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 1800 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

In most ecosystems, plant species normally coexist and share their floral visitors with other species Waser et al. (1996). From the plants' perspective, pollinator sharing can be positive for some plants Carvalheiro et al. (2014) or negative for others Pauw (2013), depending on the facilitation gradient. 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); Magrach et al. (2017). 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).

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 0 to 75 percent. However, 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 specific case 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 general trend or to identify the extent to which other plant traits are critical to heterospecific pollen impacts.

Plant traits are crucial to understand heterospecific pollen effect but the multifactorial nature of the traits that are involve in the pollen-pistil interaction make difficult to unravel what are the main traits in driving the effect. These traits can be seen from a male perspective of both donor and recipient where pollen size, pollen aperture number and pollen allelopathy are key components to understand the outcome of foreign pollen arrival Murphy and Aarssen (1995); Ashman and Arceo-Gómez (2013). In Ashman and Arceo-Gómez (2013) small pollen is predicted to cause a greater fitness decrease, although this can be true there are also other possibilities to consider which can obscure a predictive framework like big pollen can clogg small stigmas with fewer pollen grains, bigger stigmas are less likely to be clogged by small pollen grains and bigger pollen can outcompete smaller pollen grains due to faster pollen tube growth rate Williams and Rouse (1990). Moreover, to understand the different mechanical or chemical effects of pollen also the female traits of the pollen recipient have to be considered, from the literature these main traits are: stigma size, style length, number of ovules, incompatibility system and also a structural trait such as flower morphology Montgomery and Rathcke (2012); Ashman and Arceo-Gómez (2013); Tong and Huang (2016). For example, greater stigmatic area is positively correlated with greater heterospecific pollen deposition Montgomery and Rathcke (2012) and therefore possibly with an increase in negative effect. For species that are self-incompatible the barriers towards heterospecific pollen are stronger than self-compatible species 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 variablity in mating systems and predictions of effect could be more realistic (see figure 1 from Whitehead et al. (2018)). Although past research has progress in the understanding of what traits can mediate the effect as we have shown here, there are multiple traits involved and multiple possible scenarios still to be explored empirically for a full understanding of the importance of heterospecific pollen effect in nature.

For the understanding at what level or intensity the interference of pollen can occur is important to consider the relatedness of the interacting species. Closely related species are more likely to have similar traits Letten and Cornwell (2015). The similarity in traits between closely related species can lead to higher chances of ovule usurpation/abortion Arceo-Gómez and Ashman (2011) and therefore studies predict and show a greater negative effect of closely related species Ashman and Arceo-Gómez (2013); Arceo-Gómez and Ashman (2016). Few studies however, have focused on the impacts of heterospecific pollen on fitness of distantly related species Galen and Gregory (1989); Neiland and Wilcock (1999). Despite the fact that far related species are also able to decrease species fitness (REFS). 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). Understanding the role of foreign pollen from distantly related species thus deserves greater attention. The relatedness of foreign pollen gives a first snapshoot of where the pollen competition can occur and therefore could be a proxy of effect. With that said, Arceo-Gómez and Ashman (2016) is the only work which has proven a greater effect of close related species through a meta-analysis but with low sample sizes and lack of significance. Therefore, there is a need to study the effect of heterospecific pollen of far and close related species at community level beyond single pairwise interactions.

Heterospecific pollen studies in nature have the complexity added of great environmental variability which can lead to confounding interpretations. Moreover, the great diversity of floral structures and small sizes of some of them such as Asteraceae species make tedious or almost impossible the studies of heterospecific pollen on the field. For this reason, we investigated how floral reproductive traits and relatedness mediate the impact of heterospecific pollen by creating an artificial co-flowering community in a glasshouse with 10 species belonging to three different families with different traits. Our study tries to answer the following question: To what extent do (i) floral reproductive traits and (ii) relatedness, mediate the impacts of heterospecific pollen on seed set.

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 experimental design had species from three different families: Solanaceae, Brassicaceae and Convolvulaceae (**Table 1**). The different species of the study have different reproductive traits and different degree of relatedness where the reciprocal crossed between species allowed us to have multiple different scenarios of both traits and relatedness. Moreover, the species selected have fast life cycle and low structural flower complexity in order to perform the pollination treatments and grow the different species from seeds. For the purpose of the experiment all the species where 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

Family	Genus	Species
Brassicaceae	Eruca	Eruca versicaria
Brassicaceae	Sinapis	Sinapis alba
Convolvulaceae	Ipomoea	Ipomoea aquatica
Convolvulaceae	Ipomoea	Ipomoea purpurea
Solanaceae	Capsicum	Capsicum annuum
Solanaceae	Petunia	Petunia integrifolia
Solanaceae	Solanum	Solanum lycopersicum
Solanaceae	Solanum	Solanum melongena

Hand-pollination

Foreign pollen effect was studied through two different treatments, one with 50% conspecific pollen and 50% heterospecific pollen and a second one with 100% foreign pollen (N=10) in order to see if foreign pollen can trigger fruit production by itself or even seeds through ovule usurpation. Therefore, 180 different combinations were performed with N=10 per combination. Seed set was the proxy of effect for all our treatments. Moreover, hand cross pollination (between individuals of the same species), hand self pollination, apomixis (bagged emasculated flowers) and natural selfing were tested for each species (N=10). For the treatments with foreign pollen and hand cross pollination, flowers were emasculated the day prior anthesis and hand pollinated next day with a toothpick. Hand-pollination was conducted with 3-4 gentle touches on the stigma surface. For each species 20 anthers were collected and their pollen counted with a hemocytometer, each anther was counted 4 times and then an average of these counts was performed. Once, the average number of pollen grains per anther was known, the proportion of anthers per mix was calculated in order to achieve a 50-50% mix. In order to confirm that the treatments applied were the desire proportions, for each focal species the total stigmatic load of pollen was counted from one donor of each family (N=3).

Traits and evolutive distance

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

Analysis

We used the statistical language `R` (R Core Team 2018) for all our analyses. Differences of seed set between treatments and hand cross pollination for each species was tested through mixed linear models. For the following analysis we scaled the values of seed production for all the species with mean 0 and sd of 1. To test the effect of heterospecific pollen, we substracted to the seed set of hand cross pollination the seed set of heterospecific pollen treatments. In order to see correlations between heterospecific pollen effect and traits we performed Mantel test between the matrix of heterospecific pollen effect and the distance matrix of each trait (euclidean distances). Moreover, Mantel test was also conducted between heterospecific pollen effect and the square root of the matrix of phylogenetic distance due to improvement in the statistical power (Letten & Cornwell 2014). all is here, but I would break it by questions and give a bit more detail, to avoid overwhelm the reader We explored also the relations between traits and heterospecific pollen effect through generalized mixed models where the response variable was heterospecific pollen effect, the independent variable the different traits and the random effects the different treatments per species [Here I think you should think if this controls for the non independency of donors and recipients. I think not. Maybe look onto matrix regresions?). Moreover, pairwise evolutive distances were calculated with MEGA7 for two kinds of markers: 1) Internal transcribed spacer (ITS) and 2) ribulose-bisphosphate carboxylase (RBCL). The sequences of interest were downloaded from NCBI GenBank and the phylogenetic tree constructed by maximum likelihood with MEGA7.Make a section on how you contrsucted phylogeny.

I would explain three test. 0) treatment effects with GLM's, 1) Mantels: relative effects, 2) GLM's or matrix models: Absolute effects and explain them in three independent paragraphs including a rationale of why

Phylogenetic signal of traits?

RESULTS

Results of hand cross pollination, self hand pollination, natural selfing and apomixis are presented in **Table 2**. Heterospecific pollen reduced seet set signifcatively with the 50-50% heterospecific pollen treatments for 65% of the pairwise interactions p<0.05. Across families we found a very similar effect but when species where look at species level they respond differently even within the same family rephrase and maybe test statistically?, for instance for two species of the Brassicaceae family *Brassica oleracea* and *Eruca versicaria* we found very contrasting effects of foreign pollen where for the first one, all donors reduce seed set significatively and for the second, just two species did out of nine. The 100% foreign pollen treatments barely produced seeds or fruits and just for *Sinapis alba* we did not find significant differences between the hand cross pollination and one treatment with pollen from a confamilial- IB Unclear. Solanaceae species with berry fruit type developed small fruits or even normal fruits in some cases under which treatment. *S. lycopersicum* seems to produced small fruits (35% of the treatments) independently of pollen and pollen donor due to also apomictic treatments did, never normal size. *C. annuum* produced some fruits (9%) of both small and normal size and finally *S. melongena* produced seedless normal fruits with just confamilial pollen (3%), for both species seems that fruit formation was induced by pollen on the stigma because of lack of fruit production with treatments that tested for apomixis.clarify this descriptive statistics part- Also a figure with a summary of the treatments effect would be cool, or at least in the appendices

Table 2. Perecentage of seeds produced per ovule for the ten species used in the experiment. The treatments presented are hand cross pollination, hand self pollination, natural selfing and apomixis (emasculated flowers). turn into a figure somehow?

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
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

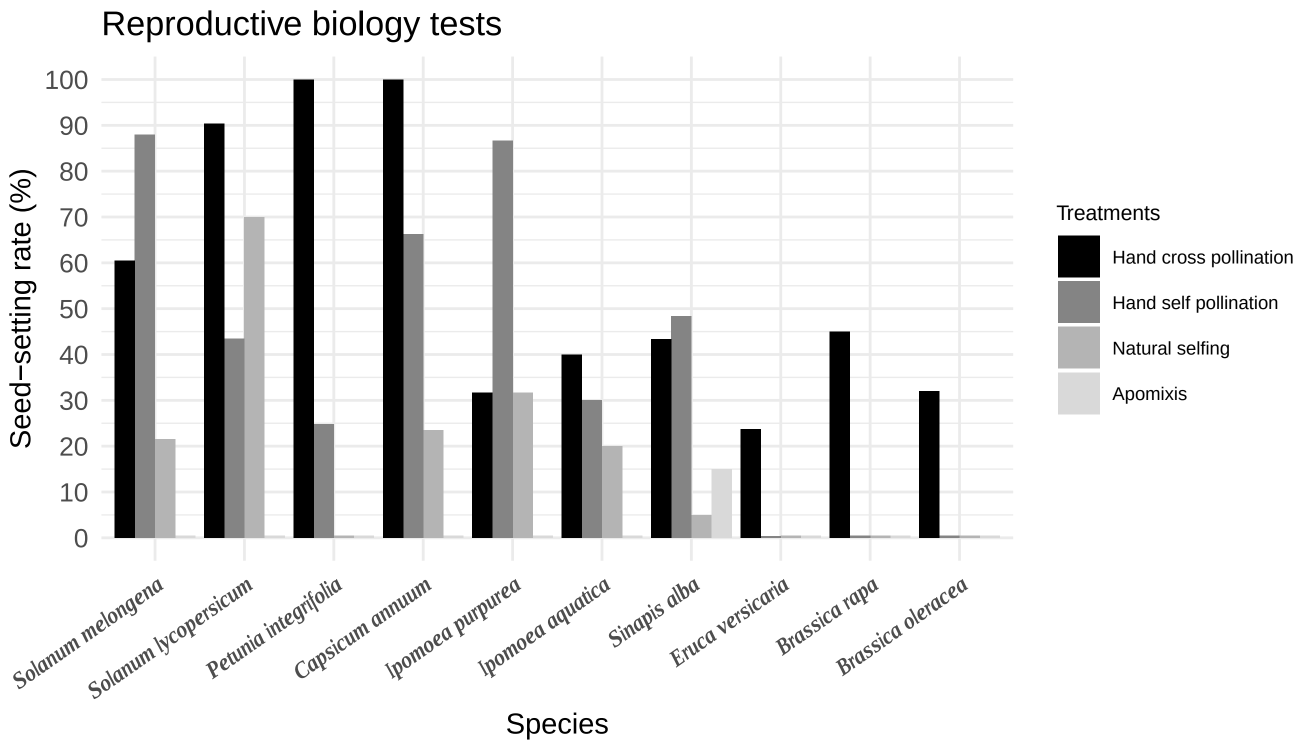

Figure 1: Barplot with the different treatments that provide information of the reproductive biology of the ten species. The y axis is the proportion of ovules converted to seed in percentage. The different treatments (N=10) which are presented in the legend are, hand cross pollination, hand self pollination, natural selfing and apomixis. More information about these treatments can be found in Methods and Appendices.

Mantel test indicates that a possible?? It exists! correlation exist between heterospecific pollen effect and the evolutive relative distances, for ITS and RBCL markers we had r coefficients of 0.29 and 0.25 respectively $p<0.05$ think on a figure - maybe using NMDS. Moreover, Mantel test indicates that also a possible?? correlation between stigma width and stigma type exist (stats??). Trait correlations were also explored with GLMM

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

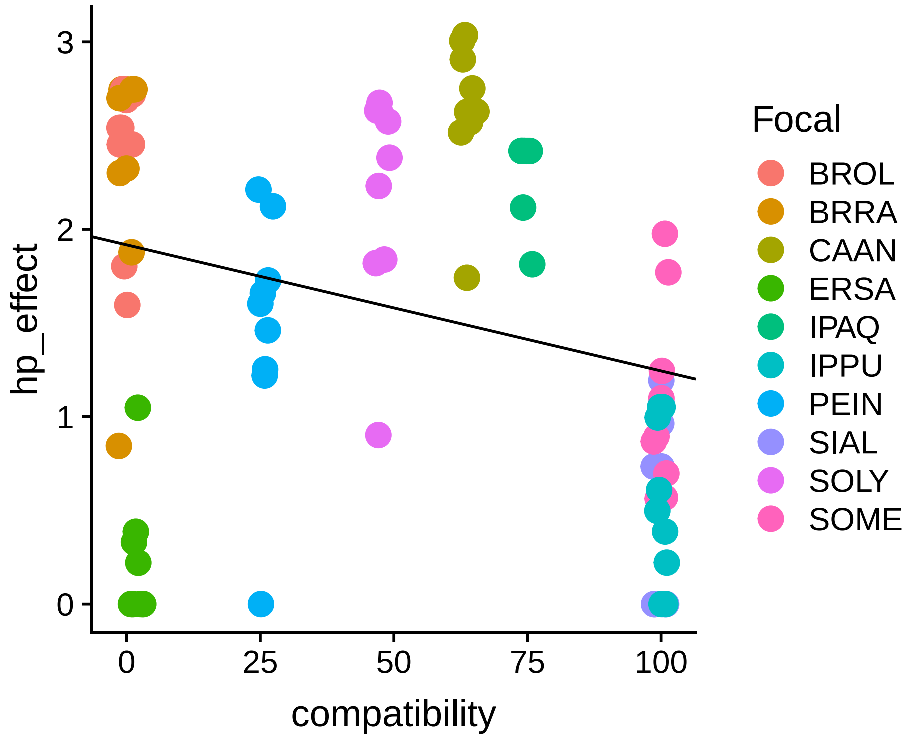

Figure 2: The effect of heterospecific pollen (scaled see set) is represented in function of the compatibility system (self/cross*100) for the the different species. Each coulored dot represents the interaction of a focal species with a different pollen donor.

190 Compatibility index don´t multiply per 100 from Lloyd

DISCUSSION

Discussion

Herbs vs tress, annual vs perennial... Many flowers vs few flowered species; structural composition on a system

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: 1) Donor pollen size $<$ Recipient pollen size 2) Donor pollen size = Recipient pollen size 3) Donor pollen size $>$ Recipient pollen size

Now I try to develop each part

1) Donor pollen size $<$ Recipient pollen size

Effect:

- Donor's pollen could clogg the stigma
- Chemical inhibition

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 embeded

IB: Think also on using tree analysis to test if hp effect depends on complex trait combinations. Tree analysis are great when two different strategies lead to the same outcome. This would never been pick up by GLMs. The r package is party{} . You can see an example applied to birds is Sol et al 2010 Science. Ask me if you want more details or code examples.

CONCLUSIONS

ACKNOWLEDGEMENTS

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