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

(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 Barrett (1988). This later acting pollen recognition mechanism is associated with greater negative effect than sporophytic recognistion 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)). 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 interpretated as a reduction of pollen exported 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.

This last bit of the paragraph is still under development

Paragraph 4 Maybe connect with paragrph above?

Comments on it

Species with similar traits are more closely related XXXXXXXXX. (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 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

Family	Genus	Species
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. 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). 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

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, 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. Solanaceae species with berry fruit type developed small fruits or even normal fruits in some cases. *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.

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).

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

Mantel test indicates that a possible correlation exist between heterospecific pollen effect and the evolutive distances, for ITS and RBCL markers we had r coefficients of 0.29 and 0.25 respectively $p<0.05$. Moreover, Mantel test indicates that also a possible correlation between stigma width and stigma type exist. 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

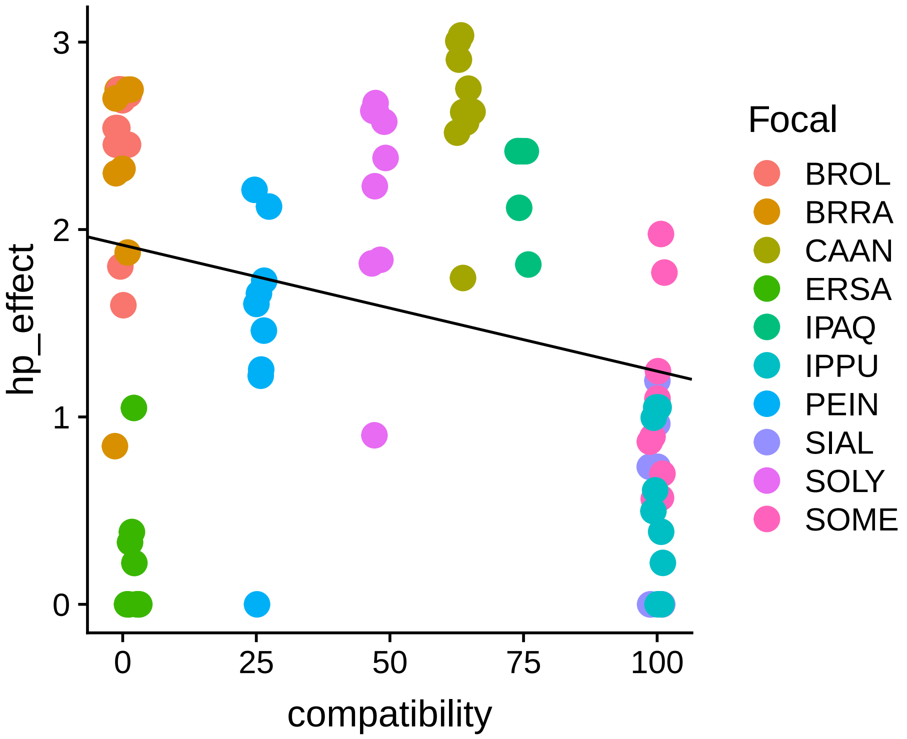

Figure 1: The effect of heterospecific pollen (scaled see set) is represented in function of the compatibility system (self/cross*100) for the the different species. Each coulored dot represents the interaction of a focal species with a different pollen donor.

171 Compatibility index don´t multiply per 100 from Lloyd

DISCUSSION

Discussion

What are the implications of the findings?

Other idea based on the paper of Aizen 2007:

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

$S = dO(1 - e^b p)$

$S = dO(1 - e^b p(HpEffect))$

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

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

CONCLUSIONS

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