

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 * ^{1,2,3}

¹US 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

²Big Name University, Department of R, City, BN, 01020, USA

³Estacion Biologica de Donana (EBD-CSIC), E-41092 Sevilla, Spain

* corresponding author: 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 most ecosystems, plant species normally coexist and share their floral visitors with other species Bascompte et al. (2003). From the plants' perspective, this pollinator sharing can be positive due to facilitation Carvalheiro et al. (2014) or negative due to competition at the pre-pollination stage Pauw (2013). An increasing number of visits often correlates with higher chances of fertilization Engel and Irwin (2003). However this is not always the case, among these possible flower visitors there are also

nectar robbers and pollen thieves Inouye (1980). Receiving both sufficient quantity and quality deposited on the stigma is thus highly relevant to the pollination success of the plant Aizen and Harder (2007).

Paragraph 2 Introducing topic and knowledge gap

By visiting many plant species, many pollinators are responsible for conspecific pollen loss and the transport of foreign pollen, both of which can have important detrimental effects on species fitness Morales and Traveset (2008); Ashman and Arceo-Gómez (2013); Arceo-Gómez and Ashman (2016). Foreign pollen arrival can play an important role in plant species fitness but outcomes are variable and appear to be context dependent as there is not always a decrease in fitness Morales and Traveset (2008). Some of this variation is likely due to the enormous variability of foreign pollen transferred across systems ranging from between 0 and 75 percent but most studies report ranges of heterospecific pollen between 0 and 20 percent of the total pollen load Bartomeus et al. (2008) Montgomery and Rathcke (2012); Ashman and Arceo-Gómez (2013); Fang and Huang (2013), yet even these relatively low amounts of heterospecific pollen transferred can decrease fitness greatly Thomson et al. (1982). While we now have some understanding of the impacts of heterospecific pollen quantity, we have less understanding of other factors that could be driving the variation in impacts upon fitness. Ashman and Arceo-Gómez (2013) postulated the first predictive framework that identifies a need to understand how plant traits might mediate heterospecific pollen effect, whereby mating system and pollen size were predicted to potentially mediate the impact of foreign pollen transfer on plant fitness. This concept is supported by other studies, such as Tong and Huang (2016) that demonstrate an asymmetrical effect in 6 species of *Pedicularis* whereby the pollen of long styled species was able to grow the full length of the style on short styled species but not vice versa. While this suggests that the impacts of heterospecific pollen may differ among pollen donor and recipient, few studies have been conducted to ascertain whether this pattern is in fact a trend or to identify the extent to which other plant traits are critical to heterospecific pollen impacts.

Paragraph 3 Expanding ideas with examples

Given the large variability in mating systems across populations Whitehead et al. (2018), it is difficult to determine potential impacts upon HP transfer yet incompatibility system is another plant trait that

appears to play an important role in foreign pollen effect whereby species that are self incompatible have stronger barriers to heterospecific pollen than self-compatible species Ashman and Arceo-Gómez (2013). The type of incompatibility, (i.e. whether sporophytic or gametophytic) is related to the location of pollen recognition; sporophytic incompatibility relates to signaling at the stigma surface while gametophytic occurs within the style. This later acting pollen recognition mechanism is associated with greater negative effect than sporophytic recognition Barrett (1988). Nonetheless, an effect of foreign pollen is a bit obscured by the variability within species, however species that are strong selfers or strong outcrossers have less variability in mating systems and predictions of effect could be more realistic (see figure 1 from Whitehead et al. (2018)). Moreover, other traits such as number of pollen grains per flower and number of ovules have been traditionally associated with the type of incompatibility system where species with higher pollen ovule ratios are predicted to be xenogamous and species with low pollen ovule ratios autogamous (REF). Selfer species are known to have a reduction of herkogamy (REF) and less pollen production per ovule (REF) which can be interpreted as a reduction of pollen exported into the community. Other morphological traits, like stigma size can be determinant for the total pollen quantity that a stigma can receive and therefore related to do that pollen size would also play an important role. Example with pollen here.

Paragraph 3 Maybe connect with paragraph above?

Species with similar traits are more closely related XXXXXXXXXX. (Refs? Brown and Mitchell (2001) Arceo-Gómez et al. (2016) Tong and Huang (2016)). Several studies predict that the impact of HP transfer is likely to be greater for closely related species (Ashman and Arceo-Gómez (2013)). Few studies however, have focused on the impacts of heterospecific pollen of distantly related species Thomson et al. (1982) Galen and Gregory (1989) Neiland and Wilcock (1999). Yet, most insects and most stigmas have been found to carry multiple species of foreign pollen with little attention to degree of relatedness (Arceo-Gómez and Ashman (2016); Fang and Huang (2013) ; also cite studies from pollen transfer networks here such as). Further, a majority of plant species are generalist and thus receive visits from multiple different pollinators. Given these are generally the ones that receive greater loads of heterospecific pollen Fang and Huang (2013) and unrelated species are more likely to coexist with other species due to less niche overlap (Ref), understanding the role of foreign pollen from distantly related species thus deserves greater attention in understanding coexistence blah blahXXXXX

92 refs.. Notwithstanding, the effect of heterospecific pollen of far and close related species at community
 93 level remains to be explored beyond single pairwise interactions.

94 **Paragraph 4** Introducing our experiment

95 In this study we investigated how floral reproductive traits and relatedness mediate the impact of HP
 96 transfer by asking the following research questions : To what extent do (i) floral reproductive traits and
 97 (ii) relatedness, mediate the impacts of heterospecific pollen on seed set. We do this by creating an
 98 artificial co-flowering community with 10 species belonging to three different families with different
 99 traits.

100 **METHODS**

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

109 **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

Family	Genus	Species
Solanaceae	Capsicum	Capsicum annuum
Solanaceae	Petunia	Petunia integrifolia
Solanaceae	Solanum	Solanum lycopersicum
Solanaceae	Solanum	Solanum melongena

110 Hand-pollination

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

121 Traits and evolutive distance

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

130 Analysis

131 We used the statistical language R (R Core Team 2018) for all our analyses. To test the effect of
132 heterospecific pollen, we subtracted to the seed set of hand cross pollination the seed set of
133 heterospecific pollen treatments. Therefore, small values mean low effect and viceversa. To be able to
134 compare among species, seed set was previously scaled with mean 0 and standard deviation of 1. In
135 order to see correlations between heterospecific pollen effect and traits we performed Mantel test
136 between the matrix of effect and the distance matrix of each trait (euclidean distances). Moreover,
137 Mantel test was also conducted between heterospecific pollen effect and the square root of the matrix of
138 phylogenetic distance due to improvement in the statistical power (Letten & Cornwell 2014). We
139 explored also the relations between traits and heterospecific pollen effect through generalized mixed
140 models where the response variable was heterospecific pollen effect, the independent variable the
141 different traits and the random effects the different treatments per species. Moreover, pairwise evolutive
142 distances were calculated with MEGA7 for two kinds of markers: 1) Internal transcribed spacer (ITS)
143 and 2) ribulose-bisphosphate carboxylase (RBCL). The sequences of interest were downloaded from
144 NCBI GenBank and the phylogenetic tree constructed by maximum likelihood with MEGA7.

145 Phylogenetic signal of traits?

146 RESULTS

147 Results of hand cross pollination, self hand pollination, natural selfing and apomixis are presented in
 148 **Table 2.** Heterospecific pollen reduced seed set significantly with the 50-50% heterospecific pollen
 149 treatments for 65% of the pairwise interactions $p < 0.05$. Across families we found a very similar effect
 150 but when species were looked at species level they respond differently even within the same family, for
 151 instance for two species of the Brassicaceae family *Brassica oleracea* and *Eruca versicaria* we found
 152 very contrasting effects of foreign pollen where for the first one, all donors reduce seed set significantly
 153 and for the second, just two species did out of nine. The 100% foreign pollen treatments barely
 154 produced seeds or fruits and just for *Sinapis alba* we did not find significant differences between the
 155 hand cross pollination and one treatment with pollen from a confamilial. Solanaceae species with berry
 156 fruit type developed small fruits or even normal fruits in some cases. *S. lycopersicum* seems to
 157 produced small fruits (35% of the treatments) independently of pollen and pollen donor due to also
 158 apomictic treatments did, never normal size. *C. annuum* produced some fruits (9%) of both small and
 159 normal size and finally *S. melongena* produced seedless normal fruits with just confamilial pollen (3%),
 160 for both species seems that fruit formation was induced by pollen on the stigma because of lack of fruit
 161 production with apomictic treatments.

162 **Table 2.** Percentage of seeds produced per ovule for the ten species used in the experiment. The
 163 treatments presented are hand cross pollination, hand self pollination, natural selfing and apomixis
 164 (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

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

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

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

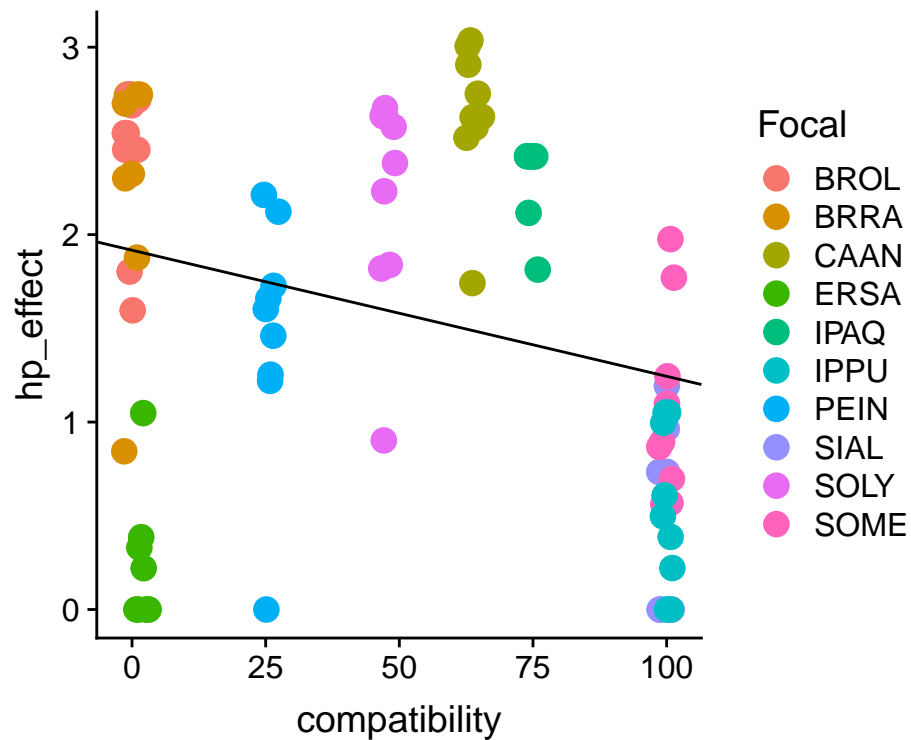


Figure 1: The effect of heterospecific pollen (scaled see set) is represented in function of the compatibility system (self/cross*100) for the the different species. Each coulored dot represents the interaction of a focal species with a different pollen donor.

DISCUSSION

Discussion

1. What are the implications of the findings?

Ideas about pollen size in heterospecific pollen effect. (still have to develop it more...)

Let's classify pollen size in three groups in order to understand the interaction between pollen donor and recipient. Donor's pollen: 1) Donor pollen size < Recipient pollen size 2) Donor pollen size = Recipient pollen size 3) Donor pollen size > Recipient pollen size

Small pollen can clogg the stigma. YES. Big pollen doesn't fit in very small stigmas. YES.

Is small pollen likely to affect bigger pollen. Ideas: It can be compensated with, less number of ovules to fertilize and faster pollen tube growth. So generally speaking, when the differences in pollen size are

181 evident we expect a similar outcome.

182 CONCLUSIONS

183 ACKNOWLEDGEMENTS

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223 **List of Figures**

224	1	The effect of heterospecific pollen (scaled see set) is represented in function of the	
225		compatibility system (self/cross*100) for the the different species. Each coulored dot	
226		represents the interaction of a focal species with a different pollen donor.	9