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

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

**Paragraph 2** Introducing topic and knowledge gap

Recent studies have advanced in the ecological understanding of heterospecific pollen effect Morales and Traveset (2008) Ashman and Arceo-Gómez (2013) Arceo-Gómez and Ashman (2016). A general overview of foreign pollen arrival is that it can play an important role on species fitness but seems to be context dependent and not always produce a decrease in fitness Morales and Traveset (2008). Part of this unpredictability is due to the enormous variability of foreing pollen transferred in nature, where levels between 0 and 75 percent are seen, but most commonly values ranges 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), being the generalist species the ones that receive greater loads of heterospecific pollen Fang and Huang (2013). Surprisingly, this low ranges of heterospecific pollen have been shown to decrease fitness greatly Thomson et al. (1982). Although heterospecific pollen quantity is fundamental to understand the outcome of the interaction so is the different traits of both pollen donor and recipient. Ashman and Arceo-Gómez (2013) postulated the first predictive framework for traits of heterospecific pollen effect, where different traits such as compatibility system and pollen size among others seems to be crucial to understand foreing pollen effect. Moreover, in Tong and Huang (2016) an assymetric effect was shown in a crossing experiment between 6 species of the genus *Pedicularis* where the pollen of long styled species was able to grow the full length of the style on short styled species but not viceversa. Despite these recent caveats, we still lack empirical evidence to affirm what are the main traits that drive heterospecific pollen effect for both pollen donor and recipient at seed production level. Interestingly, to comprehend how these traits interact is also crucial to look at the phylogenetic relatedness of the species. There is a considerable amount of literature of crosses between close related species Brown and Mitchell (2001) Arceo-Gómez et al. (2016) Tong and Huang (2016) but few works focused on heterospecific pollen of far related species Thomson et al. (1982) Galen and Gregory (1989) Neiland and Wilcock (1999) which also show a noteworthy fitness decrease. Although the effect of close related species is predicted to be greater Ashman and Arceo-Gómez (2013) the presence of pollen of non related species on multiple species Arceo-Gómez and Ashman (2016) and the higher chances to coexist with a species that has less niche overlap (Ref) make foreign pollen from far related species also an important subject of study in order to understand the importance of heterospecific pollen in natural systems. Notwithstanding, the effect of heterospecific pollen of far and close related species at community level remains to be explored beyond single pairwise interactions.

**Paragraph 3** Expanding ideas with examples

Interestingly, incompatibility system seems to play an important role in foreign pollen effect where species that are self incompatible would have stronger barriers towards heterospecific pollen than self compatible species Ashman and Arceo-Gómez (2013). The type of incompatibility, sporophytic or gametophytic is related with the place of pollen recognition where the former take place at the sitgma level and the latter occurs within the style, this last late acting pollen recognition mechanism is associated with greater negative effect Barrett (1988). Remarkably, there is a great variability in mating systems across populations Whitehead et al. (2018) and therefore predict 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)). Moreover, other traits such as number of pollen grains per flower and number of ovules have been tradittionally 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 would have a reduction of herkogamy (REF) and less pollen production per ovule (REF) which can be interpretated as a reduction of pollen expoorted into the community. Other morphological traits, like stigma size can be determinant for the total pollen quatity that a stigma can receive and therefore related to do that pollen size would also play an important role. Example with pollen here.

**Paragraph 4** Introducing our experiment

The great environmental variability in natural systems and complexity of floral structures make 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 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 |
| 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). Therefore, 180 different combinations were perform with N=10. Seed set was the proxy of effect for all our treatments. Moreover, hand cross pollination, hand self pollination, apomixis (bagged emasculated flowers) and natural selfing were tested for each species (N=10). 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. The mixes of pollen were realized on an eppendorf based on the pollen counts maded with Neubaeur chamber (each anther was counted 4 times for 20 different anthers per species). In order to confirm that the treatments applied were 50-50 percent pollen, 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 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 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. To test the effect of heterospecific pollen, we substracted to the seed set of hand cross pollination the seed set of heterospecific pollen treatments. Therefore, small values mean low effect and viceversa. To be able to compare among species, seed set was previosly scaled with mean 0 and standard deviation of 1. In order to see correlations between hetereospecific pollen effect and traits we performed Mantel test between the matrix of 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). We explored also the 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. 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.

Phylogenetic signal of traits?

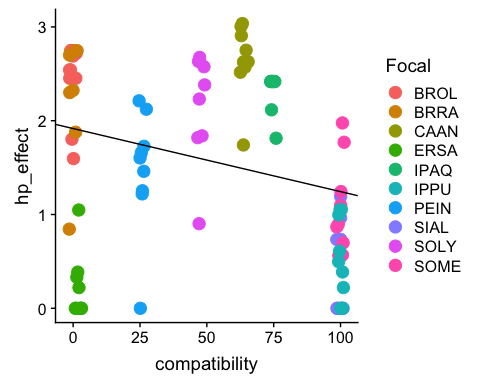
# RESULTS

Heterospecific pollen reduced seet set signifcatively with the 50-50% heterospecific pollen treatments for 65% of the paiwise 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, for instance for two species of the Brassicaceae family Brassica oleracea and Eruca sativa we found very contrasting effects of foreign pollen where for the first one all donors reduce seedt set significatively and for the second one just two species did out of nine.

Mantel test gave significant values p<0.05 for both types of evolutive distances considered, with r coefficients of 0.29 and 0.25 for the distances of ITS and RBCL markers respectively. Moreover, Mantel test gave a significant correlation between stigma width and stigma type with xxxx. Trait correlations were also explored with … and we found that…

Fix mantel test selfing rates and change it for compatibility index…

Fix this to GLMM? Yep I have to…



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?

# CONCLUSIONS

# ACKNOWLEDGEMENTS

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