

INTRODUCTION

In most ecosystems, plant species normally coexist and share their floral visitors with other species Waser et al. (1996); Carvalheiro et al. (2014). From the plants' perspective, pollinator sharing can be positive for some plants as an increasing number of visits often correlates with higher chances of fertilization Engel and Irwin (2003). Yet, among these possible flower visitors there are also nectar robbers, pollen thieves Inouye (1980); Magrach et al. (2017), and inconstant pollinators that transfer foreign pollen from other plants Pauw (2013). 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). 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). 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, Bosch, and Vilà (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, Andrews, and Plowright (1982).

While we have some understanding of the impacts of heterospecific pollen quantity, we have little knowledge of the factors that could be driving the variation in pollen quality upon fitness. Plant traits are crucial to understand heterospecific pollen effect but the multifactorial nature of the traits that are involved in the pollen-pistil interaction make difficult to unravel exactly which traits are driving the effect. 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.

The concept of trait driven mechanisms is not new and is supported by system specific studies. Pollen size, pollen aperture number and pollen allelopathy are thought to be key components in understanding the outcome of foreign pollen arrival Murphy and Aarssen (1995); Ashman and Arceo-Gómez (2013). For example, small pollen is predicted to decrease plant fitness because is more likely to clogg the stigmatic surface. Yet, large pollen can outcompete smaller pollen grains due to faster pollen tube growth rate E. Williams and Rouse (1990). Hence, understanding the different mechanical or chemical effects of pollen requires knowledge

of the female traits of the pollen recipient to also be considered 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 likely to result in an greater negative effect upon plant fitness. Further, species that are self-incompatible are thought to be more resistant to the negative impacts of heterospecific pollen than self-compatible species Ashman and Arceo-Gómez (2013). When both donor and recipient traits are considered together, other combinations of traits are also likely to impact plant fitness. For example, large pollen grains could potentially clog small stigmas with fewer pollen grains, and larger stigmas are less likely to be clogged by small pollen grains Galen and Gregory (1989). Yet, few studies have considered how effects might differ among donor and recipient species. Tong and Huang (2016) demonstrate an asymmetrical effect in 6 species of *Pedicularis* whereby foreign 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.

It is challenging to identify general patterns with respect to the mechanisms driving foreign pollen impacts as results are often obscured by the variability within and among 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) hence, greater negative effects of heterospecific pollen are thought to be associated with more 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) and those that have, often report low sample sizes and a 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. Given that pollen carried on many insects and stigmas has 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

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 heterogeneous reproductive traits. Our study addressed the following questions:

1. To what extent does the amount of foreign pollen applied to stigmas impact plant reproductive fitness (i.e. 50% and 100% foreign pollen ratio.

64 2. How do floral reproductive traits and plant relatedness mediate the impacts of heterospecific pollen on
65 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: Brassicaceae, Convolvulaceae and Solanaceae (**Table 1**). The species of the study had different reproductive traits and different degree of relatedness (see phylogenetic tree, **Figure 1**) where the reciprocal crosses between species allowed us to have multiple different scenarios of both traits and relatedness. Moreover, the species selected had 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 were considered as pollen recipient and as pollen donor. Species were watered once or twice per day and fertilized weekly (NPK 23: 3.95: 14) and the rooms of the glasshouse were temperature controlled with temperature oscillations between day and night.

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 in order to see if foreign pollen can trigger fruit production by itself or even seeds through ovule usurpation. We performed 180 different heterospecific treatments in total (N=10). 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. The anthers were mixed in tubes of 0.5/1.5 ml and shaken with an electric toothbrush without the head on the outside bottom part of the tube for a correct mix. For the species with buzz-pollination, *S. lycopersicum* and *S. melongena*, same procedure with an electric toothbrush was used in order to extract the pollen directly into the tubes, the application of it was at the bottom of the corolla/receptacle. To confirm that the treatments applied were the desired proportions, the total stigmatic load of pollen was counted and the proportions calculated between the two species of the mix. Because pollen from the same family was difficult to distinguish, and we expected similar properties in mixing, we counted pollen from just one randomly selected species within each

donor family different to the focal's family (N=3).

Traits and evolutive distance

The traits measured for each species were pollen per anther, pollen size, number of ovules and stigma, style, ovary width and length. For the stigma, the stigmatic area was also measured and moreover the stigmas were divided in wet/dry type with the help of the stereomicroscope. All the morphometrical measurements were performed with a stereophotomicroscope with the exception of pollen size that was carried out with a light microscope. 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 a stereomicroscope and a small grid over a petri dish from 15 randomly selected flowers. Fruits per number of flowers treated were counted for just Solanaceae species with fleshy fruit. For all the species we counted the number of seeds produced per average number of ovules. Levels of self-incompatibility were estimated by dividing the seed set of hand self-pollination by hand cross-pollination Lloyd and Schoen (1992).

Analysis

To evaluate heterospecific pollen effect on seed production we performed linear mixed models. The distinct heterospecific pollination treatments were compared through relevelling each variable with the cross pollination treatment which was our control for optimum seed production for all the species. The different replicates of each treatment were considered as random effects. Seed production was scaled for all the species with mean 0 and standard deviation of 1 prior to the analysis. All the analysis were conducted with the statistical language R (R Core Team 2018).

To compare the magnitude of effect of heterospecific pollen across species we conducted standarized Hedges' d [(mean of mixed 50% mix - mean of cross pollination)/pooled SD] with *effsize* package. We did in three different ways: effect sizes of each donor per focal species; effect sizes per family of the different donors per focal species; effect sizes of all the donors grouped per focal species.

We conducted mantel test to check for correlations between heterospecific pollen effect and phylogenetic distance. Due to improvents in statistical power we used the square root of the phylogenetic distance (Letten & Cornwell 2014). Two different phylogenetic distances were used from two kinds of markers: 1) Internal transcribed spacer (ITS) and 2) ribulose-bisphosphate carboxylase (RBCL). The sequences were obtained from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>, accessed 20 Oct. 2018). The sequences were aligned with clustal omega and the pairwise evolutive distances calculated with MEGA7.

In order to test the relative effect of traits on seed production with foreign pollen we performed Mantel test in R (*vegan* package, Euclidean distance) between the assymetrical matrix of heterospecific pollen effect (10 by 10 matrix) with the distance matrix of traits. Furthermore, the same analysis was conducted trait by trait in order to disentangled the specific effect of each trait. The correlation between heterospecific effect and traits was also looked at family level for Brassicaceae and Solanaceae species. The family Convolvulaceae did not have enough species to test correlation with traits though Mantel test. Heterospecific pollen effect was obtained through the subtraction of seed production by hand cross-pollination minus seed production of the different heterospecific pollen treatments. To find a model with the best explanatory traits for heterospecific pollen effect we used the function *bioenv* from R which finds the maximum rank of correlation among a subset of variables. We also conducted Mantel test between the matrix of heterospecific pollen effect and the distance matrix from all the traits. Moreover, we explored also the correlation between traits and heterospecific pollen effect through generalized mixed models where the response variable was heterospecific pollen effect and the explanatory variable the different traits. In addition, we tested the correlation between the total amount of pollen deposited on the stigma with heterospecific pollinations and the stigma size through Pearson's correlation.

RESULTS

Results of hand cross-pollination, hand self-pollination, natural selfing and apomixis are presented for all the species in **Figure 1** (see **Appendix 1** for table with values). Heterospecific pollen reduced seed set significantly with the 50-50% heterospecific pollen treatments for 65% of the pairwise interactions $p < 0.05$. Brassicaceae and Solanaceae species had less foreign pollen than expected, the ratios of pollen of recipient and donor respectively for both families were 80-20% and 60.5-39.5%. In contrast, Convolvulaceae species had just 28% recipient pollen and 72% of donor pollen. In addition, for the 100% heterospecific pollen treatments we did not find almost seed production (see *Table 3* for 100% treatments that produced seeds). However, for just one species (*S. alba*) the control pollination and the heterospecific pollination with pollen from a confamilial had similar seed production (non significant differences). Moreover, for two Solanaceae species *S. melongena* and *C. annuum* 100% pollen treatments produced few seedless fruits (3% and 9% respectively) and they did not for the apomictic treatments indicating that foreign pollen can be responsible of seedless fruit production.

The grouped effect sizes of the nine donors per species differed across species and families (see **Figure 2**), having clear statistical differences for species within and across families with not overlapping confidence

intervals. Interestingly, despite some variability in the effect of the different pollen donors per species, in general terms the effect of heterospecific pollen from the distinct nine treatments per species was homogeneous with unclear statistical differences (see **Figure 3**). Just for four species out of ten, just one donor in each did not have overlapping confidence intervals with the rest of the donors. Therefore, none of the donors had a clear stronger or weaker effect across species. When the donors were grouped by family we found a similar effect too, just for *S. lycopersicum* the confidence intervals of Brassicaceae and Convolvulaceae did not overlap (see **Figure 4**).

Matrices of heterospecific pollen effect and traits (Distance matrix of traits, euclidean distance) are positively and significantly correlated ($r=0.4$, one-tailed Mantel test $p<0.05$). The variable/s with maximum rank correlation (through *Bioenv* function) was incompatibility index by itself within a group of 16 variables calculated with euclidean distances. The same analysis of Mantel test between heterospecific pollen effect and the traits one by one gave a positive and significant correlation for stigma type and stigma measurements (length, width and area). When the effect of heterospecific pollen was looked at family level, for Solanaceae species we found a positive and significant correlation between heterospecific pollen effect and pollen-ovule ratio ($r=0.74$, one-tailed Mantel test $p<0.05$). Moreover, it seems to be a strong negative correlation between style length and heterospecific pollen effect although lack of significance (Pearson's correlation= 0.93 , $p=0.07$) (see **Figure 7**). For Brassicaceae species, the distance matrix of ovules and heterospecific pollen effect gave a positive and significant correlation ($r=0.4$, one-tailed Mantel test $p<0.05$). Separate to this, the stigmatic area and pollen grain number per stigma were positively correlated, (pearson's correlation= 0.57 , $p<0.01$).

Results of Mantel test between heterospecific pollen effect and phylogenetic distance gave a low positive correlation for both markers ($p<0.05$). The correlations with ITS and RBCL markers was respectively of 0.29 and 0.25 . We found a significant phylogenetic signal of traits for pollen size, stigma measurements and style length ($p<0.05$). Although with a lack of a significant correlation Pagel's lambda values were also relatively high (>0.45) for incompatibility index, ovary length and levels of selfing.

add table with morphometrical traits to appendix (Jose) NMDS to appendix?

DISCUSSION

The uniform effect of the different donors across the ten different species is a novel result to highlight in the understanding of heterospecific pollen effect. Traditionally, the nature of the pollen donor has been considered highly relevant to understand the outcomes of the pairwise interaction. However, this result support the idea that the pollen recipient traits are the main determinats of the effect and not the donor ones.

Caveats:

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

supporting ideas: 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)).

CONCLUSIONS

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