

# working title Compatibility system and stigma size of pollen recipient as main predictors of heterospecific pollen effect

Jose B. Lanuza, Ignasi Bartomeus, Tia-Lynn Ashman, Romina Rader \* <sup>1,2,3</sup>

<sup>1</sup>US Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Atlantic Ecology Division, 27 Tarzwell Drive Narragansett, RI, 02882, USA

<sup>2</sup>Big Name University, Department of R, City, BN, 01020, USA

<sup>3</sup>Estacion Biologica de Donana (EBD-CSIC), E-41092 Sevilla, Spain

\* corresponding author: [barragansljose@gmail.com](mailto:barragansljose@gmail.com)

---

Pollinator sharing can have negative consequences for species fitness with the arrival of foreign pollen. However, the costs of heterospecific pollen are not yet well understood. For this reason, we have conducted a glasshouse experiment where we try to understand how phylogenetic relatedness and the different traits of these species are involved in this process. We experimentally crossed 10 species belonging to three different families: Brassicaceae, Solanaceae and Convolvulaceae. Overall, more than 4000 crosses were done and seed set and pollen tubes were considered as proxy of effect. We found that for all species foreign pollen (50% or less) reduced seed set. Moreover, the seed set reduction is not dependent on the degree of relatedness of the pollen donor. However, the effect is governed by the degree of relatedness and the traits of the species recipient. Our results show that the outcome of heterospecific pollen deposition is determined in greater degree by the traits of the pollen recipient than the pollen donor and that certain traits such as compatibility system are crucial to understand the costs of heterospecific pollen.

---

**Keywords:** heterospecific pollen, plant reproduction, fitness, interspecific competition, phylogenetic distance.

## INTRODUCTION

**Paragraph 1** General idea to our concept

In natural systems plant species normally coexist and share their floral visitors with other species Bascompte et al. (2003). This pollinator sharing from the plant perspective at the pre-pollination stage can be negative due to competition Pauw (2013) or positive due to facilitation Carvalheiro et al. (2014). Once the floral visitor has arrived to the flower, pollen deposition on the stigma can take place and hence ovule fertilization. An increasing number of visits generally correlates with higher chances of

35 fertilization Engel and Irwin (2003). However this is not always the case, among these possible flower  
36 visitors we find also nectar robbers and pollen thieves Inouye (1980) and the quality of pollen that is  
37 deposit on the stigma is also highly relevant to the pollination succes Aizen and Harder (2007).  
38 Moreover, other less study issues in the pollination process are conspecific pollen loss and the arrival of  
39 foreign pollen which can have important detrimental effects on species fitness Morales and Traveset  
40 (2008) Ashman and Arceo-Gómez (2013).

## 41 **Paragraph 2** Introducing topic and knowledge gap

42 Recent studies have advanced in the ecological understanding of heterospecific pollen effect Morales and  
43 Traveset (2008) Ashman and Arceo-Gómez (2013) Arceo-Gómez and Ashman (2016). A general  
44 overview of foreign pollen arrival is that it can play an important role on species fitness but seems to be  
45 context dependent and not always produce a decrease in fitness Morales and Traveset (2008). Part of  
46 this unpredictability is due to the enormous variability of foreing pollen transferred in nature, where  
47 levels between 0 and 75 percent are seen, but most commonly values ranges between 0 and 20 percent  
48 of the total pollen load Bartomeus et al. (2008) Montgomery and Rathcke (2012) Ashman and  
49 Arceo-Gómez (2013) Fang and Huang (2013), being the generalist species the ones that receive greater  
50 loads of heterospecific pollen Fang and Huang (2013). Surprisingly, this low ranges of heterospecific  
51 pollen have been shown to decrease fitness greatly Thomson et al. (1982). Although heterospecific  
52 pollen quantity is fundamental to understand the outcome of the interaction so is the different traits of  
53 both pollen donor and recipient. Ashman and Arceo-Gómez (2013) postulated the first predictive  
54 framework for traits of heterospecific pollen effect, where different traits such as compatibility system  
55 and pollen size among others seems to be crucial to understand foreing pollen effect. Moreover, in Tong  
56 and Huang (2016) an assymetric effect was shown in a crossing experiment between 6 species of the  
57 genus *Pedicularis* where the pollen of long styled species was able to grow the full length of the style on  
58 short styled species but not viceversa. Despite these recent caveats, we still lack empirical evidence to  
59 affirm what are the main traits that drive heterospecific pollen effect for both pollen donor and recipient  
60 at seed production level. Interestingly, to comprehend how these traits interact is also crucial to look at  
61 the phylogenetic relatedness of the species. There is a considerable amount of literature of crosses  
62 between close related species Brown and Mitchell (2001) Arceo-Gómez et al. (2016) Tong and Huang  
63 (2016) but few works focused on heterospecific pollen of far related species Thomson et al. (1982)

64 Galen and Gregory (1989) Neiland and Wilcock (1999) which also show a noteworthy fitness decrease.  
65 Although the effect of close related species is predicted to be greater Ashman and Arceo-Gómez (2013)  
66 the presence of pollen of non related species on multiple species Arceo-Gómez and Ashman (2016) and  
67 the higher chances to coexist with a species that has less niche overlap (Ref) make foreign pollen from  
68 far related species also an important subject of study in order to understand the importance of  
69 heterospecific pollen in natural systems. Notwithstanding, the effect of heterospecific pollen of far and  
70 close related species at community level remains to be explored beyond single pairwise interactions.

### 71 **Paragraph 3** Expanding ideas with examples

72 Interestingly, incompatibility system seems to play an important role in foreign pollen effect where  
73 species that are self incompatible would have stronger barriers towards heterospecific pollen than self  
74 compatible species Ashman and Arceo-Gómez (2013). The type of incompatibility, sporophytic or  
75 gametophytic is related with the place of pollen recognition where the former take place at the stigma  
76 level and the latter occurs within the style, this last late acting pollen recognition mechanism is  
77 associated with greater negative effect Barrett (1988). Remarkably, there is a great variability in  
78 mating systems across populations Whitehead et al. (2018) and therefore predict an effect of foreign  
79 pollen is a bit obscured by the variability within species, however species that are strong selfers or  
80 strong outcrossers have less variability in mating systems and predictions of effect could be more  
81 realistic (see figure 1 from Whitehead et al. (2018)). Moreover, other traits such as number of pollen  
82 grains per flower and number of ovules have been traditionally associated with the type of  
83 incompatibility system where species with higher pollen ovule ratios are predicted to be xenogamous  
84 and species with low pollen ovule ratios autogamous (REF). Selfer species would have a reduction of  
85 herkogamy (REF) and less pollen production per ovule (REF) which can be interpreted as a  
86 reduction of pollen exported into the community. Other morphological traits, like stigma size can be  
87 determinant for the total pollen quantity that a stigma can receive and therefore related to do that  
88 pollen size would also play an important role. Example with pollen here.

### 89 **Paragraph 4** Introducing our experiment

90 The great environmental variability in natural systems and complexity of floral structures make  
91 heterospecific pollination studies a daunting task. Moreover, variation in sampling effort have been

shown to be determinant to characterize pollen transfer interactions Arceo-Gómez et al. (2018). Although plant-pollinator network and pollen network studies can give a first picture of the importance of foreign pollen is necessary to address how its effect is shaped with both traits and relatedness of the species. For this reason, in this study we have created an artificial co-flowering community with 10 species belonging to three different families with different traits where we try to test the following questions: 1) Does heterospecific pollen reduce seed set, if so, 2) Does heterospecific pollen effect depend on any floral trait? 3) Does heterospecific pollen effect depend on the relatedness of the species.

## METHODS

The study was conducted in a glasshouse at University of New England (Armidale, Australia) from November 2017 to March 2018. Rooms were temperature controlled depending on the requirements of the species with day and night temperature differences. The species selected (**Table 1**) belonged to three different families, Solanaceae, Brassicaceae and Convolvulaceae. The criteria of species/family selection was based on close/distant related species (see phylogenetic tree for relatedness fig 1), heterogeneous traits, low structural flower complexity and fast life cycle. For the purpose of the experiment all the species were considered as pollen recipient and as pollen donor (see interaction matrix, fig 2). Species were watered once or twice per day and fertilized weekly (NPK 23: 3.95: 14).

**Table 1**

Family	Genus	Species
Brassicaceae	Brassica	Brassica rapa
Brassicaceae	Brassica	Brassica oleracea
Brassicaceae	Eruca	Eruca versicaria
Brassicaceae	Sinapis	Sinapis alba
Convolvulaceae	Ipomoea	Ipomoea aquatica
Convolvulaceae	Ipomoea	Ipomoea purpurea
Solanaceae	Capsicum	Capsicum annuum
Solanaceae	Petunia	Petunia integrifolia

Family	Genus	Species
Solanaceae	Solanum	Solanum lycopersicum
Solanaceae	Solanum	Solanum melongena

## 109 Hand-pollination

110 Foreign pollen effect was studied through two different treatments, one with 50% conspecific pollen and  
111 50% heterospecific pollen and a second one with 100% foreign pollen (N=10). Therefore, 180 different  
112 combinations were performed with N=10. Seed set was the proxy of effect for all our treatments.  
113 Moreover, hand cross pollination, hand self pollination, apomixis (bagged emasculated flowers) and  
114 natural selfing were tested for each species (N=10). Flowers were emasculated the day prior anthesis  
115 and hand pollinated next day with a toothpick. Hand-pollination was conducted with 3-4 gentle  
116 touches on the stigma surface. The mixes of pollen were realized on an eppendorf based on the pollen  
117 counts made with Neubauer chamber (each anther was counted 4 times for 20 different anthers per  
118 species). In order to confirm that the treatments applied were 50-50 percent pollen, for each focal  
119 species the total stigmatic load of pollen was counted from one donor of each family (N=3).

## 120 Traits and evolutive distance

121 The traits measured for each species were pollen per anther, number of ovules, stigma width and length  
122 and stigmatic area, style width and length, ovary width and length. Moreover stigma type was tested.  
123 Pollen was counted for 20 anthers of each species with 4 replicates per sample with an hemocytometer.  
124 Previously anthers were squashed on a known solution with the pipette tip and homogenized with a  
125 vortex for 30 seconds. Ovule number was counted with the help of an stereomicroscope and a small  
126 grid over a petri dish from 15 randomly selected flowers. The different morphometrical traits were  
127 measured with a digital stereomicroscope. Levels of self incompatibility were estimated by dividing the  
128 the fruit set of hand self pollination by hand cross pollination Lloyd and Schoen (1992).

## 129 Analysis

130 We used the statistical language R (R Core Team 2018) for all our analyses. To test the effect of

131 heterospecific pollen, we subtracted to the seed set of hand cross pollination the seed set of  
132 heterospecific pollen treatments. Therefore, small values mean low effect and viceversa. To be able to  
133 compare among species, seed set was previously scaled with mean 0 and standard deviation of 1. In  
134 order to see correlations between heterospecific pollen effect and traits we performed Mantel test  
135 between the matrix of effect and the distance matrix of each trait (euclidean distances). Moreover,  
136 Mantel test was also conducted between heterospecific pollen effect and the square root of the matrix of  
137 phylogenetic distance due to improvement in the statistical power (Letten & Cornwell 2014). We  
138 explored also the relations between traits and heterospecific pollen effect through generalized mixed  
139 models where the response variable was heterospecific pollen effect, the independent variable the  
140 different traits and the random effects the different treatments per species. Moreover, pairwise evolutive  
141 distances were calculated with MEGA7 for two kinds of markers: 1) Internal transcribed spacer (ITS)  
142 and 2) ribulose-bisphosphate carboxylase (RBCL). The sequences of interest were downloaded from  
143 NCBI GenBank and the phylogenetic tree constructed by maximum likelihood with MEGA7.

144 Phylogenetic signal of traits?

## 145 RESULTS

146 Results of hand cross pollination, self hand pollination, natural selfing and apomixis are presented in  
 147 **Table 2.** Heterospecific pollen reduced seed set significantly with the 50-50% heterospecific pollen  
 148 treatments for 65% of the pairwise interactions  $p < 0.05$ . Across families we found a very similar effect  
 149 but when species were looked at species level they responded differently even within the same family, for  
 150 instance for two species of the Brassicaceae family *Brassica oleracea* and *Eruca versicaria* we found  
 151 very contrasting effects of foreign pollen where for the first one, all donors reduce seed set significantly  
 152 and for the second, just two species did out of nine. The 100% foreign pollen treatments barely  
 153 produced seeds or fruits and just for *Sinapis alba* we did not find significant differences between the  
 154 hand cross pollination and one treatment with pollen from a confamilial. Solanaceae species with berry  
 155 fruit type developed small fruits or even normal fruits in some cases. *S. lycopersicum* seems to  
 156 produced small fruits (35% of the treatments) independently of pollen and pollen donor due to also  
 157 apomictic treatments did, never normal size. *C. annuum* produced some fruits (9%) of both small and  
 158 normal size and finally *S. melongena* produced seedless normal fruits with just confamilial pollen (3%),  
 159 for both species seems that fruit formation was induced by pollen on the stigma because of no  
 160 production of fruit in apomictic treatments.

161 **Table 2.** Percentage of seeds produced per ovule for the ten species used in the experiment. The  
 162 treatments presented are hand cross pollination, hand self pollination, natural selfing and apomixis  
 163 (emasculated flowers).

Species	Cross	Self	Natural_selfing	Apomixis
Brassica oleracea	32.06897	0.0000000	0.00000	0
Brassica rapa	44.97041	0.0000000	0.00000	0
Eruca versicaria	23.75000	0.4166667	0.00000	0
Sinapis alba	43.33333	48.3333333	5.00000	15
Ipomoea aquatica	40.00000	30.0000000	20.00000	0
Ipomoea purpurea	31.66667	86.6666667	31.66667	0
Capsicum annuum	100.00000	66.2240664	23.48548	0

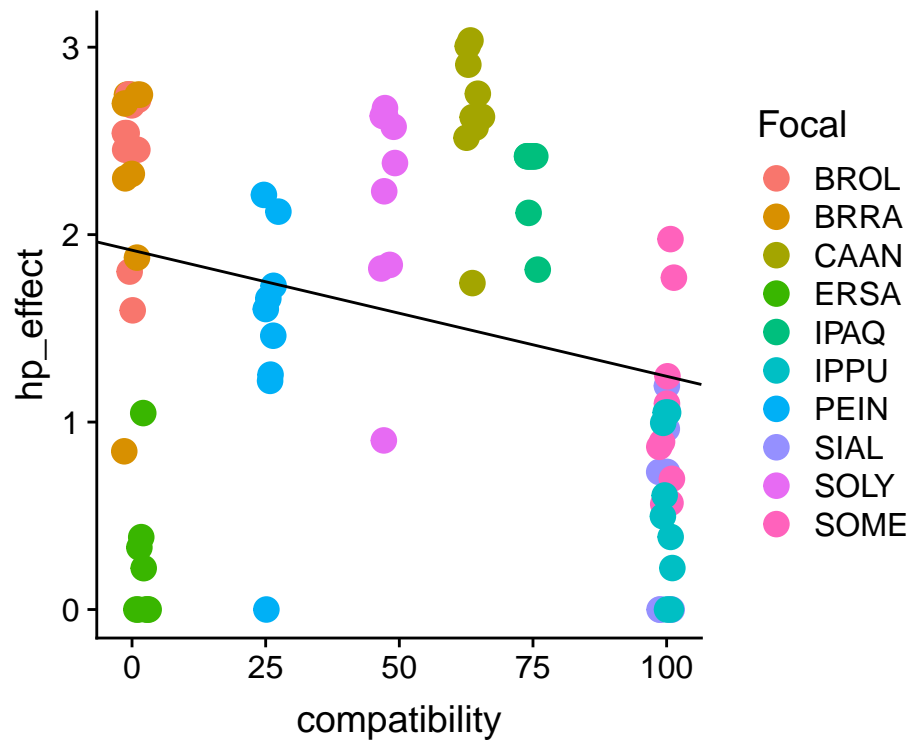
Species	Cross	Self	Natural_selfing	Apomixis
Petunia integrifolia	100.00000	24.7727273	0.00000	0
Solanum lycopersicum	90.38043	43.4782609	70.00000	0
Solanum melongena	60.47525	87.9702970	21.56436	0

164 Mantel test indicates that a possible correlation exist between heterospecific pollen effect and the  
165 evolutive distances, for ITS and RBCL markers we had r coefficients of 0.29 and 0.25 respectively  
166  $p < 0.05$ . Moreover, Mantel test indicates that also a possible correlation between stigma width and  
167 stigma type exist. Trait correlations were also explored with ... and we found that...

168 Fix mantel test selfing rates and change it for compatibility index...

169 Fix this to GLMM? Yep I have to...





## 173 CONCLUSIONS

## 174 ACKNOWLEDGEMENTS

## 175 REFERENCES

- 176 Aizen, M. A., and L. D. Harder. 2007. Expanding the limits of the pollen-limitation concept: Effects of  
177 pollen quantity and quality. *Ecology* 88:271–281.
- 178 Arceo-Gómez, G., C. Alonso, T.-L. Ashman, and V. Parra-Tabla. 2018. Variation in sampling effort  
179 affects the observed richness of plant–plant interactions via heterospecific pollen transfer: Implications  
180 for interpretation of pollen transfer networks. *American journal of botany* 105:1601–1608.
- 181 Arceo-Gómez, G., and T.-L. Ashman. 2016. Invasion status and phylogenetic relatedness predict cost  
182 of heterospecific pollen receipt: Implications for native biodiversity decline. *Journal of Ecology*  
183 104:1003–1008.
- 184 Arceo-Gómez, G., R. A. Raguso, and M. A. Geber. 2016. Can plants evolve tolerance mechanisms to  
185 heterospecific pollen effects? An experimental test of the adaptive potential in clarkia species. *Oikos*  
186 125:718–725.
- 187 Ashman, T.-L., and G. Arceo-Gómez. 2013. Toward a predictive understanding of the fitness costs of  
188 heterospecific pollen receipt and its importance in co-flowering communities. *American Journal of*  
189 *Botany* 100:1061–1070.
- 190 Barrett, S. C. 1988. The evolution, maintenance, and loss of self-incompatibility systems. *Plant*  
191 *reproductive ecology: patterns and strategies*:98–124.
- 192 Bartomeus, I., J. Bosch, and M. Vilà. 2008. High invasive pollen transfer, yet low deposition on native  
193 stigmas in a carpobrotus-invaded community. *Annals of Botany* 102:417–424.
- 194 Bascompte, J., P. Jordano, C. J. Melián, and J. M. Olesen. 2003. The nested assembly of plant–animal

mutualistic networks. *Proceedings of the National Academy of Sciences* 100:9383–9387.

Brown, B. J., and R. J. Mitchell. 2001. Competition for pollination: Effects of pollen of an invasive plant on seed set of a native congener. *Oecologia* 129:43–49.

Carvalho, L. G., J. C. Biesmeijer, G. Benadi, J. Fründ, M. Stang, I. Bartomeus, C. N. Kaiser-Bunbury, M. Baude, S. I. Gomes, V. Merckx, and others. 2014. The potential for indirect effects between co-flowering plants via shared pollinators depends on resource abundance, accessibility and relatedness. *Ecology letters* 17:1389–1399.

Engel, E. C., and R. E. Irwin. 2003. Linking pollinator visitation rate and pollen receipt. *American Journal of Botany* 90:1612–1618.

Fang, Q., and S.-Q. Huang. 2013. A directed network analysis of heterospecific pollen transfer in a biodiverse community. *Ecology* 94:1176–1185.

Galen, C., and T. Gregory. 1989. Interspecific pollen transfer as a mechanism of competition: Consequences of foreign pollen contamination for seed set in the alpine wildflower, *polemonium viscosum*. *Oecologia* 81:120–123.

Inouye, D. W. 1980. The terminology of floral larceny. *Ecology* 61:1251–1253.

Lloyd, D. G., and D. J. Schoen. 1992. Self-and cross-fertilization in plants. i. functional dimensions. *International Journal of Plant Sciences* 153:358–369.

Montgomery, B. R., and B. J. Rathcke. 2012. Effects of floral restrictiveness and stigma size on heterospecific pollen receipt in a prairie community. *Oecologia* 168:449–458.

Morales, C. L., and A. Traveset. 2008. Interspecific pollen transfer: Magnitude, prevalence and consequences for plant fitness. *Critical Reviews in Plant Sciences* 27:221–238.

Neiland, M., and C. Wilcock. 1999. The presence of heterospecific pollen on stigmas of nectariferous and nectarless orchids and its consequences for their reproductive success. *Protoplasma* 208:65–75.

Pauw, A. 2013. Can pollination niches facilitate plant coexistence? *Trends in ecology & evolution*

219 28:30–37.

220 R Core Team. 2018. R: A language and environment for statistical computing. R Foundation for  
221 Statistical Computing, Vienna, Austria.

222 Thomson, J. D., B. J. Andrews, and R. Plowright. 1982. The effect of a foreign pollen on ovule  
223 development in *diervilla lonicera* (caprifoliaceae). *New Phytologist* 90:777–783.

224 Tong, Z.-Y., and S.-Q. Huang. 2016. Pre-and post-pollination interaction between six co-flowering  
225 *pedicularis* species via heterospecific pollen transfer. *New Phytologist* 211:1452–1461.

226 Whitehead, M. R., R. Lanfear, R. J. Mitchell, and J. D. Karron. 2018. Plant mating systems often vary  
227 widely among populations. *Frontiers in Ecology and Evolution* 6:38.



## 229 List of Figures

230	1	The effect of heterospecific pollen (scaled see set) is represented in function of the	
231		compatibility system (self/cross*100) for the the different species. Each coulored dot	
232		represents the interaction of a focal species with a different pollen donor. . . . .	9