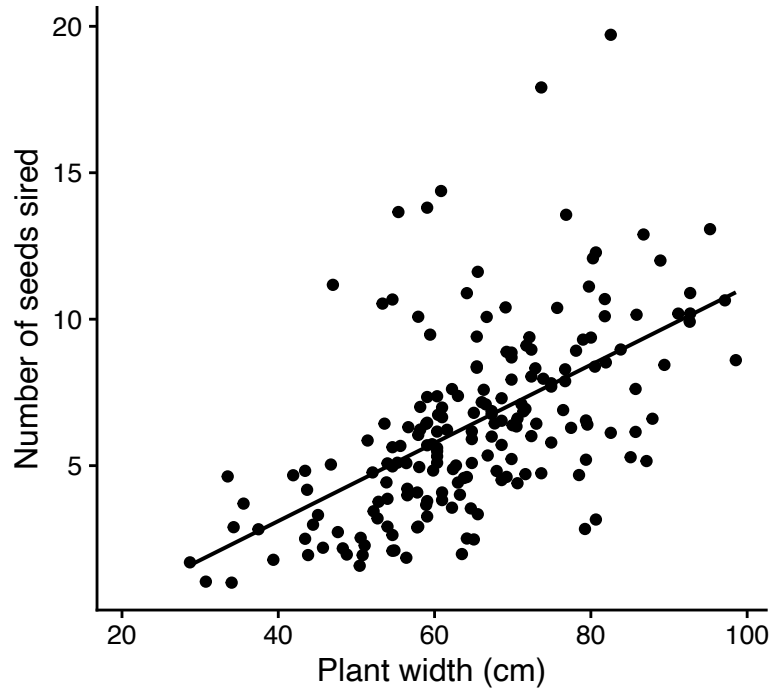
Figure S1

Figure S2

Figure S3

Table S1. Characteristics of the seven microsatellite loci used for paternity analysis. GenBank Accession, number of alleles, expected heterozygosity (H_E), observed heterozygosity (H_O), and PCR conditions for amplification are provided. All PCR reactions included a final volume of 10µl containing approximately 10-20ng of genomic DNA, 2µl of 5X Taq buffer, 0.5µl of 25mM $MgCl_2$, 1µl of 0.25mM of each dNTP, 0.1µl of 10µM of each forward and reverse primers and 0.5U of Taq Polymerase.

Locus	GenBank Accession	Number of alleles	H_E	H_O	PCR Profile
Amb15	AY849309	16	0.90	0.64	95°C for 5m; 40 cycles of: 95°C for 30s, 50°C for 30s; 65 °C for 4m; 72°C for 10m
Amb82	AY849312	28	0.92	0.73	
Ambart09	FJ595151	12	0.88	0.67	
Ambart18	FJ595153	28	0.91	0.51	95°C for 5m; 40 cycles of: 95°C for 30s, 52°C for 30s; 65 °C for 4m; 72°C for 10m
Ambart21	FJ595154	21	0.90	0.62	
Ambart27	FJ595156	32	0.91	0.87	
Amb30	AY849311	16	0.84	0.67	95°C for 5m; 40 cycles of: 95°C for 30s, 51°C for 30s; 72 °C for 30s; 72°C for 10m