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8   Reproductive biology of three wildflower species: Implications of floral compatibility

9   system and pollen limitation for prairie restoration

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22   ABSTRACT. We conducted a hand pollination experiment to test the effects of

23 supplemental pollen and pollen identity on subsequent reproductive success in three

24   American wildflower species: Chamaecrista fasciculata, Gaillardia pulchella, and Salvia

25   coccinea. These species are commonly used in ecological restoration plantings and

26   vary in pollination syndrome and compatibility system. We hypothesized that for all

27   three species, treatments that supplemented pollen would yield greater fruit and seed

28   set. Plants were germinated in a closed greenhouse until flowering, then divided into

29   three treatments: "control", with no supplemental pollen added; "self", with

30   supplemented self pollen; and "outcross", with supplemented outcross pollen. Treated

31   flowers were bagged and allowed to develop to mature fruit stage, and any resulting

32   fruits and seeds were counted and weighed. Results varied between species. Outcross

33   C. fasciculata flowers had significantly higher fruit set than self or control treated

34   flowers, while S. coccinea flowers showed no difference in fruit set between treatments.

35   For S. coccinea, self and outcross flowers tended to produce heavier and more

36   abundant seeds than control flowers, although results were not significant at the p=0.05

37   level. G. pulchella produced no fruits or seeds in any treatment. Our results highlight the

38   importance of considering the breeding system and the pollination needs of plants

39   chosen for prairie restorations. If wild pollinators or genetically diverse plant populations

40   are not initially abundant, plantings may need to be monitored or possibly re-seeded for

41   long-term establishment success.

42   Key Words: restoration, compatibility systems, pollination, pollen limitation

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44   Across the U.S., prairie restoration is increasingly conducted in urban,

45   agricultural, and degraded natural lands to enhance native biodiversity and ecosystem

46   function. To accomplish this, areas are often seeded with a mix of native grasses and

47   forbs, with the hopes that these plant species will form self-sustaining populations

48   (Godefroid et al. 2011; Menges 2008), reviving key ecological processes by supporting

49   plant, animal, fungal, and microbial communities previously absent from the disturbed

50   habitat (Sutherland 2008). Although many restoration managers are knowledgeable

51   about the seed germination requirements of plant species included in their projects,

52   other aspects of plant reproductive biology remain ignored or unknown. One key aspect

53   of plant reproductive biology is the plant's pollination needs, which are strongly driven

54   by its compatibility system.

55   The majority of flowering plant species are pollinated by animals; with nearly 90%

56   of all species exhibiting reproductive output benefited by animal visitation, which can

57   facilitate either self or outcross pollen deposition (Ollerton et al. 2011; Waser 2006).

58   Overall, a plant's level of dependence on pollen dispersal and animal visitation is related

59   to its compatibility system, which is defined by the type of pollen accepted in order for

60   fertilization to occur. In general, a plant species' compatibility falls along a spectrum

61   delimited by two end points, self-compatible (SC) and self-incompatible (SI).

62   Approximately 40% of prairie species are SI (Molano-Flores 2004), where stigma

63   acceptance requires receipt of outcross, not self, pollen (Richards 1997). An SC plant's

64   ability to self-pollinate without the aid of an external vector can allow for reproduction in

65   areas where plant genetic diversity is low or conspecifics are rare (Busch and Schoen

66   2008), or when effective animal pollinators are not present or have low visitation rates

67   (Wilcock and Neiland 2002). However, most SC plants still gain reproductive benefits

68   from animal visitation (Aguilar et al. 2006), and thus SC plants may still require animal

69   pollination in the long-term, as high levels of self-pollination can lead to lowered

70   reproductive success or population fitness (Stephenson et al. 2000). Thus, many plant

71   species have evolved SI systems as a safeguard against the potentially deleterious

72   effects of inbreeding and genetic drift (Takayama and Isogai 2005), though as a result,

73   these species are more dependent on animal-mediated pollen dispersal for reproductive

74   success.

75   In disturbed or fragmented habitats with low pollinator diversity and long

76   distances between genetically diverse conspecific plant populations, restored SI plant

77   communities can face the dual challenge of limited pollinator visitation and limited

78   outcross pollen deposition. Thus, SC plants frequently exhibit higher levels of

79   reproduction in fragmented landscapes than SI plants (Aguilar et al. 2006), and pollen

80   limitation in animal-pollinated plants in fragmented landscapes is often high, limiting

81   plant reproduction in successive years (Aguilar et al. 2006; Godefroid et al. 2011).

82   Considering that plant introductions often have low establishment rates (Godefroid et al.

83   2011), it is important to create habitats that will promote reproductive success. Despite

84   this, plant reproductive compatibility systems and levels of pollen limitation are often

85   ignored when selecting wildflower species for prairie restoration (Molano-Flores 2004).

86   In this study, we conducted a hand pollination experiment to measure the effects

87   of supplemental pollen and pollen identity on the reproductive success of three

88   wildflower species: Chamaecrista fasciculata (Michx.) Greene (Fabaceae), Gaillardia

89   pulchella Foug.(Asteraceae), and Salvia coccinea Buc'hoz ex Etl.(Lamiaceae). These

90   three species are commonly used in restoration plantings (e.g. Dickson and Busby

91   2009; Martin and Wilsey 2006), and have different compatibility mechanisms and

92   pollination needs: C. fasciculata is self-compatible and reliant on visitation by buzz-

93   pollinating bees (Arceo-Gómez et al. 2010; Fenster 1991), G. pulchella is self-

94   incompatible (Heywood 1993; Stoutamire 1977) and visited by a variety of generalist

95   insect pollinators (Ritchie et al. 2016), and S. coccinea is thought to be self-compatible,

96   though cultivars differ in their ability to set seed (Haque and Ghoshal 1981) and is

97   visited by a variety of animal pollinators (Harker et al. 1999). Despite their frequent

98   utilization as restoration plants, to our knowledge no greenhouse studies have

99   simultaneously examined self-compatibility and quantified pollination success of C.

100   fasciculata, G. pulchella, and S. coccinea from cultivated seed mixes, which are more

101   commonly used than wild-collected seeds for ecological restoration projects. We

102   predicted that for all three species, 1) individual flowers (C. fasciculata, S. coccinea) or

103   compound flowering heads (G. pulchella) that received supplementary pollen would

104   produce greater fruit and seed set than flowers receiving no supplemental pollen

105   (control group) and 2) flowers or compound heads receiving outcross pollen would

106   produce greater fruit and seed set than flowers receiving self pollen.

107   MATERIALS AND METHODS

108   The study was carried out in a greenhouse facility at the University of Texas at

109   Austin from February to August of 2014. Wildflower seeds of C. fasciculata, G.

110   pulchella, and S. coccinea were obtained from the Native American Seed Company in

111   Junction, Texas (www.seedsource.com, item numbers 1016, 1005, and 3073

112   respectively). Seeds were planted in Promix soil trays in February of 2014 and were

113   watered every 2-3 days. Seedlings were thinned and potted separately into gallon-

114   sized pots in April 2014. Twenty-nine individuals of C. fasciculata, 112 individuals of G.

115   *pulchella*, and 53 individuals of S. coccinea reached reproductive maturity and were

116   included in the experiment.

117   A hand pollination experiment was conducted in May, June, and early July, and

118   harvesting of mature fruits began in late July and ended in late August. Plants were

119   observed daily, and flowers or compound heads were enclosed in bridal mesh bags

120   (Uline S-10647) just before blooming. Once plants started to flower, they were randomly

121   assigned to one of three treatment groups: 1) "control", where individual flowers or

122   compound heads received no manually supplemented pollen and no further

123   manipulation beyond bagging; or one of two treatments with supplemented pollen,

124   either: 2) "self"; or 3) "outcross". Because each species has different floral structures

125   (Figure 1a-c), they required different methods for the self and outcross pollen

126   treatments. For C. fasciculata we simulated the high frequency vibrations produced by

127   buzz-pollinating bees using a vibrating tuning fork. The tuning fork was brought in

128   contact with anthers for 10 seconds causing the release of pollen which was collected in

129   a petri dish held under the flower (Kearns and Inouye 1993). For the self treatment,

130   pollen was collected from the flower selected for treatment and deposited back onto its

131   own stigma by gently brushing the collected pollen onto the stigma for 10 seconds. For

132   the outcross treatment, pollen was collected from flowers on two other C. fasciculata

133   donor plants, mixed in a petri dish, and then brushed onto the treated flower's stigma for

134   10 seconds. For G. pulchella., because of the impracticality of performing controlled

135   crosses on individual florets within a composite head, anthers from six florets were

136   removed with forceps from the same head (self) or from the compound heads of two

137   other pollen donor plants (outcross), and rubbed for 10 seconds over the entire

138 compound head (sensu Heywood 1993). For $S.$ coccinea, flowers for the self and

139 outcross treatments were first emasculated by removing all pollen-bearing anthers.

140 These anthers were then rubbed onto the same flower's stigma for 10 seconds (self), or

141 were discarded, and all pollen bearing anthers from two other donor plants were

142 brushed directly onto the stigma of the treated flower for 10 seconds (outcross).

143 Forceps, tuning fork, and petri dishes were cleaned with 100% ethanol between each

144 use. After treatment was applied, individual flowers or composite heads were re-

145 bagged to prevent any pollen contamination. This resulted in 256 total treated $C.$

146 fasciculata flowers (81 control, 100 self, and 74 outcross), 483 total treated $G.$ pulchella

147 compound heads (148 control, 159 self, and 176 outcross), and 134 total treated $S.$

148 coccinea flowers (54 control, 39 self, and 42 outcross).

149 Fruits were then allowed to mature fully until harvesting. Mature fruits of each

150 species vary in form (Figure 1 d-f): $C.$ fasciculata fruits are typical legumes containing

151 up to 21 seeds (Fenster 1991); $G.$ pulchella fruits are surrounded by persistent chaff

152 and contained in a compound head, with each floret in the head producing a single-

153 seeded achene (Stoutamire 1977); and $S.$ coccinea fruits consist of up to 4 single-

154 seeded nutlets contained within a persistent calyx (Haque and Ghoshal 1981).

155 Individual legume pods, calyces, and compound heads were harvested and placed in

156 individual $4^{1}/_{16}$ x $2\,^{5}/_{8}$ x $7\,^{7}/_{8}$ inch paper bags (Uline, S-13237) and then stored in a

157 drying chamber for a minimum of two weeks before counting and weighing. As a proxy

158 measurement of full reproductive output, we first weighed the fruit (if any) of each

159 individual treated flower or compound head (including any outer seed pod, pappus,

160 chaff, or calyx; as well as any seeds, achenes, or nutlets; hereafter called "fruit weight").

161   Any seeds, achenes, or nutlets were then separated from the respective outer seed

162   pod, chaff, or calyx and were counted and weighed (hereafter called "seed count" and

163   "seed weight", respectively). Though seed count and weight do not directly assess seed

164   viability, they are common and valuable measures of plant reproductive success

165   (Ne'eman et al. 2010).

166   RESULTS

167   Chamaecrista fasciculata. Fruiting success of C. fasciculata flowers varied

168   markedly between treatment groups: 0% of the control group, 12% of the self pollen

169   group, and 29.7% of the outcross group set fruit, respectively. Outcross treated flowers

170   showed a significantly higher probability of setting fruit compared to self treated flowers

171   (Chi-square, χ^2= 7.413, df=1, p=0.006). Of flowers that set fruit, mean fruit weight

172   (± standard error) was 0.078 (± 0.024) g in the self group and 0.106 (± 0.021) g in the

173   outcross group, with no significant effect of treatment on fruit weight (ANOVA, df=1,

174   F=0.683, p=0.415; Figure 2a). Mean total dry seed weight was 0.025 (± 0.010) g in the

175   self group and 0.030 (± 0.008) g in the outcross group, and mean seed count was 4.16

176   (±1.56) seeds in the self group and 4.68 (± 1.16) seeds in the outcross group. There

177   was no significant effect of treatment on total seed weight (ANOVA, df=1, F=0.164,

178   p=0.688; Figure 2b), nor did the two treatment groups significantly differ in seed count

179   (GLM, df=1, χ^2= 0.463, p=0.496; Figure 2c).

180   Gaillardia pulchella. G. pulchella failed to produce fruit or seed in any of the

181   three treatment groups.

182   Salvia coccinea. More than 80% of S. coccinea flowers produced fruit across all

183   treatment groups: 81.0% of the control group, 82.0% of the self group, and 85.7% of the

184   outcross group set fruit, with no significant difference in probability of fruit set between

185   treatments (Chi-square, χ^2= 0.811, df=2, p=0.666). Of the flowers that set fruit, mean

186   fruit weight was 0.526 (± 0.035) mg in the control group, 0.615 (± 0.0.039) mg in the

187   self group, and 0.614 (± 0.047) mg in the outcross group. Mean seed weight was 0.160

188   (± 0.025) mg in the control group, 0.240 (± 0.030) mg in the self group, and 0.243

189   (± 0.031) mg in the outcross group.

190   Although there was no significant effect of treatment on fruit weight (ANOVA,

191   df=2, F=1.74, p=0.18, Figure 3a), there was a marginally significant effect of treatment

192   on total seed weight (ANOVA, df=2, F=2.95, p=0.0564; Figure 3b). Post-hoc Tukey

193   tests revealed tendencies for higher seed weight in both the self and outcross

194   treatments when compared to the control group, although, after correcting for multiple

195   comparisons, differences were not significant at the p=0.05 level. There was no

196   difference in seed weight between the outcross and self-pollinated groups.

197   Mean total seed count was 1.61 seeds (± 0.22) seeds in the control group, 2.28

198   (± 0.24) seeds in the self group, and 2.22 (± 0.25) seeds in the outcross group. There

199   was also a marginally significant effect of treatment on seed count (GLM, df=2, χ^2= 5.75,

200   p=0.0563; Figure 3c). Similar to the result for S. coccinea seed weight, post-hoc Tukey

201   tests revealed that the control treatment tended to produce fewer seeds than both the

202   self pollen and the outcross groups, although differences were not significant at the

203   p=0.05 level after correcting for multiple comparisons. Seed counts between the

204   outcross and self treatments were nearly identical.

205

206   DISCUSSION

207   Our results indicate that $C.\ fasciculata$ clearly requires animal pollination in order

208   to reproduce. This was not surprising, as this species exhibits a buzz-pollination system,

209   where poricidal anthers require buzzing for pollen release (De Luca and Vallejo-Marín

210   2013; Proença 1992). Because honeybees cannot produce this action, buzz pollinators

211   in the U.S. are largely native bees: including bumblebees, large carpenter bees, and

212   sweat bees (Buchmann 1983; King et al. 2013). Thus $C.\ fasciculata$ may be more

213   susceptible to reduced reproductive success in restorations with low native bee

214   visitation (e.g. Liu and Koptur 2003), even if managed or feral honeybees are abundant.

215   Our results also showed that the outcross treatment had a significantly higher

216   probability of fruit set than the self treatment, with over twice as many outcross flowers

217   setting fruit as compared to the self treated flowers. Thus, our results suggest that

218   populations of $C.\ fasciculata$ are able to produce fruit and seeds if visited by an effective

219   buzz pollinator, and will be even more likely to reproduce if the pollinator is also carrying

220   outcross pollen.

221   Because no $G.\ pulchella$ plant in our experiment produced fruit, we were unable

222   to evaluate any differences between the control and supplemental pollen treatments on

223   fruit or seed production. However, this result is important for restoration managers to be

224   aware of, as genetic diversity of transplanted species is often a concern for both short-

225   term population growth and long-term reproductive success (Williams 2001). This SI

226   species has been shown to be highly susceptible to inbreeding depression, and exhibits

227   lower reproductive success if its parent plants are too genetically similar (Heywood

228   1993; Stoutamire 1977). Wildflower seeds from Native American Seed, our supplier, are

229   generally all harvested from the same field within a single year (personal

230   communication, Native American Seed), and not mixed with different ecotypes,

231   suggesting that the plants grown from our seed mix may have been close relatives. It is

232   possible that other factors (such as greenhouse conditions, etc.) could have limited

233   reproductive output, but we believe this is unlikely, given our success in fruit and seed

234   production for the remaining two species in the greenhouse. G. pulchella is often

235   suggested as an effective native restoration plant, as it has been shown to outcompete

236   highly invasive exotic species (Simmons et al. 2007). However, if plants are unable to

237   reproduce in successive years, due to low genetic diversity in the transplant population

238   and/or low conspecific density in the broader landscape, restoration projects relying on

239   this species may have lower success rates.

240   For S. coccinea, flowers set fruit in approximately equal proportions regardless

241   of treatment type, contrary to our initial hypotheses. Past studies of the genus have

242   suggested an ability of bagged flowers to 'spontaneously fruit', (Aximoff and Freitas

243   2010; Haque and Ghoshal 1981), which was also reflected in our results. However, we

244   did see evidence for benefits of supplemental pollen receipt in the species, as total seed

245   weight and count tended to be higher in both supplemental pollen treatments than in the

246   control. This result suggests that supplemental addition of pollen (regardless of pollen

247   identity) could contribute to increased seed production in this species. Therefore, it is

248   possible that animal visitation in the field may play an important role in increasing total

249   reproductive output and thus enhancing genetic diversity, even if selfing rates are high.

250   Implications for prairie restoration. Our results suggest that it is important to

251   consider the basic biology of potential plant restoration species in order to make

252   appropriate management decisions regarding species selection for prairie restoration.

253 While self-pollinating species can produce seed with reduced or absent pollinators,

254 several generations of self-pollinated reproduction can potentially reduce a population's

255 fitness and diminish offspring's ability to survive in other environments (Aizen et al.

256 2002; Levin 2012), suggesting that a combination of both SI and SC plants could be the

257 most effective long-term strategy for restoring depleted areas. In other words,

258 restoration projects may still benefit from planting SI species that are attractive to

259 pollinators in the first year of planting, even if reproductive success for the species is

260 low in subsequent years, because other members of the plant community can still

261 benefit from the increased pollinator activity (Devoto et al. 2012). Future field-level

262 studies would be helpful in determining the relative levels of pollinator attractiveness of

263 each species over time in prairie restoration projects.

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361

362   FIGURE CAPTIONS

363   Figure 1. Photographs showing the whole floral (a-c) and fruit (d-f) forms of

364   Chamaecrista fasciculata (a,d), Gaillardia pulchella (compound form; b,e), and Salvia

365   coccinea (c,f). Photo credits: a-d © Lady Bird Johnson Wildflower Center; e,f ©

366   University of Texas at Austin (Plant Resources Center, Bio406d)

367

368   Figure 2. Effect of treatment on reproductive output of Chamaecrista fasciculata. None

369   of the "control" treated flowers produced fruit or seeds, and so are not shown in the

370   plots.   For "self" and "outcross" treatments, only data for flowers that actually set fruit

371   are shown. Standard boxplots show the first and third quantiles at the borders of the

372   box, with the horizontal line in the center representing the median value of a) fruit

373   weight, b) total seed weight, and c) total seed count. There was no significant effect of

374   treatment on fruit weight, seed weight, or seed count.

375

376   Figure 3. Effect of treatment on reproductive output of Salvia coccinea. For all three

377   treatments, only data for flowers that actually set fruit are shown. Standard boxplots

378   show the first and third quantiles at the borders of the box, with the horizontal line in the

379   center representing the median value of a) fruit weight, b) total seed weight, and c) total

380   seed count. There was no significant effect of treatment on fruit weight. There were

381   marginally significant effects of treatment on seed weight (ANOVA, df=2, F=2.95,

382   p=0.056), and seed count (GLM, df=2, χ^2= 5.75, p=0.056).