

working title Compatibility system and stigma size are the main predictors of heterospecific pollen effect

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Pollinator sharing can have negative consequences for plant fitness with the arrival of foreign pollen. However, the costs of heterospecific pollen are not yet well understood. We conducted a glasshouse experiment to understand how phylogenetic relatedness and plant traits mediate the impacts of heterospecific pollen transfer. We conducted 4XXXX crosses by experimentally transferring pollen (50% and 100% ratio) with reciporcal crosses between 10 species belonging to three different families: Brassicaceae, Solanaceae and Convolvulaceae. Seed set was used as proxy of plant fitness. We found that for 65% of the treatments with 50% mix reduced seed set. Moreover, the reduction in seed set was dependent on the degree of relatedness and reproductive traits of the pollen recipient and not the pollen donor. Our results show that certain traits, particularly compatibility system, are critical in understanding 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

34 (2007).

35 **Paragraph 2** Introducing topic and knowledge gap

36 By visiting many plant species, many pollinators are responsible for conspecific pollen loss and the
37 transport of foreign pollen, both of which can have important detrimental effects on species fitness
38 Morales and Traveset (2008); Ashman and Arceo-Gómez (2013); Arceo-Gómez and Ashman (2016).
39 Foreign pollen arrival can play an important role in plant species fitness but outcomes are variable and
40 appear to be context dependent as there is not always a decrease in fitness Morales and Traveset (2008).
41 Some of this variation is likely due to the enormous variability of foreign pollen transferred across
42 systems ranging from between 0 and 75 percent but most studies report ranges of heterospecific pollen
43 between 0 and 20 percent of the total pollen load Bartomeus et al. (2008) Montgomery and Rathcke
44 (2012); Ashman and Arceo-Gómez (2013); Fang and Huang (2013), yet even these relatively low
45 amounts of heterospecific pollen transferred can decrease fitness greatly Thomson et al. (1982). While
46 we now have some understanding of the impacts of heterospecific pollen quantity, we have less
47 understanding of other factors that could be driving the variation in impacts upon fitness. Ashman and
48 Arceo-Gómez (2013) postulated the first predictive framework that identifies a need to understand how
49 plant traits might mediate heterospecific pollen effect, whereby mating system and pollen size were
50 predicted to potentially mediate the impact of foreign pollen transfer on plant fitness. This concept is
51 supported by other studies, such as Tong and Huang (2016) that demonstrate an asymmetrical effect in
52 6 species of *Pedicularis* whereby the pollen of long styled species was able to grow the full length of the
53 style on short styled species but not vice versa. While this suggests that the impacts of heterospecific
54 pollen may differ among pollen donor and recipient, few studies have been conducted to ascertain
55 whether this pattern is in fact a trend or to identify the extent to which other plant traits are critical to
56 heterospecific pollen impacts.

57 **Paragraph 3** Expanding ideas with examples

58 Given the large variability in mating systems across populations Whitehead et al. (2018), it is difficult
59 to determine potential impacts upon HP transfer yet incompatibility system is another plant trait that
60 appears to play an important role in foreign pollen effect whereby species that are self incompatible
61 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 Barrett (1988). This later acting pollen recognition mechanism is associated with greater negative effect than sporophytic recognition Ashman and Arceo-Gómez (2013). 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.

[This last bit of the paragraph is still under development](#)

Paragraph 4 Maybe connect with paragraph above?

[Comments on it](#)

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

Paragraph 5 Introducing our experiment

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

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

Family	Genus	Species
Convolvulaceae	Ipomoea	Ipomoea purpurea
Solanaceae	Capsicum	Capsicum annuum
Solanaceae	Petunia	Petunia integrifolia
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 a 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 a 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. Differences of seed set
131 between treatments and hand cross pollination for each species was tested through mixed linear models.
132 For the following analysis we scaled the values of seed production for all the species with mean 0 and sd
133 of 1. To test the effect of heterospecific pollen, we subtracted to the seed set of hand cross pollination
134 the seed set of heterospecific pollen treatments. In order to see correlations between heterospecific
135 pollen effect and traits we performed Mantel test between the matrix of heterospecific pollen effect and
136 the distance matrix of each trait (euclidean distances). Moreover, Mantel test was also conducted
137 between heterospecific pollen effect and the square root of the matrix of phylogenetic distance due to
138 improvement in the statistical power (Letten & Cornwell 2014). We explored also the the relations
139 between traits and heterospecific pollen effect through generalized mixed models where the response
140 variable was heterospecific pollen effect, the independent variable the different traits and the random
141 effects the different treatments per species. Moreover, pairwise evolutive distances were calculated with
142 MEGA7 for two kinds of markers: 1) Internal transcribed spacer (ITS) and 2) ribulose-bisphosphate
143 carboxylase (RBCL). The sequences of interest were downloaded from NCBI GenBank and the
144 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 responded 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 treatments that tested for apomixis.

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 GLMM

169 I have done it at the moment just for Compatibility system Also I have to fix from mixed linear model
170 to GLMM, just realize that

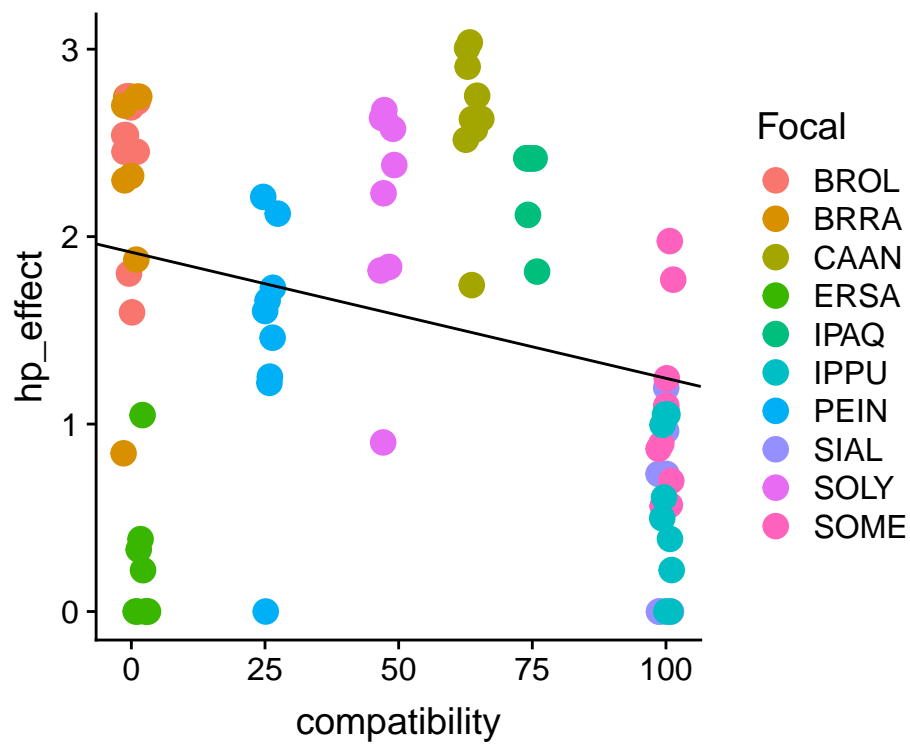


Figure 1: The effect of heterospecific pollen (scaled see set) is represented in function of the compatibility system (self/cross*100) for the the different species. Each coulored dot represents the interaction of a focal species with a different pollen donor.

171 Compatibility index don't multiply per 100 from Lloyd

172 DISCUSSION

173 Discussion

174 What are the implications of the findings?

175 Other idea based on the paper of Aizen 2007:

176 He explains seed set in this way. O total number of ovules, d fraction of ovules that become seed set, b
177 proportion of pollen grains that reach ovules, p number of pollen grains.

178
$$S = dO(1 - e^{-bp})$$

179
$$S = dO(1 - e^{-bp(HpEffect)})$$

180 but this Hp effect maybe can be divided at the same time in the interaction between recipient-donor
181 with the main traits that drive the effect. Moreover, this should consider quantity of hp. LINEAR
182 EFFECT? How to model this maybe talk with nacho.

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

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

187 Now I try to develop each part

188 1) Donor pollen size < Recipient pollen size

189 Effect:

190 • Donor's pollen could clogg the stigma

191 • Chemical inhibition

192 Traits associated with bigger pollen of the recipient:

- Recipient's pollen have faster pollen tube growth (example with my data)
 - Reduction in number of ovules (Also with my species)
 - Big differences in pollen size can be traduced in low relatednes therefore less likely of pollen germination on a far related stigma.
- 2) Donor pollen size = Recipient pollen size
- Very relatedness dependant this point
 - Similar probabilities of taken space on the stigma
- 3) Donor pollen size > Recipient pollen size

Effect:

-In small stigmas big pollen grains can occupy great part of the stigmatic area.

-small pollen grains can get embedded

CONCLUSIONS

ACKNOWLEDGEMENTS

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245 **List of Figures**

246 1 The effect of heterospecific pollen (scaled see set) is represented in function of the
247 compatibility system (self/cross*100) for the the different species. Each coulored dot
248 represents the interaction of a focal species with a different pollen donor. 9