The effect of pollen origin and pollen load on the stigmas in the female reproductive success of Dalechampia species

A thesis submitted in partial fulfilment of the requirements for the award of the degree of Master of Philosophy of the University of Portsmouth

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Abstract

In *Dalechampia* blossoms, anther-stigma distance (ASD) can influence self-pollination rates; blossoms with small anther-stigma distance values will experience more self-pollination. Self-pollination will cause the exposure of deleterious alleles then natural selection will purge from the popualtion. Additionally, when the small ASD can result in more pollen on the stigmas, it may increase pollen competition and thereby reduce inbreeding depression in offspring as more superior pollen will achieve fertilisation, The ASD (herkogamy) is thought to indicate a population's mating history, and, if habitual inbreeders purge their genetic load, then the ASD should influence levels of inbreeding depression. This project aimed to assess if two self-compatible species, *Dalechampia heteromorpha* and *D. scandens,* displayed inbreeding depression and the level to which pollen competition could reduce its negative effects. In these species, hand-pollination experiments were performed controlling the amount of pollen load and the source of pollen (self vs. outcross). In *D. scandens*, four populations were classified as 'predominant selfer' (smallest ASD), 'selfer' (small ASD) and as 'outcrosser' (large ASD). The populations received hand-pollination experiments with the amount of pollen load varied. It was hypothesised that the 'predominant selfer' and 'selfer' populations have experienced a long inbred history whereas 'outcrosser' populations require pollinator services. The results from *D. heteromorpha* and *D. scandens* revealed that the offspring suffered from outbreeding depression at the early traits (pre-germination); in *D. heteromorpha,* inbreeding depression was detected in the later traits (post-germination). Increased pollen competition (higher pollen loads) increased the early fitness traits; especially for cross-pollination treatment, however, in the later traits saw a decrease in fitness. It was hypothesis that stigma blocking occurred, reducing the chance of superior pollination. Overall, pollen competition performed weaker than expected, it is likely to be occurring but it is mild and difficult to detect. The floral morphology for *D. heteromorpha* and *D. scandens* displayed a reduced ASD suggesting they are inbred. These findings agree that inbred species exhibit less inbreeding depression. The results from the comparison of the *D. scandens* populations show; that seed weight was lower in the 'predominant selfer' and 'selfer' populations. In addition, germination rate was lowest in the 'predominant selfer', but highest in the 'selfer' and the 'outcrosser' populations fell in between the two. Increased pollen competition improved fitness in the 'predominant self' yet it was not detected in the other populations. The lack of strong positive effects from pollen competition in the 'outcrosser' populations, could suggest that, if floral morphology affects self-pollination rates, a certain level of inbreeding may be beneficial for the offspring. In addition, this study detected that there was a positional affect in *D. scandens*; the central pistil frequently produced greater numbers of seeds, heavier seeds and increased chance of germination, regardless of treatment. Further to this, it was also found that two simultaneous pollinated blossoms on different branches were not independent. When two blossoms were pollinated with the same pollen origin there was an increased likelihood of abortion, suggesting that *D. scandens* can detect and favour increased genetic diversity of the offspring between blossoms.

Contents

Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

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Abbreviations

ASD – Anther-Stigma Distance

Predominant selfer – refers to the Chamlea population

Selfer – refers to the La Mancha population

Outcrossers – refers to the Maria la Gorda and Puerto Morelos populations

Heather Margaret Bulpett

1955-2013

This is dedicated to Heather. A good friend who helped teach me to read as a child. She supported and helped with homework, essays and reports over the years.

She saw me achieve my ambition to go to university and was delight when I graduated with a 2:1 degree

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Dissemination

Poster

Floral Architecture and Inbreeding Depression in *Dalechampia scandens (Euphorbiaceae).*

Present at the University of Portsmouth Postgraduate Day

Chapter 1: Introduction

Introduction

The mutualistic relationships between plants and pollinators are believed to have begun in the early Cretaceous period (135 million years ago); by the end of the Cretaceous period (65 million years ago) angiosperms had risen to dominance (Crane & Lidgard, 1989; Lupia et al., 1999). Darwin recognised the relationship between angiosperms and their pollinators and started to describe co-evolution briefly in 'On the Origin of Species' (1859) and described it more in detail later in 'On the various contrivances by which British and foreign orchids are fertilised by insects, and on the good effects of intercrossing' (1892). Co-evolution causes species to constantly adapt to each other with each generation (Zimmer; 1988, 2002) and is usually thought to have caused a great diversification within the angiosperms.

Mating systems

The mating system in hermaphroditic self-compatible plants is the frequency of self- vs. cross-pollination with an unrelated individual (Eckert et al., 2009), within a species or population. This section will provide evidence of the relationships between plants and pollinators in relation to blossom morphology, with particularly reference to the distance between anthers and stigmas and their effect on the mating system.

Self-pollination can take two forms: autogamy and geitonogamy. Autogamy occurs when pollen fertilises the ovules from the same flower; geitonogamy occurs when the pollen comes from a different flower from the same plant. Darwin (1876) stated that "nature abhors perpetual self-fertilisation"; however, with the widespread occurrence of self-pollinating species, either predominantly self-pollination or as part of a mixed mating system, selfing is clearly adaptive under some conditions (see Schemske & Lande, 1985). The existence of a flower is to produce seed for the individual to continue their line, whether fertilisation occurred by self- or cross-pollination is secondary (Darwin, 1876, p. 302). Self-pollination can be seen as a short-term solution, for example, when pollinators are scarce or absent, or when plants colonise a new area (Stebbins, 1970; Chang & Rausher, 1999; Bailey & McCauley, 2006). At the same time, self-pollination can have negative consequences for plants, such as inbreeding depression (as described below).

Many plants are self-compatible yet possess morphological mechanisms to prevent self-pollination (Darwin, 1876). One morphological mechanism is herkogamy, the distance between the anther and stigma. The level of herkogamy usually correlates with the size of the flower;

larger flowers usually display greater herkogamy than small flowers, and belong to predominantly cross-pollinating population (Igic & Bush, 2013). Larger flowers increase advertisement and rewards which promotes visitation (Armbruster et al., 2005) and this increases pollen exportation at the same time the increased herkogamy decreases autogamy (Takebayshi et al. 2006; Dai & Galloway, 2010). In *Datura stramonium* large variation in herkogamy was found to influence outcrossing rates (Motten & Antonovics, 1992); outcrossing rate decreased with reduced herkogamy. Webb and Lloyd (1986) also suggests that herkogamy is a way to reduce sexual interference. Another morphological mechanism is stylar polymorphism (Barrett, 2002). Floral dimorphic populations exhibit two different floral forms, usually different positions between anthers and stigmas. The underlying mechanism is the promotion of cross-pollination between the different morphs, by means of negative frequency-dependent selection (Pannell et al., 2005). Style dimorphism frequently occurs in the genus *Narcissus* (Barrett & Harder, 2005), that possess long and short styled morphs. Dichogamy; the separation of the presentation of the anthers and stigmas in time is another mechanism. Protandrous plants have the anthers mature first whereas protogynous have the stigmas developed first. *Jatropha curcas*, has been observed to display protandry (Raju & Ezradanam, 2002; Chang-wei et al. 2007a) and protogyny (Negussie et al., 2014). However, the development of fruits and seed yield indicated that this species benefitted from pollinator services (Negussie et al., 2014). Lloyd and Webb (1986) also suggest that dichogamy is a way to reduce sexual interference. Another strategy is that some species are self-incompatible. Many flowers avoid selfing by chemical self-recognition of the pollen (Richards, 1986). In *Impomoe Wolcottiana*, self-pollination retarded the development of subsequent cross-pollination (Parra-Tabla & Bullock, 2005). However, the loss of self-incompatibility has in itself minimal effects on the morphological traits of the blossom (Stebbins, 1970). *Leptosiphon jepsonii* shows self-incompatibility for 24-48 hours after opening and then becomes self-compatible (Goodwillie et al., 2004). The most obvious mechanism is self-incompatibility, however many species are not incompatible. These species posses various mechanisms which promote cross-fertilisation, meanwhile allowing reproductive assurance from self-pollination (Kalisz & Vogler, 2003) and potentially reducing sexual interferences which increase the fitness of both the female and male functions (Lloyd & Webb, 1986; Webb & Lloyd, 1986).

There is abundant evidence showing that selfing rates correlate with variation in floral traits, allowing the historical patterns of mating based on the floral traits and morphology to be extrapolated (Parker et al., 1995). Large flowers tend to attract more pollinators, which results in high visitation rates, and therefore higher levels of pollen export and import from many donors to the stigmas. The opposite occurs in small flowers; selfing rates are higher than outcrossing rates, and seed production might be more reliant on self-pollination (Armbruster, 1985; Armbruster et al., 2002; Armbruster et al., 2005). Motten and Antonovics (1992) suggested that a reduction in flower size could modify the floral morphology, such as herkogamy, placing anthers and stigmas very close, which can increase the rates of self-pollination. In addition, the benefits of self-pollination may outweigh those from cross-pollination. For example, the reduction in flower size can reduce energy invested in the flower's development and reward. In *Collinsieae,* Armbruster et al. (2002) found that small flowered populations were highly self-pollinating whereas larger flowered populations had higher outcrossing rates. Populations with small flowers (2 mm space) had early anther-stigma contact and early stigma receptivity. However, populations with large flowers (20 mm space) presented delayed anther–stigma contact and delayed stigma receptivity.

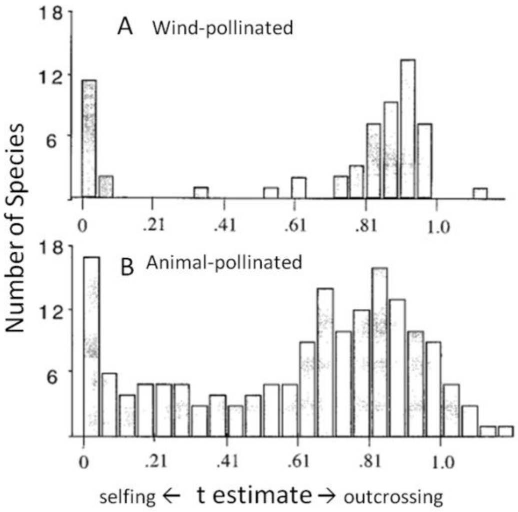


Figure 1. Modified from Vogler & Kalisz' (2001). Shows distributions of the cross-pollination rates for 59 wind- and 169 animal-pollinated species. The frequency histograms with data classes of 0.05 intervals ranked t estimated data.

Lande and Schemske's (1985) classic model of the mating system in plants suggests that populations are bimodal: populations can be either predominantly self- or cross-pollinating. The mating system that is present in the population will depend largely on the mating history. Populations that are predominantly self-pollinating would be expected to have experienced bottlenecks, reduction or even absence of pollinators on a regular basis and/or purging of the genetic load, resulting in relatively little inbreeding depression (Lande & Schemkse, 1985; Charlesworth & Charlesworth, 1987; Kephart et al., 1999). Predominantly cross-pollinating populations are expected to possess substantial genetic load and suffer inbreeding depression upon self-pollination and they possess mechanisms to prevent self-pollination (Lande & Schemkse, 1985; Charlesworth & Charlesworth, 1987; Kephart et al., 1999). Predominantly cross-pollinating populations can evolve into predominantly self-pollinating populations much more readily than the other way around (Lande & Schemske, 1985). When the mating system becomes predominantly self-pollinating, the population can become isolated from other populations and can be generally considered to be the end of the evolutionary line (Stebbins, 1970; Lande & Schemske, 1985; Takebayashi & Morrell, 2001). The evolution from a self-pollinating to cross-pollinating population is discussed at length by Igic and Busch (2013), the authors conclude that the current hypothesis is that there are genetic and environmental barriers that stop this; however, the authors draw doubt on this and suggest that empirical evidence is needed. Reversal of self-pollinating populations to cross-pollinating populations would need to retrace the

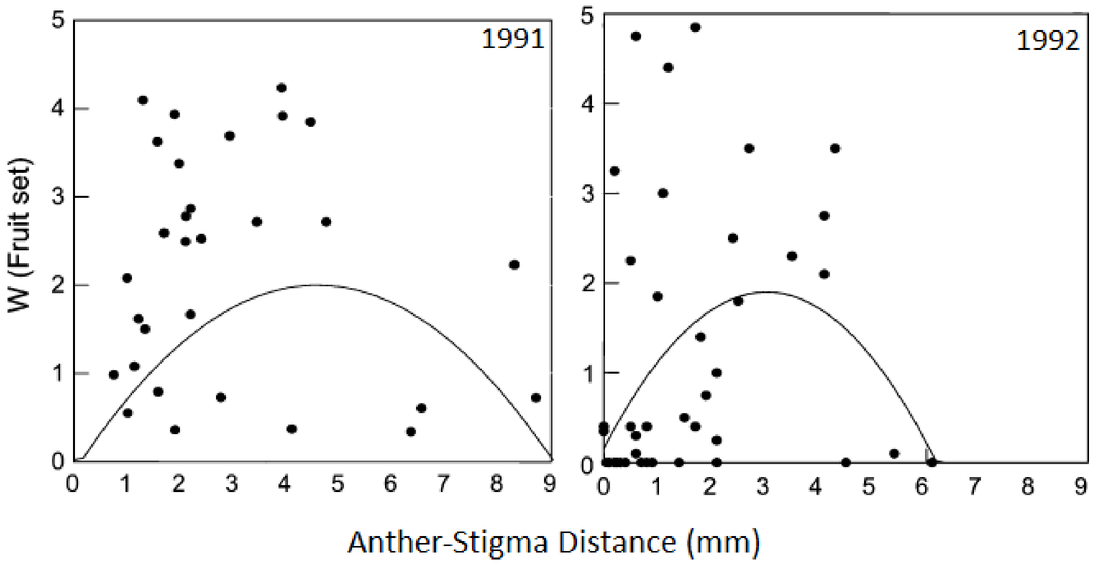


Figure 2. Modified from Para-Tabla & Bullock (2005). The effects of Anther-Stigma Distance on fruit set, an index of relative female fitness of trees of *Ipomoeas wolcottiana* in two consecutive year. The continuous lines represents the fitted models of stabilizing (γ′) selection. In 1991 γ′ = -1.086 ± 0.3, p = 0.002 and R squared = 0.196. In 1992 γ′ = -1.9 ±0.78, p = 0.003 and R squared = 0.157. In 1992 there was , in part, due to unusual rainfall which disrupted flower visitation.

evolutionary shifts, at the same pathway (Goldberg & Igic, 2008). Evolution of a selfing populations into an outcrossing population, requires new mutations that increase outcrossing (Nasrallah et al. 2004). In spite of this classic model, many populations exhibit both-combinations self- and cross-pollination, not fitting the proposed bimodal model (Vogler & Kalisz, 2001; Goodwille, et al., 2005). Vogler & Kalisz' (2001) reanalysis of data taken from three major surveys that estimated the outcrossing rates: Schemske & Lande, 1985; Barrett & Eckert, 1990 and Barret et al., 1996. They concluded that wind pollinated species exhibited a bimodal model and mixed mating in animal pollinated species was common (see Figure 1). Aide (1986) suggested that the use of a biotic agent in pollination increases unpredictability and causes the mating system to become less bimodal. Mixed mating in wind pollinated species may be brief as it transitions between strategies, whereas animal-pollinated species may be in a state of instability due to highly variable biotic effects between years, making the optimal trait value change across years. For example, Parra-Tabla & Bullock (2005) stated that weather conditions affecting the main pollinator changed the optimal value of the herkogamy between years(see Figure 2). If factors such as weather are highly variable, then a mixed mating system will be beneficial as it will guarantee seed set due to self-pollination in the years that the pollinators are adversely affected and allow cross-pollination in years when pollinators are abundant. Recent declines in pollinating insects (pre vs. post 1980) due to climate and habitat change have caused outcrossing plant species to decline alongside their pollinators (Biesmeijer et al., 2006). The strong effect of floral morphology on outcrossing rates suggests that the mixed mating strategy may be stable rather than in transition to complete self-pollination (Motten & Antonovics, 1992). In *Collinsieae,* Armbruster et al. (2002) found that floral traits were continuous between small and large flowered populations, which suggests that the size and self-pollination rates are continuous, agreeing with the continuous model of mating strategies suggested by Vogler & Kaliz (2001). Goodwillie *et al*. (2005) suggested that mixed mating systems could be stable due to biparental inbreeding, pollen discounting, pollen competition and resource allocation but more research would be needed to provide empirical evidence. Yet, the stability of the mixed mating system is still not fully understood (Goodwillie & Knight, 2006). Thus, the evolutionary dynamics of inbreeding have become central in attempts to account for the remarkable diversity of plant mating systems (Charlesworth & Charlesworth, 1987; Holsinger, 1992).

Inbreeding depression

The mating system will affect the level of inbreeding which may influence inbreeding depression. Inbreeding depression is “the reduction in fitness of progeny derived from inbreeding relative to those derived from outcrossing” (Husband & Schemske, 1996). In contrast, Charlesworth and Charlesworth (1987) gave a genetic definition as the decline in fitness with increasing homozygosity within a population. Thus, inbreeding depression can be defined as the decline in fitness with increasing homozygosity caused by the cross-pollination of closely related individuals (or, at the extreme, self-fertilisation). The consequences of inbreeding are still not yet fully understood; as inbreeding does not always lead to significant levels of inbreeding depression (see Armbruster & Rogers, 2004; Wright et al., 2007). The expression of inbreeding depression is lower in more self-pollinating populations (Barrett & Charlesworth, 1991; Dole & Ritland, 1993; Holtsford, 1996).

Fisher (1941) formulated a mathematical model, “automatic selection advantage model”, (now “transmission advantage”; Husband & Schemske, 1996; Chang & Rausher, 1999) to explain how self-pollination within a population can evolve. Alleles that promote self-fertilisation will be transmitted at a 3:2 ratio (an extra 50%) over cross-pollinating alleles. Self-pollinating alleles transmits two copies from self-pollination and one from cross-pollination whereas the cross pollinating alleles transmits one copy as the maternal plant passes it to the progeny and one copy in the export of pollen to another individuals progeny. An important limitation of this model is that it does not include the concept of inbreeding depression (Lande & Schemeske, 1985), which is given as the opposing force to the transmission advantage (Husband & Schemske, 1996; Chang & Rausher, 1999; Kephart et al., 1999) and maintenance of outcrossing if inbreeding depression is substantial. Different models for inbreeding depression have been proposed (Charleswoth & Charlesworth, 1987; 1999; Roff, 2002).

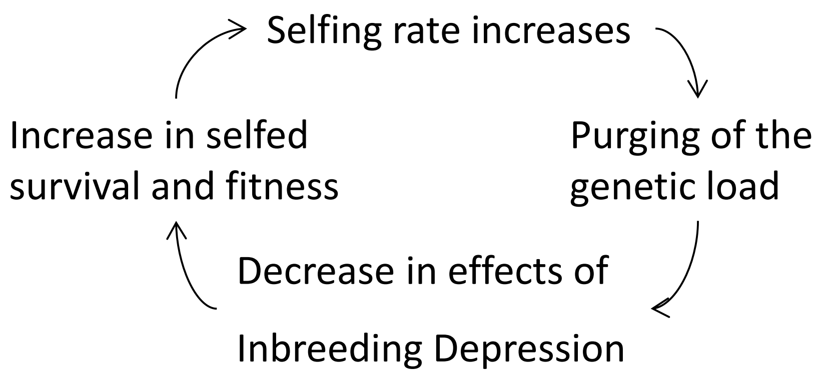


Figure 3. The suggested positive feedback mechanism. This is based on the ideas from Lande & Schemske (1985) and Goodwillie & Knight (2006).

In the "dominance model", natural selection will purge homozygote individuals expressing recessive, or partially recessive, deleterious alleles normally masked by heterozygosity, causing the fixation of alleles (Lande & Schemske, 1985; Dudash & Carr, 1998; Wright et al., 2007). These deleterious alleles will generally be present in populations at low frequencies (mutation-selection balance) (Charlesworth & Willis, 2009). The positive feedback mechanism (Figure 3) indicates how self-pollination and inbreeding depression can evolve together (Goodwillie & Knight, 2006).

In the "overdominance model", heterozygous individuals are superior to homozygous individuals because the two alleles are expressed and they have an additive effect on the fitness (Charlesworth & Willis, 2009). Inbreeding depression results from heterozygous individuals being lost from the population, and as a consequence the mean fitness will decline until heterozygosity is restored (Mitton & Grant, 1984; Lande & Schemske, 1985; Wright et al., 2007). These alleles are maintained at intermediate frequencies by balancing selection (Charlesworth & Willis, 2009) with no purging from the population (Land & Schemske, 1985; Wright et al., 2007).

In the pseudo-overdominance model, complementation can occur between unlinked deleterious alleles when heterozygous; when homozygous this complementation cannot occur. Thus, giving the appearance of overdominance at loci or in genomic regions (Charlesworth & Willis, 2009).

The dominance model is generally considered to be the major cause of inbreeding depression (Dudash & Carr, 1998; Wright et al., 2007; Charlesworth & Willis, 2009). Inbreeding depression based on the dominance model will be caused by deleterious alleles; these can be purged by natural selection. In the sixth generation of inbreeding in *Ipomea purpurea*, Darwin (1876) found a plant with surprising vigour, which he called "Hero". It is now interpreted that these occasional superior performances from an inbred line to be the result of the genetic load being purged (Land & Schemske, 1985; Charlesworth & Charlesworth, 1987; Charlesworth et al., 1990). In contrast, inbreeding depression based on the overdominace model cannot be purged without lowering the population's mean fitness (Lande & Schemske, 1985; Charlesworth & Charleworth, 1987;

Charlesworth et al., 1990). Byers and Waller (1999) suggest that much evidence for purging exists, yet there is no consistency and it is not substantial enough in most cases to favour fully inbred forms of mating. However, if not all loci acted in a dominate manner, but some in an overdominant (Dudash & Carr, 1998; Chareslworth & Willis, 2009) and psudeo-overdominace manner (Charlesworth & Willis, 2009) then purging would be inconsistent.

Mitton and Grant (1984) suggested that heterozygosity (this would include both dominance and overdominance models) may not be the only factor to influence fitness. They concluded that 70-80% of the variation in growth and developmental stability can be attributed to heterozygosity, 15-20% from specific gene combination (i.e. pseudo-overdominance and epistatic interactions) and the rest remained unidentified. Yet, specific values varied among species and populations.

Inbreeding depression can be expressed throughout the life cycle (Charlesworth & Charlesworth, 1987; Husband & Schemske, 1996). Early expression is due to recessive lethal alleles resulting in embryonic abortion or seedling death, in contrast late expression is a cumulative build up of faintly recessive deleterious alleles (Charlesworth & Charlesworth, 1987; Husband & Schemske, 1996) with longer lived individuals having a longer period of time for the accumulation to build up (Ishida, 2008). Deleterious alleles may differ in their expression due to the degree of deleteriousness in their effect (Lankinen & Armbruster, 2007). In addition, more genetic material is expressed during reproduction and seed maturation compared to early life cycle stages (Husband & Schemske, 1996); thus inbreeding depression can be seen during reproduction.

Outbreeding depression

Despite the benefits of outcrossing, several authors have highlighted that mating between genetically different individuals is not always beneficial and that outbreeding can have negative effects on the progeny (Ellstand & Elam, 1993; Waser & Price, 1994; Fenster & Galloway, 2000a; Tallmon et al., 2004; Galloway & Etterson, 2005; Becker et al., 2006 Bailey & McCauley, 2006; Grindeland, 2008). Outbreeding depression can take two forms; maladaptive outbreeding depression and genetic-breakdown outbreeding depression.

Maladaptive outbreeding depression (Ellstand & Elam; 1993) suggests that a small population might be unable to adapt to a local environment due to gene flow from a larger population. The foreign genes disrupt life history traits resulting in a reduced fitness (Edmands, 2007). Maladaption is generally seen in the F1 generation and can be attributed to the progeny being suited to neither environment of the parents. In *Plantao lanceolata* crosses between populations from dissimilar environments and geographical distance decrease the fitness of the offspring within a population (Crémieux et al., 2010). In addition, Jourdan-Pineau et al. (2012) have shown that small fragmented populations of *Pelodytes punctatus* with high levels of heterozygosity (due to gene flow from other populations) can exhibit a reduced fitness. These foreign genes stop the populations becoming locally adapted, as hypothesised in the Dobzhansky-Muller Model (see below).

The second cause of outbreeding depression would be due to the genetic-breakdown of the offspring. Initially, a population must first gain positive association within its own genetics. The Dobzhansky-Muller model suggests that isolated populations gradually accumulate neutral or advantageous mutations over time (as in Edmands, 2007). The selection for positive epistasis may result in the development of unique co-adapted gene complexes within isolated populations which if crossed would cause disruption/non-compatibility between these mutations (Whitlock et al., 1995; Fenster et al., 1997). Fenster and Galloway (2000a, b) proposed models that indicate that F1 generations would not show outbreeding depression, although F2 or later generations would exhibit evidence of outbreeding depression. If an inbred population crossed with other populations, then the F1 progeny can be more fit due to the reduction of inbreeding depression. However, as recombination of the DNA occurs within the population a deterioration of co-adapted gene complexes and/or epistatic interactions can occur (Mayr, 1963; Shields, 1982; Fenster & Dudash, 1994, p.40; Edmands, 1999; Chang & Rausher, 1999; Fenster & Galloway, 2000a,b; Luijten et al., 2002; Bailey & McCauley, 2006). Moreover, it can even bring together mutations from each population that have not encountered each other, increasing their harmful effect (Orr 1996; Turelli et al., 2001). In *Campanula americana*, F1 hybrids between distant populations performed poorly compared to their parents. The experiment was conducted in a greenhouse and the authors argued that the effects seen were due to genetic rather than ecological factors. This was the loss of additive-by-additive epistasis, thus the removal of local adaptation (Galloway & Etterson, 2005). This also shows that the F1 generation can show outbreeding depression.

Outbreeding depression has been found to be more pronounced at later stages in the life cycle (Waser & Price, 1994) and more noticeable in reproductive traits (Pélabon et al., 2005) such as reduced flower production (Grindeland, 2008) but has also been detected in juvenile traits: probability of germination, days to germination and blossom size (Galloway & Etterson, 2005). The effects are also stronger with increased genetic distance between parental sources (Pélabon et al., 2005; Galloway & Etterson, 2005), which is usually correlated with increased geographical distance.

Optimal outcrossing distances

Due to potential negative consequences of both self- and cross-pollination, a theory of optimal outcrossing distance has arisen (Waser & Price, 1994; Grindeland; 2008). Intermediate distances were found to show no outbreeding depression (Fenster & Galloway, 2000a; Galloway & Etterson, 2005; Grindeland, 2008). The closer the optimal distance between parental sources suggests that the population has had a history of self-pollination (Fenster & Galloway, 2000a; Bailey & McCauley, 2006). With increasing genetic distance between parents, epistatic interactions become progressively more affected by genetic divergence (Pélabon et al., 2005).

Outbreeding depression has been seen over great and small distances: the progeny from>1000 km crosses performed worse than their parents although the progeny from <1000 km crosses outperformed their parents in *Chamaecrista fasciculata* (*Fabaceae*) (Fenster & Galloway, 2000); the progeny from 550 km crosses had lower fitness than progeny from 1.5 km crosses in *Campanula americana* (Gallow & Etterson, 2005); progeny from 111-124 km displayed outbreeding depression at the same time as progeny from 3-10km crosses experienced inbreeding depression in *Stylidium hispidum* (Hufford et al., 2012). However, in *Gentianella germanica* (*Gentianaceae*) the progeny from 10 m crosses possessed higher fitness than the fitness of progeny from natural, 1 m (inbreeding depression) and between-population crosses (outbreeding depression) (Fisher & Matthies, 1997). A similar pattern was detected in *Delphinium nelsonii,* where the progeny from crosses of 3 m and 10 m exhibited a similar fitness which possess increased fitness compared to 1 m (inbreeding depression) and 30 m (outbreeding depression) crosses (Waser & Price, 1994). In *Digitalis purpurea* progeny from 6 m crosses were superior to progeny from 30 m crosses and self-pollination (Grindeland, 2008).

Pollen competition

The number of pollen grains present on the style can influence fertilisation and progeny performance (Mulcahy, 1974), not just the genetic origin of the pollen. It has been shown that pollen competition is a mechanism that can reduce the negative effects of inbreeding (Richardson & Stephenson, 1992; Quesada et al., 1993; Husband & Schemske, 1996; Armbruster & Rogers, 2004; Lankinen & Armbruster, 2007; Lankinen et al., 2009). Pollen competition promotes fertilisation by more compatible or genetically superior pollen, which in turn improves the fitness of the progeny (Richardson & Stephenson, 1992; Quesada et al., 1993; Husband & Schemske, 1996; Armbruster & Rogers, 2004; Lankinen & Armbruster, 2007; Madjidian et al., 2012). Presumably, this mechanism can also reduce outbreeding depression, but it may cause the underestimation of outbreeding depression (Lankinen & Armbruster, 2007). In *Collinsia heterophylla*, pollen competition increased fitness in self-pollinated offspring and inbreeding depression was lower when large pollen loads were applied relative to the low pollen load treatment (Lankinen & Armbruster, 2007).

For pollen competition to occur, the number of pollen grains must first exceed ovules. This suggests that there will be a minimum number of pollen grains needed before pollen competition can begin. Yet, it is known that not all pollen grains will fertilise the ovules even when ovules exceed pollen grains (Cruzan, 1990; Nemeth & Smith-Huerta, 2002). Ganeshaiah & Uma Shaanker (1988a) found that a critical number of pollen grains were needed otherwise pollen tube growth was inhibited. Parra-Tabla & Bullock (2005) needed a minimum of 10 pollen grains in *Ipomoea wolcottiana* before fertilisation could take place. Requiring a minimum number suggests that pollen load is important, yet this might be an adaptation against pollen limitation. Fertilisation can still be pollen limited if pollen number exceeds ovules and ergo may not experience pollen competition as pollen tubes do not always reach the ovules (Bertness & Shumway, 1992; Björkman, 1995).

A large proportion of the paternal genetic material is expressed during pollen germination allowing maternal assessment of the pollen and facilitates growth of more compatible pollen (Mulcahy, 1974). It is thought that faster growing pollen tubes will fertilise the ovules (Mulcahy, 1974; Snow, 1990; Snow & Spire, 1991; Pasonen et al., 1999; Skogsmyr & Lankinen, 2002), especially under intense pollen competition (Mulcahy, 1974). Faster pollen tubes are generally a sign the pollen is more vigorous and results in more vigorous offspring (Mulcahy, 1979). Yet, this is disputed, as pollen growth rate have been found to be highly plastic and responds to many factors, such as: temperature (Elgarsma et al., 1989), low nutrients during pollen development (Young & Stanton, 1990) and herbivory and environmental factors (Delph et al., 1997). Larger pollen loads may have a beneficial effect not only from pollen competition; the maternal plant may allocate more resources, based on pollen quantity (Charlesworth, 1988). Pollen mentoring is a benign or indirect action between pollen grains; initially a critical number of pollen grains open a path between the stylar cells (Ganeshaiah & Uma Shaanker, 1988a), which is beneficial to all pollen grains present. This positive effect can lead to more intense competition (Pasonen & Käpylä, 1998) and increases fertilisation success (Esser, 1953 as cited in Björkman, 1995; Ter-avanesian, 1978 as cited in Björkman, 1995).

Pollen competition is expected to result in the greatest benefit for cross-pollination and self-pollination within a predominantly outcrossing population (Husband & Schemske, 1996; Armbruster & Rogers, 2004) because the pollen load possesses a high level of genetic variation. Following on from this, multiple pollen donors will benefit the maternal plant in terms of pollen competition (Marshall & Ellstrand, 1986). However, Nemeth & Smith-Huerta (2002) and Cruzan (1990) found evidence of pollen inhibition with two pollen donors, especially with increasing contact between the two pollen sources. Despite the interaction, the most compatible pollen had the greatest chance of fertilisation (Lankinen & Skogsmyr, 2001).

Armbruster and Rogers (2004) used a spatial method for varying pollen competition in *Dalechampia scandens*: high pollen dispersion (low pollen competition) and low pollen dispersion (high pollen competition) on the stigmatic surface without varying the number of pollen grains. The more intense pollen competition treatment produced heavier seed and seedlings with faster-growing radicles compared to the less intense pollen competition treatments. It was shown that pollen competition reduced inbreeding depression, and the experimental design reduced a possible sampling effect from high pollen load by chance selection of superior pollen.

Resource allocation

Plants need to invest many resources for successful sexual reproduction. Sexual reproduction may cause a detrimental effect on competing functions, as allocation removes resources from investment in other life history traits (Richardson & Stephenson, 1992; Obeso 2002; Bañuelos & Obeso, 2004; Obeso, 2004). The concept of the maternal plant directing limited resources towards the desired areas was described by Bloom et al., (1985). Resource allocation can lead to conflict between; 1) the maternal plant and her offspring and 2) between siblings. Plants have evolved mechanisms to balance the respective interests of both the maternal plant and her offspring (Uma Shaanker & Ganeshaiah, 1997). For example, the maternal-offspring conflict arises as the embryos demand maximal resources (Trivers, 1974), which can result in rivalry among the offspring (Uma Shaankaer et al., 1988; Uma Shaanker & Ganeshaiah, 1997). Despite this, the maternal plant aims to achieve a well-balanced optimal level of resources for the offspring. This balance will allow the maternal plant to create the maximal number of seeds with the optimal resources for each seed (Smith & Fretwell, 1974). A healthy embryo can obtain more resources due to being a stronger sink resulting in differently weighted seeds and fratricide in extreme cases (Stephenson, 1981; Arathi et al., 1996; Mock & Parker, 1997; Uma Shaanker & Ganeshaiah, 1997). The strongest sinks among young fruits and seeds typically have the highest invertase activity (the breakdown of sucrose), greatest respiration rate, and most rapid cell division (Huang et al., 1992; Koch, 2004; Ruan et al., 2012).

The abortion of seeds is one way the maternal plant can allocate resources; it has been demonstrated in several studies as a non-random process indicating that maternal choice is an important factor (Stephenson & Bertin, 1983; Bookman, 1984; Stephenson, 1984; Stephenson & Winsor, 1986; Obeso, 1993b). Seed abortion has been linked to two hypotheses. The first hypothesis is that the maternal plants will favour embryos that are genetically superior (Lloyd et al., 1980; Obeso, 2004). Certain pollen donors have higher abortion rates when paired with particular maternal plants (Bertin, 1982), this would be a maternal-paternal interaction. The second hypothesis is that flower and fruit abortion is much more likely to be due to the maternal assessment of the fruit rather than the individual seeds within (Nakamura, 1986). Thus, resource allocation is more likely to occur to the whole fruit (Nakamura, 1986). The level of pollen competition can influence the number of seeds within a fruit, which in turn influences the chance of fruit maturation; the greater the number of seeds within a fruit the greater the chance that fruit will mature (Stephenson, 1981; Schemske & Fenster, 1983; Winsor et al., 1987). The timing of the abortion of flowers and fruits will occur early in reproductive development (Gurusamy & Bal, 2000) since the maternal plant will conserve maximal resources (Nakamura, 1986). In some species, slower growing fruits (weaker sinks) are much more likely to abort, these maternal investment patterns are established early on (Nakamura, 1986). In *Asclepias speciosa*, slower growing pods were aborted along with small pods, they were suggested to be weaker competitors (Bookman, 1983). An order of dominance can occur within a developing fruit and can influence seed maturation; basal seeds have a greater chance of maturing when compared to distal seeds (Ganeshaiah & Uma Shaanker, 1988b; Gurusamy & Bal, 2000; Diggle, 2003). Floral morphology has been identified as a potential causes of different weighting between seeds (Diggle, 1995), ovules located closer to the resources have a greater chance of development, or a floral morphology where ovules are places at unequal distances apart causing a staggered timing in fertilisation (Ganeshaiah & Uma Shaanker, 1988b). Gurusamy & Bal (2000) suggest that non-random seed abortion could be caused by two factors; firstly that slow growing pollen may be intrinsically less vigorous in competition with their siblings and secondly, that the embryos fertilised first secrete growth stimulated hormones, thus gaining a temporal advantage as sinks for resources (Hossaert & Valero, 1988).

After abortion, maximal resources are invested (Stephenson, 1981), to achieve an optimal number of fruits and seeds given available resources. Yet, Nakamura (1986) showed that cross-pollinations produced heavier seeds, suggesting continued maternal resource allocation. In *Fagopyrum esculentum* large pollen loads increased seed production and weight (Björkman, 1995); in contrast in *Mesua farrea* heavier seed came from fewer seeded fruits (Khan et al., 1999). In both species, the heavier seeds showed increased vigour in germination and growth, yet this increase of fitness could be genetic or maternal resource based. The production of larger seeds resulted in a higher success rate of germination and establishment of the seedling when compared to smaller seeds (Schaal, 1980; Leishman et al., 2000; Westoby et al., 2002; Moles & Westoby, 2004); however, there is a trade-off between seed mass and the number of seeds that can be produced (Smith & Fretwell., 1974; Lloyd, 1987; Leishman et al., 2000; Westoby et al., 2002; Parciak, 2002), resulting in a negative relationship between seed number and seed mass (Agren, 1989).

Dalechampia was used as the study system because there is much variation in floral morphology particularly in the level of herkogamy. This allows the comparison of various species and populations to test theories of mating systems within and between similar species. In addition, the species can be easily grown within a tropical greenhouse and encouraged to flower throughout the year giving a constant supply of ovules and pollen. Hand pollination are straightforward to carry out to a high degree of accuracy as they possess very large pollen grains (50-100 micrometers) that can be counted using a hand magnifier. There has also been a large amount of previous research conducted into *Dalechampia* as a study species with regard to pollination ecology, phylogeny, and floral evolution (Armbruster, 1984; 1985.; 1988.; 1993.; Armbruster, et al., 1995; 2002; 2005; Armbruster & Hertzig,. 1984.; Armbruster & Rogers 2004; Armbruster & Webster, 1979; Bolstad et al., 2010; Pérez-Barrales et al., 2013; Pélabon et al. 2012, 2005).

Aims & Predictions

The aim of this project consists on studying the relationship between the presence of inbreeding depression in *D. heteromorpha* and in four populations of *D. scandens* and possible mechanisms to ameliorate their negative effects on the female fitness.

Experiment 1 will assess the effect of pollen competition and inbreeding depression in the progeny. It is expected that offspring from self-pollination will possess reduced fitness than offspring from cross-pollination (prediction 1); offspring resulting from higher pollen competition will display increased fitness in both self- and cross-pollinated offspring (prediction 2). In addition, the results obtained in the two species will be compared to assess whether these species respond in a similar manner.

Experiment 2 will assess the effect of pollen competition and inbreeding depression in the progeny from four populations of *D. scandens*. It is expected that populations will not perform in a similar way to each other due to differences in the mating system based on the assessment of the anther stigma distance (ASD) (prediction 3); populations that exhibit a low ASD will exhibit reduced inbreeding depression (prediction 4); that pollen competition can reduce inbreeding depression (prediction 2); and pollen competition will be of greatest benefit to populations that exhibit larger ASD (prediction 5).

Chapter 2: Materials and Methods

Experimental Design and Research Methodology

Study system

Dalechampia (*Euphorbiaceae*) is a pantropical plant genus with most species distributed in Central and South America. *Dalechampia* species have unisexual flowers bore in pseudanthial inflorescences like a bisexual blossom (Webster & Webster, 1972). Each blossom contains three pistillate flowers, each of these with three ovules; thus each blossom can produce a maximum of 9 seeds. The pistillate flowers possess a long style with a large stigmatic surface extending from the style tips to about two thirds of the way to the style base (Armbruster et al., 1995). Each inflorescence usually contains 8-10 staminate flowers. The blossoms are functionally protogynous: during the first 2-3 days the bracts open and resin is secreted only the pistilate flowers are receptive. During the subsequent 5-6 days, the pistils remain receptive and the staminate flowers open 1-2 at a time (Armbruster & Herzig, 1984). Thus, seed production can result from both natural self- and cross-pollination (including geitonogamy). Associated with the staminate flowers is a gland-like complex of bractlets which secretes a sticky triterpenoid resin (Armbruster, 1984). Euglossine bees (Apidae), and *Hypanthidium* (Megachilidae) bees pollinate the flowers while collecting resin. This resin is collected by female bees for nest construction (Armbruster & Webster, 1979; Armbruster, 1984; Armbruster & Herzig, 1984). The bees land with the head contacting the gland and, if the bee is large enough with the thorax, legs and/or abdomen contacting the stamens and stigma flowers. Megachilid bees often collect pollen in addition to resin. Resin-collecting bees other than the megachilids appear to collect nothing else when collecting resin (Armbruster, 1984).

The study species *D. scandens* and *D. heteromorpha* are self-compatible and exhibit mixed mating systems. They were used because they display significant variation in traits related to the ability to attract pollinators and promote outcrossing at the same time as possessing traits involved in self-pollination.

Dalechampia heteromorpha

Dalechampia heteromorpha (Figure 4A) is a twining vine found in Mexico and Central America (Armbruster, 1993). The ASD range from 0.5 mm to 1.6 mm, therefore the species can be considered facultative autogamous (Armbruster, 1993).

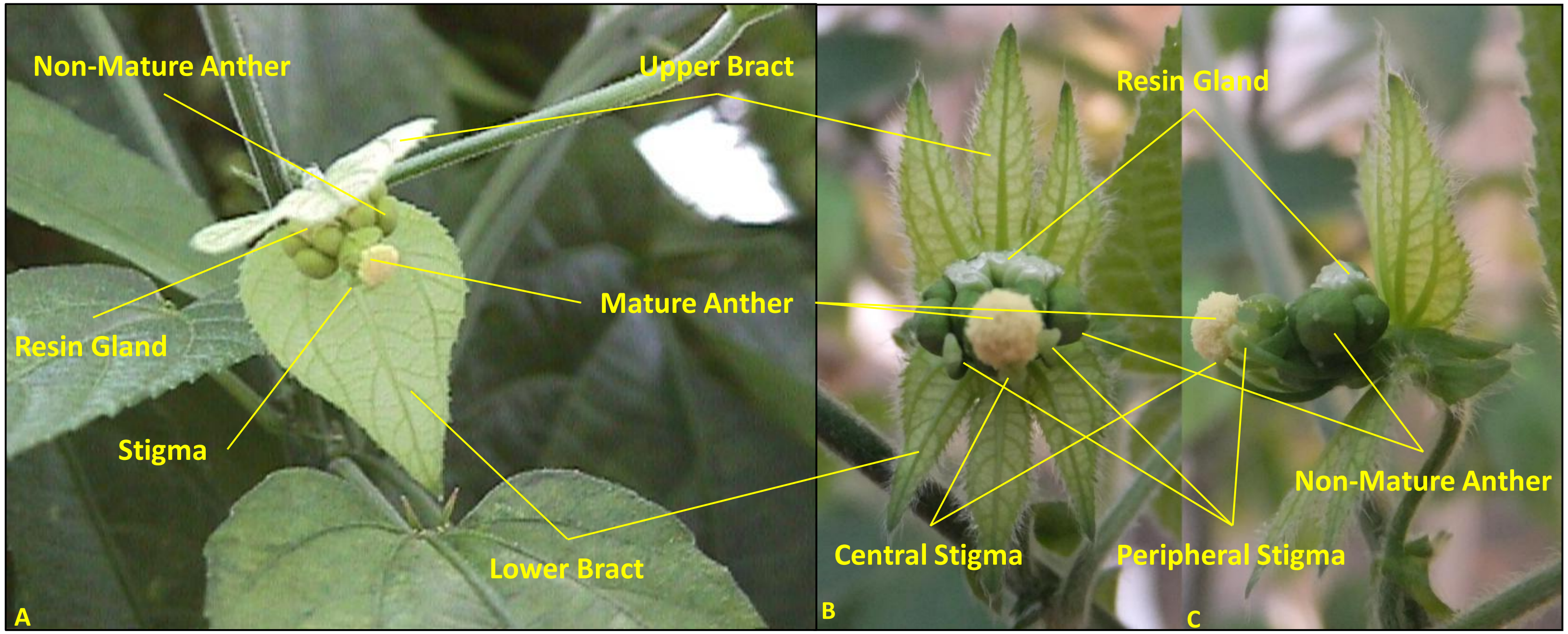


Figure 4. Left image (A) shows *D. heteromorpha*; blossom on first day of opening. Taken in the Greenhouses of the University of Portsmouth by DR. R. Pérez-Barrales. Right images (B & C) show *D. scandens* (Chamlea population); blossom on first day of opening. Forward view (B) and side view (C). Taken in the Greenhouse of the University of Portsmouth by Andrew Terry.

Dalechampia scandens

Dalechampia scandens (Figure 4B & C) is a self-compatible neotropical twining vine (Webster & Webster, 1972; Webster & Armbruster, 1991), which grows in disturbed areas (e.g. roadsides and tree falls), in the canopy of thorn woodland and dry tropical forests, and the edges of wet tropical forest (Armbruster, 1993). The species exhibits a great variation in the blossom morphology across population, and the ASD ranges between 0 and 3.72 mm (Figure 5).

Experiment 1:

Varying pollen competition and pollen origin in *D. heteromorpha* and *D. scandens*

Dalechampia heteromorpha individual plants used in the experiment came from a collection of seeds from University of Alaska, Fairbanks, Alaska. The collection probably originated from near Palenque, northern Chiapas, Mexico. The plants were derived from cuttings and/or selfed seeds produced by the original plants. *Dalechampia scandens* were grown from seeds collected in the field from Chamela, Mexico. The plants used were all first generation. The plants within the greenhouse were regularly moved around to reduce any location effect.

The experiment aimed to assess factors affecting inbreeding depression in the offspring by varying pollen competition and pollen origin in the two species. Pollen competition was achieved by applying different quantities of pollen to the stigmas, and these pollinations were either self- or cross-pollinations. In the *D. heteromorpha* cross-pollination was chosen by what was available and if repeated pollination sets occurred then a different paternal source would be used. In *D. scandens*, three predefined paternal lines were determined before the pollinations began. These lines were not used to produce seeds. Since the blossom has three pistils, each stigma received the same treatment. Before conducting hand-pollinations, blossoms were emasculated to avoid contamination of self-pollen (see below). There were certain differences between the species in the way the experiment was conducted, and details are provided below. Experiments on *D. heteromorpha* between were carried out in 2008-2009. Experiments on *D. scandens* occurred 2010-2012. A heat wave, ~50°C for 1 day, occurred within the greenhouse in 2012 during the germination experiment for *D. scandens* germination experiment and it was decided that after germination no further measurements would be taken. This will stop direct comparison after germination between the two species. It could have also impacted negatively on smaller seeds however, that data suggest that the added stress it could have increased the expression of inbreeding and outbreeding depression (Armbruster & Reed, 2005).

Experimental manipulation in *D. heteromorpha*

In *D. heteromorpha*, blossoms selected for hand pollination were emasculated; this was done to avoid natural self-pollination or dislodged pollen during the manipulation. Emasculation was achieved by the removal of the staminate flowers and the gland complex at the base when the first staminate flower matured but before it opened. A pair of forceps was used to cut the base stem.

Two levels of pollen competition were established (low and high) with this pollen either being self-pollinations or cross-pollinations. This resulted in four treatment combinations: self-low, self-high, cross-low and cross-high. Self-pollinations were achieved with pollen collected from the same genotype whereas cross-pollinations were from a single genetically different source. All cross-pollinations within a set of four pollinations used the same paternal source. However, several maternal lines received multiple sets of four pollinations; these did not use the same paternal source as a previous set of four pollinations used. The low pollen-load treatment was achieved by using a streaking loop: pollen was collected from anthers with the loop and transferred to the stigmas, and the number of pollen grains were counted until the desired amount had been transferred. The mean ± standard deviation of the low pollen load added to the stigma was 20.58 pollen grains ± 5.90 SD, N=62. The high pollen load treatment was achieved by bringing the stamen into contact with the stigmas. The pollen grains deposited on the stigma were estimated at 154.72 pollen grains ± 28.12 SD, N=62. In total, 18 maternal lines used in the experiment; of these 18 maternal lines, seven received a single set of four pollinations, 10 maternal lines received two sets of four pollinations, and one maternal line received three sets of four pollinations. In total, 30 sets of four pollinations were carried out.

Several measurements of stem growth rate and leaf number were taken; radicle growth, stem growth on day 11, 39 and 74 (mm/day) and the number of leaves present on days 39 and 88. After 95 days, plants were harvested, dried in an oven overnight and weighted.

Experimental manipulation in *D. scandens*

In *D. scandens,* three levels of pollen load were established (low, medium and high), and self-pollinations were effected with pollen collected from the same genotype whereas cross-pollinations were from three predefined paternal sources. These paternal lines were selected from across the population, before the experimental pollinations began, to reduce or avoid any paternal-maternal interactions. This resulted in six treatment combinations: self-low, self-medium, self-high, cross-low, cross-medium and cross-high. Blossoms were first emasculated. This was achieved by the removal of the maturing staminate flowers from the blossom before the anthers would open, to avoid self-pollination, whilst still allowing the remaining immature staminate flowers to mature. The maturing staminate flowers move forward as the subtending column elongates and opens to reveal the pollen. Emasculation occurred before the anthers opened as during emasculation pollen could be dislodged. To remove the staminate flower, a pair of forceps cuts the pedicle of the staminate flower. Usually 8-10 staminates are present on a blossom and emasculation would then occur over the next 4-5 day, as the males develop sequentially. Hand pollinations were made within 3 days of the first emasculation. This method of emasculation was chosen over removal of all staminate flowers and the gland (the reward structure), as many blossoms would soon die afterwards (personal observations). It was hypothesised that if blossom takes “too much damage”, the blossom is aborted. Emasculation of the maturing staminate flowers could mimic a more natural process and allows the flow of resource to continue. Hand pollination where achieved by collecting pollen from newly opened staminate flowers. The anthers were collected into an Eppendorf tube and agitated, by flicking, to loosen pollen from the anther and allow mixing for cross-pollination treatments. Self-pollinations were also done in this way. Pollen was transferred to the stigma with the aid of a plastic streaking loop. The number of pollen grains placed on the stigmas was confirmed using a hand magnifier. The amounts of pollen transferred to stigmas were as follows: low pollen load: 22.76 pollen grains ± 1.79 SD pollen grains, N=306; medium pollen load: 41.02 pollen grains ± 3.19 SD pollen grains, N=279; and, high pollen load: 81.42 pollen grains ± 3.41 SD, N= 297 pollen grains. In total 13 maternal lines (and 3 paternal lines) were used and 98 pollinations were carried out. After pollination, excess buds were removed until fruit maturation as a way to control for possible limitation or competition of resources between fruits and flowers within the plant.

Collection of data

The number of seeds produced per blossom was noted. In *D. heteromorpha* collection occurred when the fruits were ripe and dehisced (an explosive scattering) into an empty tea bag which surrounded the fruits. In *D. scandens* seeds were also noted to which pistil within the blossom produced them. The fruits were harvested before they dehisced, indicated by a change in colour associated with drying of the fruit. Tea bags were placed around fruit nearing maturity as a safeguard. Seed where then labelled and stored in a cold room at 5°C. All seeds were weighted to 4 significant figures, on the same set of balances. Once weighted, the seeds were labelled and given individual identification so the seed performance could be tracked.

To germinate the seeds they first needed to be scarified. In both species the seed coat was abraded until it could be removed without damaging the embryo inside. Scarification of the seed coat was achieved by placing the seed between two emery boards and kept in place by use of the hole at the base of a key. Gentle pressure was applied with rubbing of the seed coat. Once the seed coat had been weakened (usually denoted when the seed coat developed a red or rust coloured tinge) a fine point of forceps were pushed into the seed coat and broken away. The embryo was placed into a sterile environment either an Eppendorf tube or a well plate silicon lid and stored at 5°C. The embryos were germinated on moistened filter paper in a tray within the greenhouse. The trays were covered with cling film to increase humidity and reduce water loss for the germination period. If the seed germinated then the young plant was transplanted when the radicle root had grown to 10mm in length into a labelled pot. The compost within the 4 litre black pots were made up on site, 3:2:2 peat: vermiculite: perlite mix.

The radicle growth rate was the length of the root on the day of transfer to the pot divided by the number of days from the start of germination. The root was measured using digital callipers. Also, seeds that germinated and did not germinate were noted.

In *D. heteromorpha* several measurements of stem growth rate and leaf number were taken; stem growth on day 11, 39 and 74 (mm/day), the number of leaves present on days 39 and 88 and the dry weight. The stem growth rate was determined by measuring the length of the stem and dividing it by the number of days since germination began, the number of leaves present was a simple count of mature leaves present on the plant. The area was not taken as the size of the mature leaf does not vary much. The dry weight of the plant was determined after 95 days of growth; the plants were removed from their pots, had their roots washed to remove the soil, placed into a sheet of newspaper, pressed and placed into a drying oven overnight at 80° C. Once dry, the harvested plants were weighted.

Pollen origin and its effect on resource allocation in *D. scandens*

Offspring fitness can be influenced by the maternal plant allocating more resources to ovules fertilised by more compatible pollen. The aim of this additional experiment was to assess the ability of the maternal plant to allocate resources when given either a self– or cross-pollination at the same time a second blossom receives either the same or alternative treatment. This is a control experiment to ascertain if branches act independently or if the maternal plant allocates resource towards a favoured pollen origin.

Experimental manipulation

There were three treatments: self/self, self/cross or cross/cross. As such the plant required two blossoms at similar stages of development on separate branches. One reason for this is that the development of blossom on a branch is sequential, so two blossoms on a branch at the same stage is highly improbable. Once two blossoms were identified they were emasculated and pollinated (as described in the experimental manipulation in *D. scandens* above). One of the three treatments would be randomly allocated to this pair of blossoms. If they were located with the self/cross treatment then the pollen origin would be randomly assigned again. Self-pollinations were achieved using pollen from same genotype and the cross-pollination with pollen from the three predefined fathers (see above for more details). A high pollen load was applied to increase any effect of pollen competition and reduce pollen limitation (80.76 pollen grains ±4.25 SD, N=684). All developing buds were then removed until the fruits had matured. In total seven maternal lines were used with 76 pollinations (38 pairs).

Analysis of varying pollen competition and pollen origin in *D. scandens* and *D. heteromorpha*

A generalised linear mixed model (GZLMM) was used to assess the effect of pollen competition and pollen origin in *D. scandens* and *D. heteromorpha*. The traits were analysed at different levels: individual level, female flower level and blossom level. In the individual level analyses, seeds were treated as independent, in the female flower level analyses the traits were averaged to the pistil within a blossom and in the blossom level analyses the traits averaged to the blossom. . The analyses of the female flower level and the assessment of the pistil effect are only available for *D. scandens.* In *D. scandens*, during germination a heat wave (about 50°C for 1 day) occurred and the experiment was stopped after germination.

In the *D. heteromorpha* analysis, the dependent variables were the number of seeds, weight of the seeds, probability of germination, radicle growth rate, stem growth rate on days 11, 39 and 88 (mm/d), the number of leaves present on day 39 and 88 and the final dry weight (g) after 95 days from planting. The number of seeds was modelled as Poisson log error distribution and probability of germination was modelled as binomial log error distribution at the blossom level based on the number of seeds produced. Pollen origin, pollen load and the interaction between pollen origin and pollen load were included as fixed factors with the maternal and paternal lines included as random factors. The weight of the seeds, radicle growth rate, stem growth rate on days 11, 39 and 88, the number of leaves present on day 39 and 88 and the final dry weight used a linear error distribution at the individual level. The germination was modelled as binomial logit error response. Pollen origin, pollen load and the interaction, pollen origin by pollen load were included as fixed factors with the radicle growth rate included as a co-variate on the stem growth rate on days 11, 39 and 88 and the number of leaves present on day 39 and 88. The maternal and paternal lines included as random factors in all models.

In *D. scandens*, two different analyses were carried out. The first analysis was similar to that in *D. heteromorpha,* and this allows comparisons between species. The dependent variables were the number of seeds, weight of the seeds, probability of germination and radicle growth rate. The number of seeds was modelled as Poisson log error distribution and the probability of germination was modelled as binomial logit error response based on the number of seeds produced at the blossom level. Pollen origin, pollen load and the interaction term pollen origin by pollen load were included as fixed factors; the maternal line was included as random factor. The weights of the seeds and radicle growth rate were modelled as linear error distribution at the individual level. Pollen origin, pollen load and the interaction term pollen origin by pollen load were included as fixed factors; the maternal line and the blossom number were included as random factors. The blossom number are the seeds produced by a blossom grouped to each other, while at the female level it groups the pistils within the blossom together. The second analysis used the same dependent variables. The number of seeds was modelled as Poisson log error distribution and the probability of germination was modelled as binomial logit error response based on the number of seeds produced at the female level. Pollen origin, pollen load, female position and the interaction pollen origin by female position and the interaction pollen load by female position was included as fixed factors. The maternal line and blossom number were included as random factors. The weights of the seeds and radicle growth rate were modelled as linear error distribution at the individual level. Pollen origin, pollen load, female position and the interaction pollen origin by female position and the interaction pollen load by female position was included as fixed factors. The maternal line and blossom number was included as a random factor.

The value of inbreeding depression was evaluated by using the δ value which represents the strength of inbreeding depression which is calculated as:

$$\delta = 1 - \left(\frac{\omega_s}{\omega_o}\right),$$

where ω_s and ω_o are the fitness of selfed and outcrossed offspring respectively (Ishida 2008, Johnston & Shoen, 1994). The equation has been modified to evaluate the magnitude of inbreeding based on the pollen load (Lankinen & Armbruster, 2007):

$$\delta = 1 - \left(\frac{\omega_l}{\omega_h}\right),$$

where ω_l and ω_h are the fitness of lower pollen and higher pollen load, respectively. This has been calculated for each trait that has been analysed. In *D. heteromorpha* the value of inbreeding depression was determined between the pollen origin and pollen load for: number of seeds, seed weight, probability of germination, radicle growth rate, growth rate on days 11, 39 and 74, the number of leaves on day 39 and 88 and the final dry weight. In *D. scandens* the value of inbreeding depression was determined between the pollen origin and between the pollen loads (low v medium, low v high and medium v high) for: number of seeds, seed weight, probability of germination and radicle growth rate.

An overall estimate of inbreeding depression has be calculated using a multiplicative fitness function (W).

$$\delta = 1 - \left(\frac{W_s}{W_o}\right), \delta = 1 - \left(\frac{W_l}{W_h}\right),$$

where W_s, W_o, W_l and W_h represent overall fitness for selfed, outcrossed, lower, and higher pollen loads, respectively. Two stages will be assess, the early traits and the late traits.

$$W_{early\ traits} = \left(\frac{\text{seed number}}{9}\right)(\text{seed weight})(\text{germination})(\text{radicle growth rate})$$

$$W_{later\ traits} = (\text{g r } 11)(\text{g r } \ 39)(\text{gr } 74)(\text{leaves on day } 39)(\text{leaves on day } 88)(\text{dry weight})$$

Analysis of pollen origin and its effect on resource allocation in *D. scandens*

In the analysis, there are four statistically testable manipulations: self/self, self/cross, cross/self and cross/cross, with the first term indicating the pollination treatment as the main manipulation and the second term indicating the pollination treatment in the neighbouring manipulation. A generalised linear mixed model (GZLMM) was used to assess the effect of pollen origin and resource allocation. The dependent variables were abortion rate of the blossom and the number of seeds produced from the observations on the main blossom. Since abortion rate of the blossoms was high, the aborted blossoms were removed from subsequent traits as they caused an artefact in subsequent analyses.

Abortion rate was analysed at the blossom level and modelled as binomial log error distribution; pollen origin, neighbouring pollen origin and the interaction pollen origin by neighbouring pollen origin were included as fixed factors in the model with the maternal line as a random factor. Number of seeds produced was analysed at the female level and modelled as binomial error response. Pollen origin, neighbouring pollen origin female position, the interaction terms pollen origin by neighbouring pollen origin, pollen origin by female position and neighbouring pollen origin by female position were included as fixed factors in the model with the maternal line and blossom number as random factors.

Post-hoc comparisons were performed using pairwise sequential Bonferroni were carrired out on number of seeds, probability of germination, growth rate at day 74, number of leaves present at day 8 in *D. heteromorpha*. Post-hoc comparisons were performed using pairwise sequential Bonferroni were carried out on number of seeds per blossom using the first analyse and the number of seeds and seed weight in the second analysis. in *D. scandens*. In the resource allocation experiment in *D. scandens*, post-hoc comparisons were performed using pairwise sequential Bonferroni on the abortion rate, the number of seeds produced and the seed weight.

Experiment 2:

Can blossom morphology predict inbreeding depression? A comparison of four populations of *D. scandens*

The aim of this experiment was to compare the strength of inbreeding depression across four populations. Previous studies revealed substantial variation in the ASD, which can influence self-pollination rates (e.g. blossom with small ASD may experience more self-pollination than blossom with large ASD (Armbruster, 1988; Pérez-Barrales et al., 2013; Bolstad et al 2010). These populations were selected because they represent this variation (Figure 5).

The blossoms from all four populations were emasculated and hand-pollinated with self-pollen (as described above). Three pollen loads were applied: low (23.43 pollen grains ± 3.393 SD, N=387), medium (41.17 pollen grains ± 3.195 SD, N=405) and high (81.68 pollen grains ± 3.165 SD, N=486). The number of pollen grains laid on the stigmas was confirmed using a hand magnifier.

Study Populations

Chamela

This population (Figure 5) was established from seeds collected from the Chamela-Cuixmal Biosphere Reserve, Jalisco State, Mexico in 2009. The staminate and pistillate flowers are frequently in contact with one another, and the blossom visitation by *Hypanthidium* bees was observed to be low to absent (Personal observation). The greenhouse observations show that the population possess the smallest total bract area, gland area and ASD (Figure 6A-C). These traits suggest that it has become highly reliant on self-pollination and as such has the highest level of self-pollination to occur and they also produced the most seeds (Figure 6D&E). In comparison to the other populations the evidence suggests this is a predominantly self-pollinating species. In total 13 maternal lines were used in the pollinations.

La Mancha

This population occurs ca. 50 km north of the city of Veracruz, Veracruz State, Mexico; seeds were collected in 2006 and 2007. This population (Figure 5) exhibits an ASD and gland area that fall in between Chamela and Maria la Gorda/Puerto Morelos (which are similar; see below) (Figure 6A&B). However, the bract area is the largest in all the populations (Figure 6C). The stamens and stigmas are located close to each other and this facilities self-pollination. *Dalechampia* is generally considered to be an honest advertiser of reward available (Pélabon et al., 2012), the difference between gland and bract size could indicate that this population has recently transited to increase self-pollination and bract size has yet to evolve to represent this decrease in size of other traits. Disregarding the bract area, auto-pollination and seed production (Figure 6C&D) follow the pattern in of high level of auto-pollination and seed production, slightly less than Chamlea. In comparison to the other populations the evidence suggests this is a self-pollinating population. In total 8 maternal lines were used.

Maria la Gorda

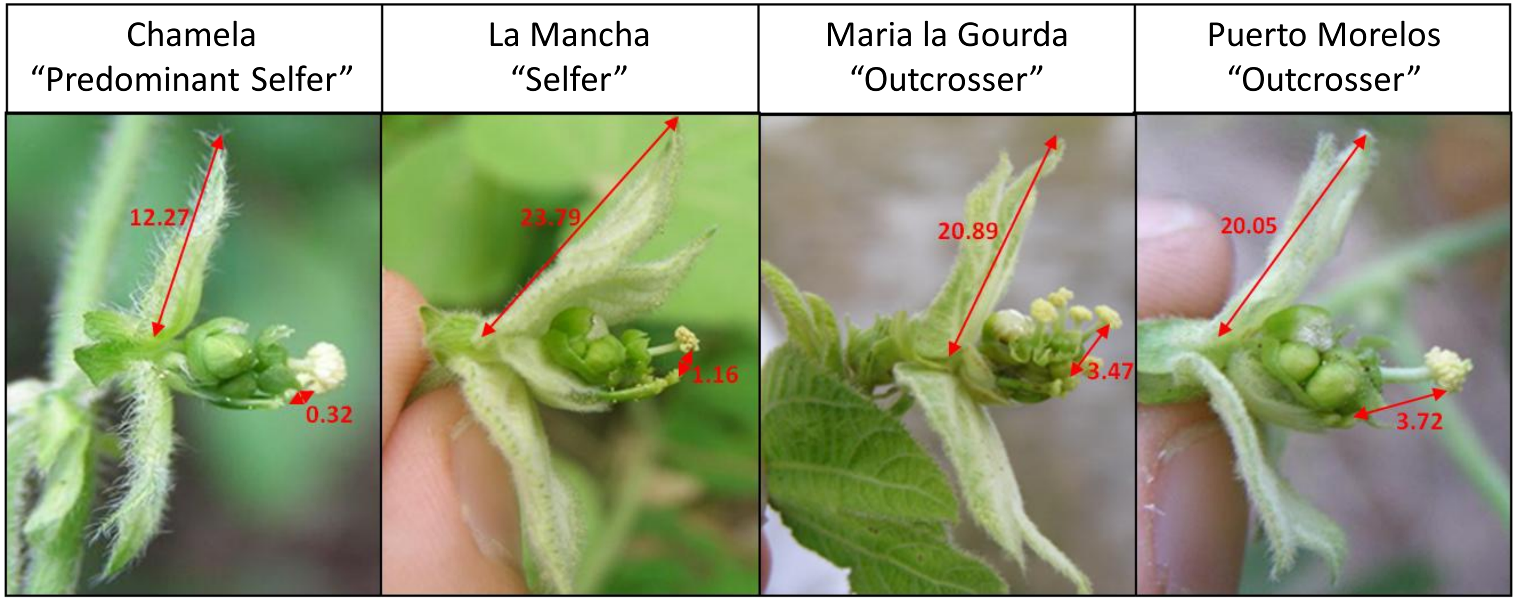
This population (Figure 5) was collected from the western tip of Cuba in 2007. The population possess the large ASD, a large gland area and a large bract area which all suggest they are outcrossers (Figure 6A-C). The auto-pollination and seed production rates were low (Figure 6D&E) as would be expected from the increased ASD. These traits indicate that it is a largely outcrossing population. In total 12 maternal lines were used.

Puerto Morelos

This population (Figure 5) was collected near Puerto Morelos, 35 km south of Cancun, Quintana Roo State, Mexico in 2006 - 2007. The population possess a large ASD, a large gland area and a large bract area which all suggests this is an outcrossers (Figure 6A-C). The auto-pollination and seed production rate was low (Figure 6D&E) as would be expected from the increased ASD. These traits indicate that it is a largely outcrossing population. In total 11 maternal lines were used.

Analysis to assess whether blossom morphology can be a predictor of inbreeding depression: a comparison of four populations of *D. scandens*

Analyses were carried out both among and within populations. The traits were analysed at different levels: individual plant, female flower and blossom level. In the individual level analyses, seeds were treated as independent, in the female flower level analyses the traits were averaged to the pistil within a blossom and in the blossom level analyses the traits averaged to the blossom. During germination a heat wave occurred and the experiment was stopped after germination.


Chamela “Predominant Selfer”

La Mancha “Selfer”

Maria la Gourda “Outcrosser”

Puerto Morelos “Outcrosser”

Figure 5. Image of a blossom from each population of *D. scandens*, each imagine shows the average upper bract length and anther stigma distance. They have been classed as ‘predominant selfer’, ‘selfer’ or ‘outcrosser’ based on the morphology they possess. Image taken in the Greenhouse of the University of Portsmouth by Andrew Terry

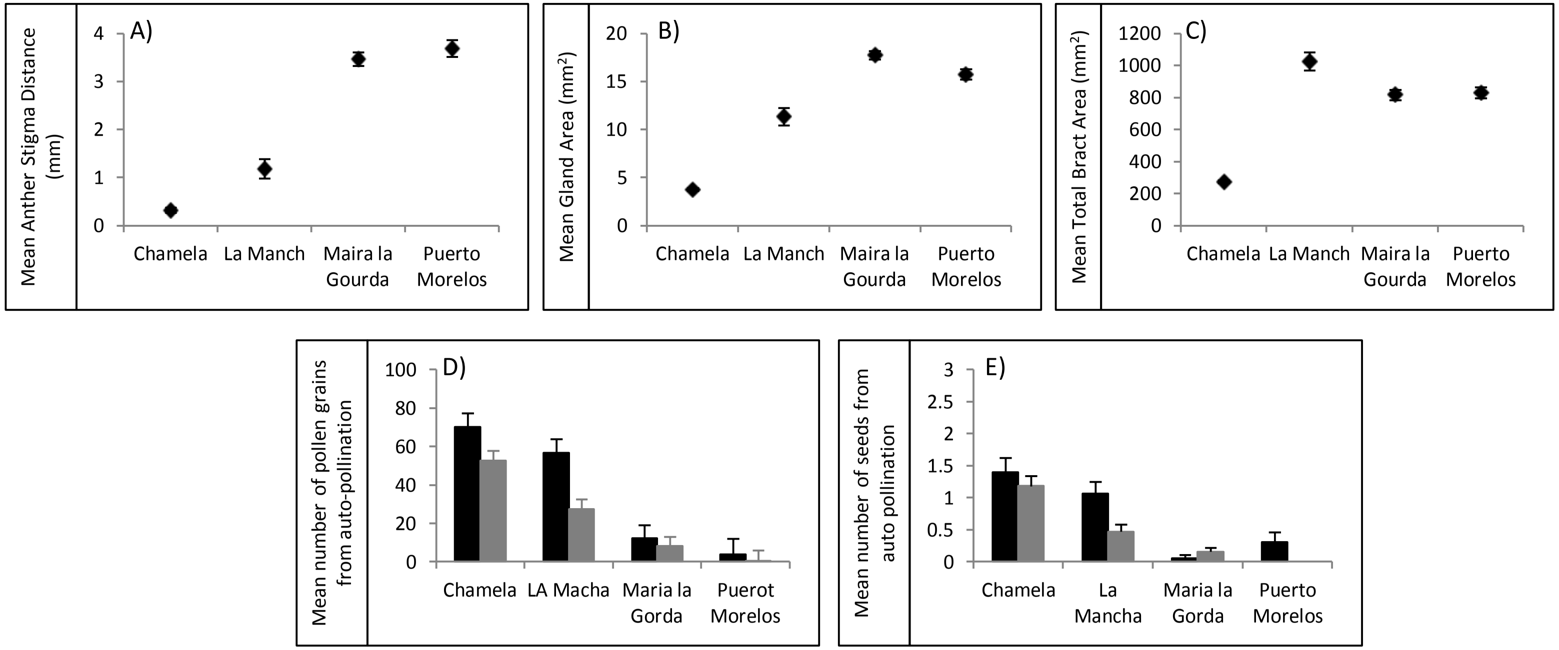


Figure 6. A-C shows three morphological measurements all measurements were taken on the first day of the bisexual phase; A) shows the mean anther stigma stigmas distance (ASD), B) shows the mean gland area and C) shows the mean total area of the bracts. D & E show pollination; D) shows the mean number of pollen grains arriving as a result of auto pollination, and. E) shows the number of seeds produced as a result of autogamy. Black represents the central female and the grey represents the peripheral females. Error bars are two standard errors.

A generalised linear mixed model (GZLMM) was used to assess the effect of pollen competition in four populations of *D. scandens*. The dependent variables were the number of days to produce fruit, the number of seeds produced, seed weight (g), probability of germination and radicle growth rate (mm/d). The number of days to produce fruit was analysed at the blossom level and modelled as normal distribution. Population, pollen load and the interaction population by pollen load were included as fixed factors and the maternal line as random factor. The number of seeds produced was analysed at the female level modelled as binomial logit error distribution and the probability of germination was modelled as binomial logit error distribution, dependent on the number of seeds produced at the female level. Population, pollen load, female position, the interaction population by pollen load and the interaction pollen load by female position were included as fixed factors. Maternal line and blossom number were included as random factors.

The seed weight (g) and radicle growth rate were analysed at the individual level and modelled as normal distribution. Population, pollen load, female position, the interaction population by pollen load and the interaction pollen load by female position were included as fixed factors. The maternal line and blossom number were included as random factors. A generalised linear mixed model (GZLMM) was used to assess the effect of pollen competition in four populations of *D. scandens*. The dependant variables were the number of days to produce fruit, the number of seeds produced seed weight (g), probability of germination and the radicle growth rate (mm/d). The number of days to produce fruit was analysed at the blossom level modelled as normal distribution. Pollen load as a fixed factor and the maternal line as a random factor. The number of seeds produced was analysed at the female level was modelled as binomial logit error distribution and the probability of germination was modelled as binomial logit error distribution, dependent on the number of seeds produced at the female level. Pollen load, female position and the interaction pollen load by female position were included as fixed factors and the maternal line and blossom number as random factors. The seed weight (g) and radicle growth rate (mm/d) were analysed at the individual level modelled as normal distribution. Pollen load, female position and the interaction pollen load by female position were included as fixed factors. The maternal line and blossom number were included as random factors. The inclusion of blossom number was a way to reduce the statistical assumption of independence.

Post-hoc comparisons were performed using pairwise sequential Bonferroni on seed weight, probability of germination, seed number and seed weight in *D. scandens* between the four population comparisons.

All the analysis carried out were done in SPSS20.

Chapter 3: Results

Varying pollen competition and pollen origin in *D. heteromorpha* and *D. scandens*

In *D. heteromorpha* the number of seeds produced differed significantly with pollen load (P < 0.001) and marginally with pollen origin (P = 0.076; Table 1). The high pollen load treatment produced more seeds ($\bar{x}$ = 7.54 seeds, SE = 0.36) than the low pollen load ($\bar{x}$ = 4.52 seeds, SE=0.33; Figure 7A). The self-pollination treatment produced more seeds ($\bar{x}$ = 6.43 seeds, SE = 0.35) than the cross-pollination treatment ($\bar{x}$ = 5.53 seeds, SE = 0.36; Figure 7A). The results from post-hoc tests indicate that there were statistically significant difference in the number of seeds per blossom (Figure 7A) between the pollen loads for the self-and cross-pollination treatments (P = 0.002 and P < 0.001, respectively). There was also a statistically significant difference between the pollen origins for the low pollen load treatment (P = 0.049, Figure 7A), yet no statistical significance was detected between the pollen origins for the high pollen load treatment. The weight of the seeds did not differ significantly between treatments. The probability of germination differed significantly between pollen loads (P = 0.031) and approached significance for pollen origin(P = 0.069 Table 1). The seeds that were produced from high pollen loads had a greater chance of germination ($\bar{x}$ = 0.41, SE = 0.07), than those from low pollen loads ($\bar{x}$ = 0.31, SE = 0.07). Those seeds produced from self-pollination had a greater probability of germination ($\bar{x}$ = 0.40, SE = 0.07) than cross-pollination ($\bar{x}$ = 0.31, SE = 0.07; Figure 7B). The results from post-hoc tests indicate that there was a statistically significant difference for the probability of seed germination (Figure7B) between the pollen origins for the high pollen load treatment (P = 0.018) but no detectable significance between the pollen origins at the low pollen load. There was also a statistically significant difference for the probability of seed germination between the pollen loads for the self pollination treatment (p=0.016) but no detectable difference between the pollen loads for the cross pollination treatment. For the seeds that germinated, the radicle growth rate was not influenced by the treatments (P = 0.228). In *D. scandens* the number of seeds produced differed significantly with pollen origin (P < 0.001), pollen load (P = 0.007) and the interaction term between them (P = 0.011; Table 1). Self-pollination produced more seeds ($\bar{x}$ = 5.04, SE = 0.43) than cross-pollination ($\bar{x}$ = 1.92, SE = 0.224). The seed production responded positively to the high pollen load ($\bar{x}_{low\ pollen\ load}$= 2.82, SE = 0.34; $\bar{x}_{medium\ pollen\ load}$ = 2.66; SE = 0.35; $\bar{x}_{high\ pollen\ load}$ = 4.02, SE = 0.42).

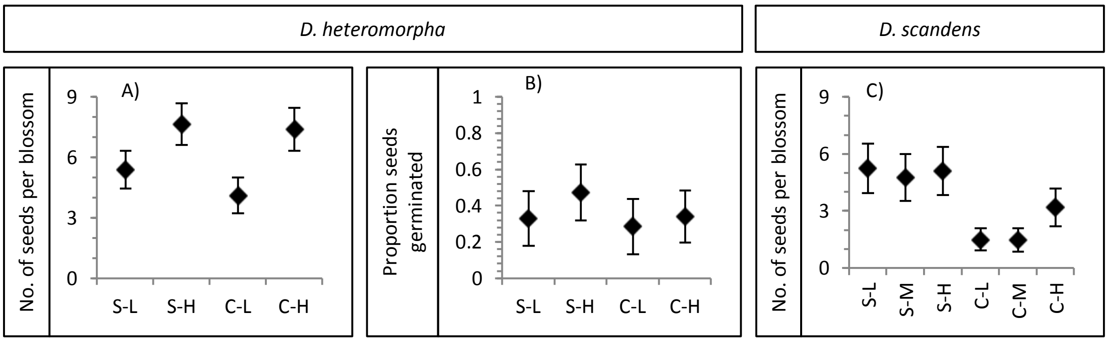


Figure 7. (A-C) Graphs showing offspring fitness in *D. heteromorpha* and *D. scandens*. Pollen origin is represented by S (self) and C (cross). Pollen load is represented by L (low), M (medium) and H (high). Error bars indicate two standard errors. See Table 1.

Table 1. Generalized Linear Mixed Model (GZLMM) for early fitness traits in D*. heteromorpha* and *D. scandens* produced from differing pollen origins (P.O.), self- or cross-pollination with varying pollen loads (P.L.). *D. heteromorpha* experienced low and high pollen loads while *D. scandens* had low medium and high.

Source of variation	F	df1	P-value	F	df1	P-value	F	df1	P-value	F	df1	P-value
	Number of seeds per blossom (*n* = 102)			Seed weight (g) (*n* = 643)			Probability seeds germinated (*n* = 102)			Radical growth rate (mm/d) (*n* = 201)		
D. heteromorpha												
P.O.	3.221	1	0.076	0.187	1	0.666	3.390	1	0.069	1.465	1	0.228
P.L.	30.869	1	0.000	0.058	1	0.810	4.773	1	0.031	0.679	1	0.411
P.O*P.L.	1.984	1	0.162	1.604	1	0.206	0.877	1	0.351	1.514	1	0.220
D. scandens	(*n* = 98)			(*n* = 342)			(*n* = 75)			(*n* = 93)		
P.O.	61.769	1	0.000	1.195	1	0.275	0.484	1	0.489	1.649	1	0.202
P.L.	5.230	2	0.007	0.070	2	0.933	1.887	2	0.159	1.225	2	0.299
P.O*P.L.	4.712	2	0.011	1.277	2	0.280	1.959	2	0.149	1.532	2	0.222

However, the interaction term shows that self-pollination produced the same number of seeds regardless of pollen load but cross-pollination is inferior but does respond positively to pollen load ($\bar{x}_{self-low}$ =5.25 seeds, SE = 1.31; $\bar{x}_{self-medium}$ = 4.77 seeds; SE = 1.24; $\bar{x}_{self-high}$ = 5.11 seeds, SE =1.26; $\bar{x}_{cross-low}$ = 1.51 seeds, SE = 0.59; $\bar{x}_{cross-medium}$ = 1.49 seeds, SE = 0.63; $\bar{x}_{cross-high}$ = 3.20 seeds, SE = 0.99; Figure 7C). The results from post-hoc tests indicate that there was a statistically significant difference for the number of seeds per blossom (Figure 7C) between the pollen origins for the low, medium and high pollen load treatments($P < 0.001$, $P < 0.001$ and $P = 0.009$, respectively). There was also a statistically significant difference for the number of seeds per blossom (Figure 7C) between the low and high pollen loads ($P = 0.006$) and between the medium and high pollen loads ($P = 0.006$) for the cross-pollination treatment but no statistically significant difference for number of seeds per blossom between the pollen loads from the self-pollination treatment.

The growth rate on day 11 (mm/d) did not differ significantly across pollen origin or pollen load, but the radicle growth rate did vary differ significantly ($P < 0.001$; Table 2). The growth rate on day 39 (mm/d) was significant for the pollen origin ($P = 0.044$) and radicle growth rate (mm/d)

Table 2. Generalized Linear Mixed Model (GZLMM) for later fitness traits *D. heteromorpha* produced from differing pollen origins (P.O.), self- or cross-pollination with varying pollen loads (P.L.), low or high. Radicle growth rate (RGR) was included as a co-variant

Source of variation	F	df1	P-value	F	df1	P-value	F	df1	P-value
	Day 11 growth rate (mm/d) (*n* = 191)			Day 39 growth rate(mm/d) (*n* = 160)			Day 74 growth rate(mm/d) (*n* = 160)		
P.O.	2.232	1	0.137	4.118	1	0.044	3.213	1	0.075
P.L.	0.259	1	0.611	0.213	1	0.645	0.045	1	0.832
P.O*P.L.	0.645	1	0.234	0.024	1	0.877	3.986	1	0.048
RGR	46.932	1	0.000	5.103	1	0.025	24.18	1	0.000
	Number of leaves on day 39 (*n* = 160)			Number of leaves on day 88 (*n* = 159)			Dry Weight (g) (*n* = 102)		
P.O.	1.430	1	0.234	2.976	1	0.087	0.029	1	0.866
P.L.	2.589	1	0.110	5.899	1	0.016	0.957	1	0.330
P.O*P.L.	0.059	1	0.808	2.987	1	0.086	1.612	1	0.207
RGR	14.605	1	0.000	11.015	1	0.001	33.752	1	0.000

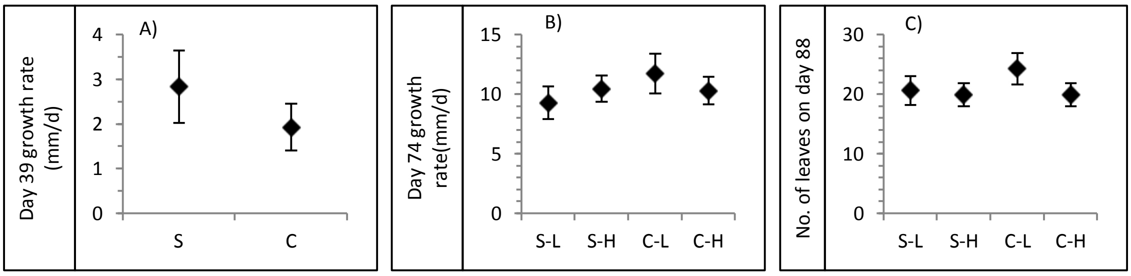


Figure 8. (A-C) Graphs showing offspring fitness in *D. heteromorpha*. Pollen origin is represented by S (self) and C (cross). Pollen load is represented by L (low), M (medium) and H (high). Error bars indicate two standard errors. See Table 2.

had a positive effect (P = 0.025; Table 2). The seeds produced from self-pollination had a faster radicle growth rate ($\bar{x}$ = 2.82 mm/d, SE = 0.41) than cross-pollination ($\bar{x}$ = 1.94 mm/d, SE = 0.26; Figure 8A). The growth rate at day 74 (mm/d) was significant for the interaction pollen origin by pollen load (P = 0.048), radicle growth rate (P < 0.001) and pollen origin approached significance (P = 0.075; Table 2). The interaction term suggests that the growth rate at day 74 (mm/d) responds positively to pollen load for the self-pollination treatment ($\bar{x}_{self-low}$= 9.30 mm/d, SE = 0.69; $\bar{x}_{self-high}$ = 10.45 mm/d; SE = 0.55), however, the pollen load responds negatively for the cross-pollination treatment ($\bar{x}_{cross-low}$ = 11.769 mm/d, SE = 0.84; $\bar{x}_{cross-high}$ = 10.33 mm/d, SE = 0.58; Figure 8B). The pollen origin for the growth rate at day 74 suggests that cross-pollination was superior, 11.065 mm/d (SE = 0.52) to self-pollination ($\bar{x}$ = 9.893 mm/d, SE = 0.45). The results from post-hoc tests indicated that there was statistical significance for the growth rate at day 74 (Figure 8B) between the pollen origins from the low pollen load treatment (P = 0.021), however there was no detectable difference for the pollen origins for the high pollen load. The number of leaves present on day 39 was significantly related to radicle growth rate (P < 0.001; Table 2). The number of leaves on day 88 differed significantly across pollen load treatment (P = 0.016) and was

Table 3. Generalized Linear Mixed Model (GZLMM) for early fitness traits in *D. scandens* produced from differing pollen origins (P.O.), self- or cross-pollination with varying pollen loads (P.L.), low, medium and high. The female position (F.P.) was included as a factor.

Source of variation	F	df1	P-value	F	df1	P-value	F	df1	P-value	F	df1	P-value
	No. of seeds produced (*n* = 294)			Seed weight (g) (*n* = 342)			Probability of germination (*n* = 149)			Radical growth rate(mm/d) (*n* = 93)		
P.O.	26.619	1	0.000	1.802	1	0.180	0.201	1	0.654	1.753	1	0.189
P.L.	2.039	2	0.132	0.079	2	0.925	1.919	2	0.151	1.057	2	0.352
F.P.	16.226	1	0.000	19.961	1	0.000	7.744	1	0.006	0.639	1	0.426
P.O.*P.L.	1.098	2	0.345	1.527	2	0.219	1.816	2	1.670	1.348	2	0.265
P.O.*F.P.	1.278	1	0.259	11.308	1	0.001	0.065	1	0.798	0.043	1	0.837
P.L.*F.P.	9.308	2	0.000	3.129	2	0.045	0.223	2	0.800	0.262	2	0.770

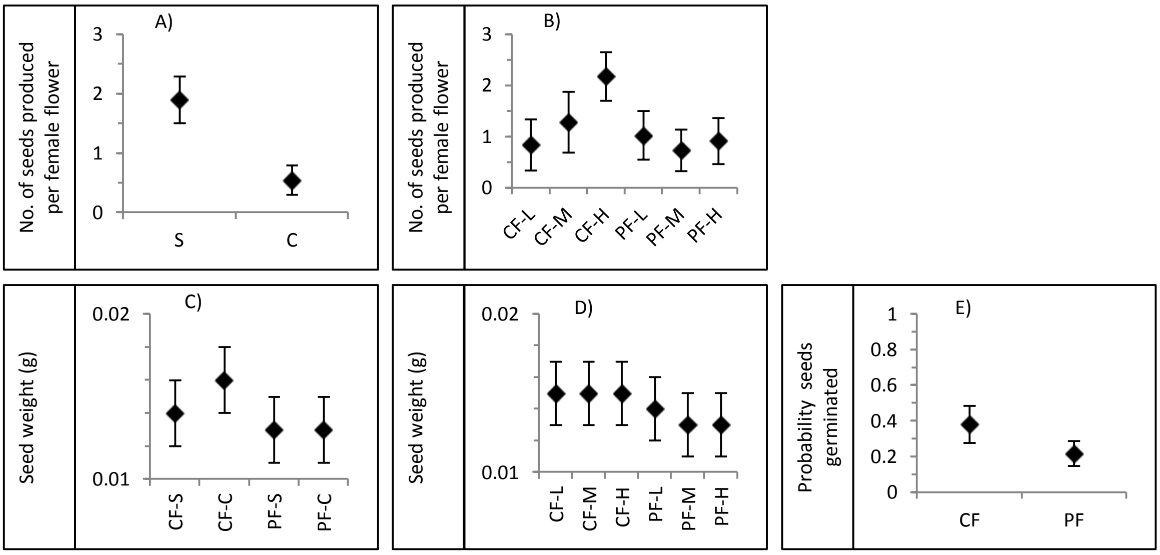


Figure 9. (A-D) Graphs showing offspring fitness in *D. scandens*. Pollen origin is represented by S (self) and C (cross). Pollen load is represented by L (low), M (medium) and H (high). The female position is represented by CF (central) and PF (peripheral). Error bars indicate two standard errors. See Table 3.

also significantly related to radicle growth rate (P = 0.001), pollen load and the pollen origin by pollen load interaction term had marginally statistically significant effects (P = 0.087 and P = 0.086, respectively). The progeny from the low pollen load treatment had more leaves on day 88 that the high pollen load ($\bar{x}_{low\ pollen\ load}$= 22.53 leaves, SE = 0.93; $\bar{x}_{high\ pollen\ load}$ = 19.96 leaves; SE = 0.74). The pollen origins effect indicates that progeny from cross pollination produced more leaves on day 88 than those produced by progeny from self-pollination ($\bar{x}_{cross-polliantion}$= 22.18 leaves, SE = 0.88; $\bar{x}_{self-pollination}$ = 20.31 leaves; SE = 0.82). The interaction term suggest that the progeny from high pollen load produced the same number of leaves regardless of pollen origin, but the progeny from low pollen loads responded positively to the cross-pollination ($\bar{x}_{self-high}$ = 19.93 leaves , SE = 0. 96; $\bar{x}_{cross-high}$ = 19.99 leaves; SE = 0. 98; $\bar{x}_{self-low}$ = 20.69 leaves, SE = 1.20; $\bar{x}_{cross-low}$ = 24.37 leaves; SE = 1.32; Figure 8C). The results from post-hoc tests indicate that there was statistically significant difference for the number of leaves present on day 88 (Figure 8C) between the pollen origins at the low pollen load treatment

Table 4. Generalized Linear Mixed Model (GZLMM) for early fitness traits in *D. scandens* produced by varying pollen origin (P.O.), self- or cross-pollination and neighbouring pollen origin (N.P.O.). The female position (F.P.) was included as a factor

Source of variation	F	df1	P-value	F	df1	P-value
	Probability of blossom abortion (n = 76)			Number of seeds produced (n = 87)		
P.O.	1.98	1	0.164	2.372	1	0.127
N.P.O	0.442	1	0.508	6.338	1	0.014
F.P.				1.378	1	0.244
P.O.*N.P.O.	5.187	1	0.026	0.323	1	0.571
P.O.*F.P.				4.508	1	0.037
N.P.O*F.P.				17.38	1	0

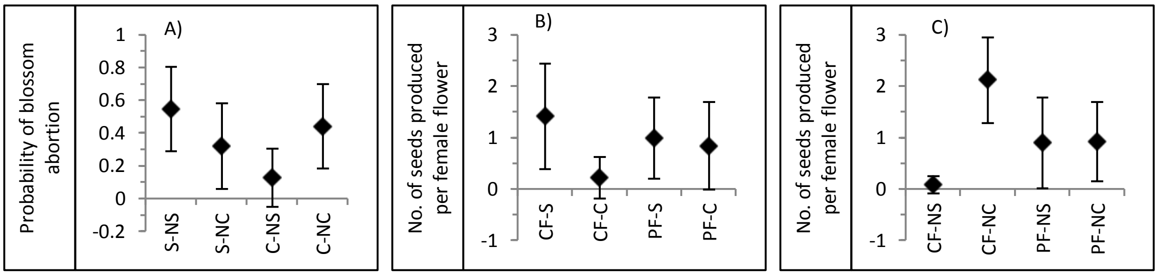


Figure 10. (A-C) Graphs showing offspring fitness in *D. scandens*. Pollen origin is represented by S (self) and C (cross) while neighbouring blossoms pollen origin is represented by NS (neighbour self) and NC (neighbour cross). The female position is represented by CF (central) and PF (peripheral). Error bars indicate two standard errors. See Table 4.

(P = 0.033) but there was no detectable difference at the high pollen load. There was also a statistically significant difference for the number of leaves present on day 88 (Figure 8C) between the pollen loads for the cross pollination treatment (P = 0.005). The dry weight of the plant after 95 days varied significantly with the radicle growth rate (P <0.001; Table 2).

In the second analysis of *D. scandens* that included the female position, the number of seeds varied significantly with pollen origin (P < 0.001), the female position (P < 0.001) and the interaction between pollen load and female position (P < 0.001; Table 3). The self-pollination treatment resulted in higher seed production ($\bar{x}$ = 1.89 seeds, SE = 0.21); than the cross-pollination treatment ($\bar{x}$ = 0.54 seeds, SE = 0.12; Figure 9A). The flowers in the central position had higher seed production ($\bar{x}$ = 1.44 seeds, SE = 0.18) than the peripheral females seed production ($\bar{x}$ = 0.90 seed, SE = 0.12). In addition, the interaction term showed that pollen load had a positive effect on seed production at the central female, but not at the peripheral females ($\bar{x}_{central-low}$ = 0.84 seeds, SE = 0.24; $\bar{x}_{central-medium}$ = 1.29 seeds, SE = 0.30; $\bar{x}_{central-high}$ = 2.19 seeds, SE = 0.36; $\bar{x}_{peripheral-low}$ = 1.02 seeds, SE = 0.24; $\bar{x}_{peripheral-medium}$ = 0.78 seeds; SE = 0.21; $\bar{x}_{peripheral-high}$ = 0.93 seeds, SE = 0.36; Figure 9B). The results from post-hoc tests indicate that there was a statistically significant difference for the number of seeds per blossom between the low and high and between the medium and high pollen loads (P < 0.001 and P = 0.040, respectively) for the central female. However, there was no detectable difference between the number of seeds produced by the pollen loads at the peripheral females (Figure 9B). There was also a statistically significant difference between the number of seeds per blossom at the central and peripheral females at the medium pollen load and the high pollen load treatment (P = 0.019 and P = 0.001, respectively, Figure 9B). The weight of the seed was significantly influenced by the female position (P < 0.001), the pollen origin by female position interaction term (P = 0.001) and the pollen load by female position interaction term (P = 0.045; Table 3). The central female produced heavier seeds ($\bar{x}$ = 0.015g, SE = 0.001), compared to the peripheral females, ($\bar{x}$ = 0.013g, SE = 0.001). The central female produced heavier seeds when cross-pollination ($\bar{x}_{central-cross}$ = 0.016g, SE = 0.001; $\bar{x}_{central-self}$ = 0.014g, SE = 0.001; $\bar{x}_{peripheral-cross}$ = 0.013g, SE = 0.001; $\bar{x}_{peripheral-self}$ = 0.013g, SE = 0.001; figure 9C). The weight of the seed was not significantly influenced by pollen load on the central female; however, the peripheral females had a negative relationship. ($\bar{x}_{central-low,medium\,\&\,high}$ = 0.015g, SE = 0.001;$\bar{x}_{peripheral-low}$ = 0.014g, SE = 0.001; $\bar{x}_{peripheral-medium}$ = 0.013g, SE = 0.001; $\bar{x}_{peripheral-high}$ = 0.13g, SE = 0.001; Figure 8D). The results from post-hoc tests indicate that there was a statistically significant difference for the weight of the seed between the pollen origins for the central female (P = 0.021, Figure 9C). There was also a statistical significance for the weight of the seed between the female positions for the cross-pollination treatment (P < 0.001, Figure 9C). Additionally, there was also a statistically significant difference for the weight of the seed between the female positions for the medium and high pollen loads (P < 0.001 and P = 0.004, respectively, Figure 9D). The probability of germination differed significantly between the female positions (Table 3). Seeds from the central female flowers had a greater chance of germinating ($\bar{x}$ = 0.38, SE = 0.05), than seeds from the peripheral flowers ($\bar{x}$ = 0.22, SE = 0.04) Figure 9E). The radicle growth rate did not differ significantly across seeds from these two types of flowers.

Level of inbreeding depression

In *D. heteromorpha*, inbreeding depression caused by the pollen origin is only present up to the germination ($\delta_{\text{number of seeds}}$ = - 0.011, $\delta_{\text{seed weight}}$ = -0.002, $\delta_{\text{probability of germination}}$ = - 0.01 overall, the $\delta_{\text{early traits}}$ = -0.042, Table 5), however after germination outbreeding depression occurs ($\delta_{\text{radicle growth rate}}$ = 0.05, $\delta_{\text{growth rate on day 11}}$ = 0.06, $\delta_{\text{growth rate day 39}}$ = 0.03, $\delta_{\text{growth rate 74}}$ = 0.06, $\delta_{\text{the number of leaves present on day 39}}$ 0.04 and $\delta_{\text{the number of leaves present on day 88}}$ = 0.04, $\delta_{\text{dry weight}}$ = 0.05 and overall, $\delta_{\text{late traits}}$ = 0.036, Table 5). The effect of pollen load suggests that the traits up to germination respond positively to high pollen load and increase fitness ($\delta_{\text{number of seeds}}$ = 0.037, $\delta_{\text{seed weight}}$ = 0.002, $\delta_{\text{probability of germination}}$ = 0.013 overall, the $\delta_{\text{early traits}}$ = 0.044, Table 5). However, the post germination traits suggested that high pollen load decrease fitness, ($\delta_{\text{radicle growth rate}}$ = -0.03, $\delta_{\text{growth rate on day 11}}$ = - 0.10, $\delta_{\text{growth rate day 39}}$ = -

Table 5. Differences in the expression of inbreeding depression in *D. heteromorpha* between self and cross-pollination ($\delta = \omega_{\text{Self pollination}} - \omega_{\text{Cross pollination}}$) and between high and low pollen loads ($\delta = \omega_{\text{Low pollen load}} - \omega_{\text{High pollen load}}$). The final two measurements are the overall estimation of the early and late traits $\delta = W_{\text{Self pollination}} - W_{\text{Cross pollination}}$ and $\delta = W_{\text{Low pollen load}} - W_{\text{High pollen load}}$).

	Pollen Origin	Pollen load
	δ	δ
Number of Seeds	-0.11	0.37
Seed Weight (g)	-0.002	0.002
Probability of Germination	-0.1	0.13
Radicle growth rate	0.05	-0.03
Day 11 Growth Rate (mm/d)	0.06	-0.10
Day 39 Growth Rate (mm/d)	0.03	-0.11
Day 74 Growth Rate (mm/d)	0.06	0.02
Day 39 Leaves Present	0.04	-0.04
Day 88 Leaves Present	0.04	-0.11
Dry Weight (g)	0.05	-0.11
Early Traits	-0.42	0.44
Late Traits	0.36	-0.67

Table 6. Differences in the expression of inbreeding depression in *D. scandens* between self and cross-pollination ($\delta = \omega_{\text{Self pollination}} - \omega_{\text{Cross pollination}}$) and between low, medium and high pollen loads ($\delta = \omega_{\text{Lower pollen load}} - \omega_{\text{Higher pollen load}}$). The final measurement is the overall estimation of the early traits ($\delta = W_{\text{Self pollination}} - W_{\text{Cross pollination}}$ and $\delta = W_{\text{Lower pollen load}} - W_{\text{Higher pollen load}}$).

	Pollen Origin	Pollen load		
	δ	δ (low-med)	δ(med - high)	δ (low-high)
Number of Seeds	-1.45	-0.03	0.24	0.22
Seed Weight (g)	0.06	0.01	0.08	0.08
Probability of Germination	-0.14	0.03	-0.08	-0.05
Radicle growth rate	0.15	0.10	-0.14	-0.03
Early Traits	-1.06	0.15	-0.16	0.01

0.011, $\delta_{\text{growth rate 74}} = 0.02$, $\delta_{\text{the number of leaves present on day 39}} = -0.04$, $\delta_{\text{the number of leaves present on day 88}} = -0.11$, $\delta_{\text{dry weight}} = -0.11$ and overall, $\delta_{\text{late traits}} = -0.67$, Table 5).

In *D. scandens*, inbreeding depression caused by the pollen origin has a strong effect on the number of seeds produced (δ = -1.45) and probability of germination (δ = -0.14), but outbreeding has also been detected in the seed weight (δ = 0.06) and radicle growth rate (δ = 0.015). Overall, the traits suggest that outbreeding is stronger (δ = -1.06).

Pollen load on the other hand suggests that overall an increases in the pollen load increased the number of seeds ($\delta_{\text{low-medium}} = -0.03$, $\delta_{\text{medium-high}} = 0.024$ and $\delta_{\text{low-high}} = 0.022$), Also with increasing pollen loads there is an increase in seed weight ($\delta_{\text{low-medium}} = 0.01$, $\delta_{\text{medium-high}} = -0.08$ and $\delta_{\text{low-high}} = 0.08$. However, the pollen load for germination does not follow this pattern and a lower pollen load is superior. The low and medium pollen loads are possess a higher germaitnion rate ($\delta_{\text{low-high}} = -0.05$ and $\delta_{\text{medium-high}} = -0.08$) however the medium pollen load is favoured over low pollen load (δ

= 0.03) suggesting that the medium pollen load has the best germination rate. The pattern is also seen in the radicle growth rate, the low and medium pollen loads are seen to be supiro to high pollen load ($\delta_{\text{low-high}}$ = - 0.03 and $\delta_{\text{medium-high}}$ = - 0.14) however, the medium pollen load is favoured over the low (δ = 0.10) suggesting that the medium pollen load has the best radicel growth rate. Overall, these early traits indicate that the medium pollen load is supiour as it out performed the low load (δ = 0.15) and high pollen load (δ = -0.16) while the high pollen load was just superior to the low pollen load (δ = 0.01).

The pollen origins are very similar in responses to both species, the number of seeds and germination are both affected strongly, but the seed weight responds, in different directions, yet this could be influenced by their number of seeds. The pollen load indicates in both species that if the pollen load is too high then the progeny will be less fit than a lower pollen load.

Pollen origin and its effect on resource allocation in *D. scandens*

The pollen origin by neighbour pollen origin interaction influenced blossom abortion rates (P = 0.026; Table 4). Specifically, a higher abortion rate occurred when both blossoms experienced the same pollination treatment ($\bar{x}_{self-self}$ = 0.55, SE = 0.13; $\bar{x}_{self-cross}$ = 0.32, SE = 0.13; $\bar{x}_{cross-self}$ = 0.13, SE = 0.09; $\bar{x}_{cross-cross}$ = 0.44, SE = 0.13; Figure 10A). The results from post-hoc tests indicate that there was a statistically significant difference for the abortion rate at a selfed neighbouring blossom for the pollen origin of the main blossom (P = 0.005, Figure 10A,). There was also a statistically significant difference for the abortion rate between the pollen origins at the neighbouring blossom for a cross-pollination on the main blossom (P = 0.036, Figure 10A). The number of seeds produced varied significantly with neighbour pollen origin (P = 0.014), the pollen origin by female position interaction (P = 0.037) and the neighbour pollen origin by female position interaction (P < 0.001; Table 4). Blossoms produced more seeds if the neighbouring blossom was cross-pollinated, ($\bar{x}$ = 1.53 seeds, SE = 0.45), than if self-pollinated, ($\bar{x}$ = 0.30 seeds, SE = 0.28). The central female produced more seed if self-pollinated than if cross-pollinated, at the same time the peripheral females produced a similar number of seeds ($\bar{x}_{central-self}$ = 1.41 seeds, SE = 1.02 seeds; $\bar{x}_{central-cross}$ = 0.24 seeds, SE = 0.39; $\bar{x}_{peripheral-self}$ = 0.99 seeds, SE = 0.78; $\bar{x}_{peripheral-cross}$ = 0.54 seeds, SE = 0.81; Figure 10B). The central female produced more seeds if the neighbouring blossom received cross-pollination, at the same time the peripheral females were not detectably affected by this treatment. ($\bar{x}_{central-neighbour\ self}$ = 0.09 seeds, SE = 0.018; $\bar{x}_{central-neighbour\ cross}$ = 2.13 seeds, SE = 0.81; $\bar{x}_{peripheral-neighbour\ self}$ = 0.90 seeds, SE = 0.90; $\bar{x}_{peripheral-neighbour\ cross}$ = 0.93 seeds, SE = 0.78; Figure 10C). The results from post-hoc tests indicate that there was a statistically significant difference for the numbered seeds produced between the pollen origins at the central female (P = 0.030, Figure 10B) but there was no statistically significant difference detected for the number of seed at the peripheral flowers. Significance was also detected between the neighbouring blossom and female position (Figure 10C), the central female responds to the pollen origin of the neighbouring blossom ($P < 0.001$) but not on the peripheral. However, there is also a difference between the central and peripheral female flowers if the neighbour is self-pollinated ($P = 0.041$) and if the neighbouring blossom is a cross-pollination ($P < 0.001$).

Can blossom morphology predict inbreeding depression? A comparison of four populations of *D. scandens*

The number of days to produce mature fruit differed significantly between the populations ($P < 0.001$; Table 7). The Chamela population required the fewest days in contrast the three other populations required similar number of days ($\bar{x}_{Chamela}$ = 22.82 days, SE = 0.99; $\bar{x}_{La\ Mancha}$ = 34.63 days, SE = 1.55; $\bar{x}_{Maria\ la\ Gorda}$ = 33.63 days, SE = 1.12; $\bar{x}_{Puerto\ Morelos}$ = 35.35 days, SE = 1.27; Figure 11A). The results from post-hoc tests indicate that there was statistically significant difference for the number of days to fruit between the populations, Chamela compared to the three other populations ($P < 0.001$). However, there were no statistically significant difference for the number of days to fruit between the other populations. The number of seeds produced differed significantly between the female position within the blossom ($P < 0.001$; Table 7). The central female produced more seeds ($\bar{x}$ = 1.92 seeds, SE = 0.21), than the peripheral females ($\bar{x}$ = 1.44 seeds, SE = 0.015; Figure 11B). The seed weight differed significantly between populations ($P < 0.001$) and between the female flower position ($P = 0.002$; Table 7). Individuals from Chamela produced the lightest seeds, followed by La Mancha. Individuals from Maria La Gorda and Puerto Morelos produced the heaviest seeds ($\bar{x}_{Chamela}$ = 0.013 g, SE = 0.002; $\bar{x}_{La\ Mancha}$ = 0.026 g, SE = 0.002; $\bar{x}_{Maria\ la\ Gorda}$ = 0.036 g, SE = 0.002; $\bar{x}_{Puerto\ Morelos}$ = 0.034 g, SE = 0.002; Figure 11C). The results from post-hoc tests indicate that there was a statistically significant difference for the seed weight between the populations (Figure 11C); Chamela was significantly different from all population ($P < 0.001$), La Mancha was significantly different between Maria la Gorda ($P = 0.001$) and Puerto Morelos ($P = 0.018$). There was no statistically significant difference in the seed weight between Maria la Gorda and Puerto Morelos. Seeds produced by the central female ($\bar{x}$ = 0.028g, SE = 0.001) were heavier that the peripheral ($\bar{x}$ = 0.027g, SE= 0.027, Figure11D). The probability of germination differed significantly among populations ($P = 0.016$) and between the female positions ($P = 0.016$; Table 7). Seeds from Chamela experienced the lowest probability of germination, followed by Puerto Morelos, Maria la Gorda and finally La Mancha ($\bar{x}_{Chamela}$ = 0.27, SE = 0.04; $\bar{x}_{La\ Mancha}$ = 0.48, SE = 0.08; $\bar{x}_{Maria\ la\ Gorda}$ = 0.45g, SE = 0.05; $\bar{x}_{Puerto\ Morelos}$ = 0.38,

Table 7. Generalized Linear Mixed Model (GZLMM) for fitness traits produced from self-pollination in four populations (POP) of *D. scandens* produced from differing pollen loads (P.L.), low, medium and high. The female position (F.P.) was included as a factor.

Source of variation	F	df1	P-value	F	df1	P-value	F	df1	P-value	F	df1	P-value	F	df1	P-value
	Days to produce fruits (*n* = 191)			Number of seeds produced (*n* = 426)			Seed weight (g) (*n* = 696)			Probability of germination (*n* = 287)			Radical growth rate (mm/d) (*n* = 269)		
POP	29.905	3	0	1.297	3	0.275	40.73	3	0.000	3.515	3	0.016	2.468	3	0.063
P.L.	1.608	2	0.205	1.081	2	0.340	1.628	2	0.197	1.17	2	0.312	0.411	2	0.664
F.P.				18.565	1	0.000	9.906	1	0.002	5.918	1	0.016	0.839	1	0.360
POP*P.L.	0.877	6	0.515	0.067	6	0.725	0.321	6	0.926	0.728	6	0.627	1.619	6	0.142
P.L.*F.P.				1.583	2	0.207	1.059	2	0.347	0.554	2	0.581	0.99	2	0.373

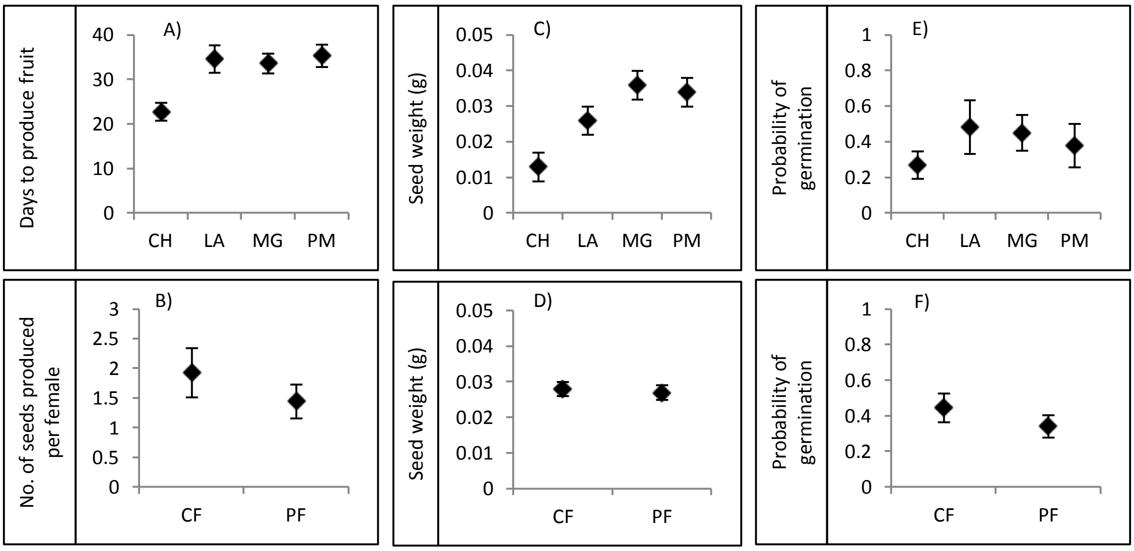


Figure 11. (A-F) Graphs showing offspring fitness in four populations of *D. scandens*. The four populations are represented by CH (Chamla), LA (La Mancha), (MG) Maria la Gorda and PM (Puerto Morelos). The female position is represented by central (CF) or peripheral (PF). Error bars indicate 2 standard errors. See table 7.

SE = 0.06; Figure 11E). The results from post-hoc tests indicate that there was a statistically significant difference for the probability of seed germination between Chamela and Maria la Gorda (P = 0.029), Chamela and La Mancha approached significance (P = 0.060, Figure 11E). The central female experienced a higher probability of germination ($\bar{x}$ = 0.45, SE = 0.04) when compared to the peripheral, ($\bar{x}$ = 0.34, SE = 0.064; Figure 11F). Radicle growth rate was not significantly affected by pollen origin, pollen load or population. Within each population, the number of days to fruiting was not significantly influenced by pollen load, position of female flower, or their interaction (Table 8). The number of seeds produced within the Chamela population differed significantly between the female positions (P < 0.001) and the female position by pollen load interaction term (P = 0.029; Table 8). The central female, ($\bar{x}$ = 2.25 seeds, SE = 0.18), had a greater chance of producing seed than the peripheral females, ($\bar{x}$ = 1.50 seeds, SE = 0.021). The interaction female position by pollen load indicates that the central female had a positive relationship with increasing pollen load at the same time the peripheral females had a negative relationship ($\bar{x}_{central-low}$ = 1.95 seeds, SE = 0.39; $\bar{x}_{central-medium}$ = 2.13 seeds, SE = 0.36; $\bar{x}_{central-high}$ = 2.55 seeds, SE = 0.24; $\bar{x}_{peripheral-low}$ = 1.86 seeds, SE = 0.36; $\bar{x}_{peripheral-medium}$ = 1.44 seeds; SE = 0.36; $\bar{x}_{peripheral-high}$ = 1.26 seeds, SE = 0.36; Figure 12A). The results from post-hoc tests indicate that there was a statistically significant difference for the number of seeds between the female positions for the medium pollen load (P = 0.022) and the high pollen load treatments (P = 0.022 and P < 0.001, respectively, Figure 12A). However there is no statistically significant difference for the number of seeds between the pollen loads at the female positions). The number of seeds produced within the Maria la Gorda population differed significantly between the female positions (P = 0.029; Table 8). The central female had a greater

Table 8. Generalized Linear Mixed Model (GZLMM) for fitness traits produced from self-pollination for each of the four populations of *D. scandens* produced from differing pollen loads (P.L.), low, medium and high. The female position (F.P.) was included as a factor.

Source of variation	F	df1	P-value	F	df1	P-value	F	df1	P-value	F	df1	P-value	F	df1	P-value
	Days to produce fruits			No. of seeds produced			Seed weight (g)			Probability of germination			Radical growth rate (mm/d)		
Chamela	(*n* = 41)			(*n* = 138)			(*n* = 234)			(*n* = 93)			(*n* = 63)		
P.L.	1.606	2	0.214	0.104	2	0.907	0.42	2	0.658	0.426	2	0.655	0.473	2	0.626
F.P.				15.075	1	0.000	1.538	1	0.216	0.531	1	0.023	1.115	1	0.296
P.L.*F.P.				3.652	2	0.029	3.046	2	0.050	0.324	2	0.724	1.043	2	0.359
La Mancha	(*n* = 17)			(*n* = 63)			(*n* = 101)			(*n* = 40)			(*n* = 50)		
P.L.	1.696	2	0.219	0.288	2	0.751	1.036	2	0.359	0.324	2	0.726	0.592	2	0.557
F.P.				2.965	1	0.091	0.454	1	0.502	2.81	1	0.103	0.624	1	0.434
P.L.*F.P.				2.103	2	0.132	1.032	2	0.360	0.661	2	0.523	0.505	2	0.607
Maria la Gorda	(*n* = 34)			(*n* = 120)			(*n* = 215)			(*n* = 91)			(*n* = 100)		
P.L.	0.218	2	0.805	0.046	2	0.955	0.06	2	0.942	1.602	2	0.208	1.993	2	0.142
F.P.				4.875	1	0.029	4.918	1	0.028	0.557	1	0.457	7.27	1	0.008
P.L.*F.P.				1.77	2	0.175	0.8	2	0.923	1.702	2	0.188	0.624	2	0.538
Puerto Morelos	(*n* = 27)			(*n* = 105)			(*n* = 146)			(*n* = 63)			(*n* = 56)		
P.L.	0.905	2	0.418	1.538	2	0.220	1.788	2	0.171	1.117	2	0.334	0.076	2	0.927
F.P.				0.023	1	0.880	1.337	1	0.250	7.01	1	0.010	3.166	1	0.081
P.L.*F.P.				0.754	2	0.473	1.367	2	0.258	1.806	2	0.174	1.183	2	0.315

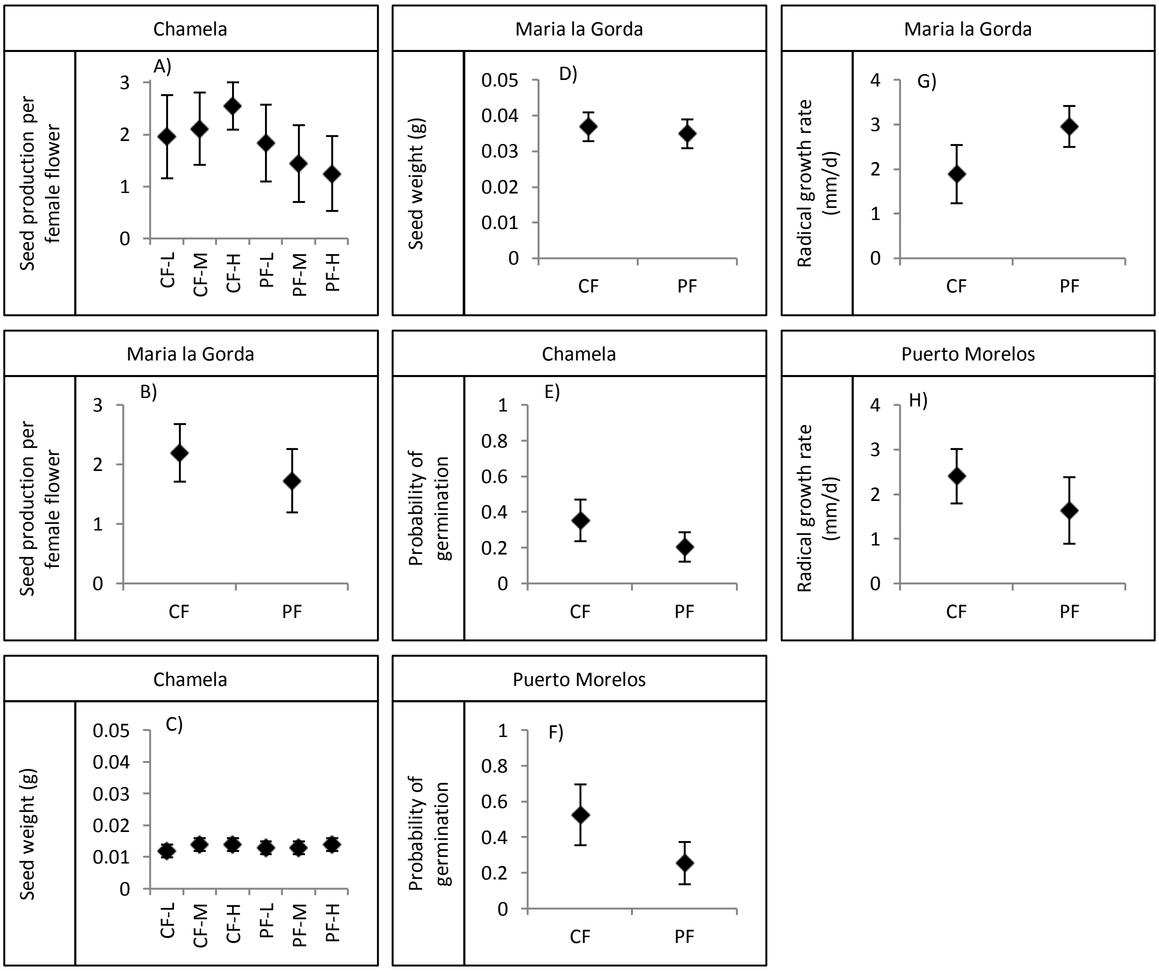


Figure 12. (A-H) Graphs showing offspring fitness between four populations of *D. scandens*. The graphs have been labeled by the populations. Pollen load is represented by L (low), M (medium) and H (high). The female position is represented by central (CF) or peripheral (PF). Error bars indicate 2 standard errors. See table 8.

chance of producing seed, ($\bar{x}$ = 2.22 seeds, SE = 0.024) than the peripheral females, ($\bar{x}$ = 1.74 seeds, SE = 0.27; Figure 12B). The number of seeds produced within the La Mancha population approached significance between the female positions (P=0.091; Table 8). The trend showed that central female had a greater chance of seed production, ($\bar{x}$ = 1.89 seeds, SE = 0.048), with peripheral, ($\bar{x}$ = 1.32 seeds, SE = 0.045). There were no detectable patterns for number of seeds produced within the Puerto Morelos population. There was a significant effect of the pollen load by female position interaction on seed weights within the Chamela population (P = 0.050; Table 8). The central female responded to pollen load between the low and medium pollen loads, the peripheral females responded between the medium and high pollen loads ($\bar{x}_{central-low}$ = 0.012g, SE = 0.001; $\bar{x}_{central-medium}$ = 0.014 g, SE = 0.001; $\bar{x}_{central-high}$ = 0.014 g, SE = 0.001; $\bar{x}_{peripheral-low}$ = 0.0012, SE = 0.001; $\bar{x}_{peripheral-medium}$ = 0.013 g; SE = 0.001; $\bar{x}_{peripheral-high}$ = 0.014 g, SE = 0.001; Figure 12C). The results from post-hoc tests indicate that there is a statistically significant difference for the weight of the seed between the female positions for the medium pollen load treatment (P = 0.019) but, there was no statistically significant difference from the pollen load at the female position. The weight of the seeds in Maria la Gorda varied significantly at the female flower position (P = 0.028; Table 8). The central female in Maria la Gorda produced heavier seeds ($\bar{x}$ = 0.037 g, SE = 0.002), compared to the peripheral females, ($\bar{x}$ = 0.035 g, SE = 0.002, Figure 12D). The weight of the seeds in La Mancha varied significantly with the position of the female flowers (P = 0.023; Table 8). The probability of germination was higher at the central female, ($\bar{x}$ = 0.36, SE = 0.06) compared to the peripheral females, ($\bar{x}$ = 0.21, SE = 0.04; Figure 12E). The probability of germination in the Puerto Morelos population varied significantly with the position of the female flowers (P = 0.001; Table 8). The probability of germination in Chamela was higher in the central female ($\bar{x}$ = 0.53, SE = 0.08), than in the peripheral females ($\bar{x}$ = 0.26, SE = 0.06; Figure 12F). The radicle growth rate differed significantly in the Maria la Gorda population between the female positions (Table 8). In the Maria la Gorda population, seedlings from the peripheral females had a higher growth rate ($\bar{x}$ = 2.96 mm/d, SE = 0.23), than those from the central female ($\bar{x}$ = 1.90 mm/d, SE = 0.33) (Figure 12G). The radicle growth rate differed significantly in the Puerto Morelos population between the female flower positions. In the Puerto Morelos population, seedlings from the central female experienced a higher radicle growth rate, ($\bar{x}$ = 2.42 mm/d, SE = 0.31), compared to the peripheral females ($\bar{x}$ = 1.64 mm/d, SE = 0.38) (Figure 12H).

Chapter 4 Discussion

Effects of pollen origin

Prediction one stated that offspring from self-pollination will possess reduced fitness than offspring from cross-pollination. It was found that overall, the early traits (number of seeds, seed weight, probability of germination and the radicle growth rate) did not support this hypothesis. Instead, the number of seeds produced and the probability of germination in *D. heteromorpha* and the number of seeds produced in *D. scandens* showed the superior outcome of self-pollination over cross-pollination (Table 1). The overall inbreeding coefficient for the early traits in *D. heteromorpha* and *D. scandens* indicated that outbreeding depression was occurring ($\delta = -0.42$, Table 5 and $\delta = -1.06$, respectively, Table 6,). However, the later traits in *D. heteromorpha* (growth rate on day 39 mm/d, growth rate on day 74 mm/d and the number of leaves present on day 88, Table 2), suggest that there is a move away from the superior outcome of the self-pollinated progeny towards the increased fitness of the cross-pollination. This is also reflected in the positive value of the overall late inbreeding depression coefficient in *D. heteromorpha* ($\delta = 0.36$, Table 5). These later traits support prediction one.

The results for *D. heteromorpha* suggest that outbreeding depression and inbreeding depression may be occurring simultaneously. The superiority of self-pollinated offspring over cross-pollinated for the earlier fitness traits could reflect outbreeding depression whereas the later traits indicate that inbreeding depression was present. Inbreeding depression could be stronger than outbreeding depression for the later traits; alternatively, outbreeding depression could have been reduced if heterosis has occurred for the later growth traits. Escobar et al. (2008) showed that a meta-population of *Physa acuta* can experience outbreeding depression and heterosis simultaneously. If the progeny of *D. heteromorpha* continued growing, it is likely that inbreeding depression would become more pronounced in older individuals due to cumulative build up. One possible reason for the reduced performance of the cross-pollination is that the pollen donors are too dissimilar from the maternal plant, i.e. from beyond the optimal distance (Waser & Price, 1994; Fisher & Matthies, 1997; Grindeland, 2008). On the other hand a possible reason for the increased fitness in the selfed progeny is that the maternal plant favours self-pollination as this allows the transfer of two copies of the maternal genes to be passed to the next generation; this hypothesis could explain the increased number of seeds produced by self-pollination in *D. scandens* and the trend in *D. heteromorpha*. Yet, it would also be expected that seed weight would increase as well, although this theory is less likely than outbreeding depression occurring in the early traits it cannot be discounted.

However, many of the early traits did not respond to the pollen origin (see Table 1 & 3). Seed weight did not respond to pollen origin in either species suggesting that seeds received the same resources to achieve an optimal seed size. There was also a lack of statistical significance for the probability of germination in *D. scandens*, while only a marginal significance in *D. heteromorpha*. The was no statistical significance in for the radicle growth rate in either species. This could suggest that there is little difference between the pollen sources or that the population has become fixed for certain traits.

Taken together, these results support the dominance model. The dominance model allows natural selection to purge homozygote individuals expressing recessive, or partially recessive, deleterious alleles normally masked by heterozygosity, causing the fixation of alleles (Lande & Schemske, 1985; Dudash & Carr, 1998; Wright et al., 2007). These results combined with the variation in ASD, suggest that these species have experienced a long history of self-pollination which can eliminate or reduce inbreeding depression (Carr & Dudash, 1997) and possibly the fixation of alleles (Charlesworth & Willis, 2009). However, highly self-pollinating populations can still experience inbreeding depression (Holtsford & Ellstand, 1990), but this would decrease over time (Holtsford & Ellstand, 1990; Dole & Ritland, 1993; Carr & Dudash, 1996). Thus, these results support the idea that *D. heteromorpha* and *D. scandens* have purged a significant amount of their genetic load over time.

The overdominace model is where heterozygote individual are more fit then homozygote individual (Charlesworth & Willis, 2009), the initial disadvantage of cross-pollination would indicate that the overdominance model is incorrect as there is no evidence of heterosis in the early stages, but it cannot be discounted. Outbreeding depression has been detected in the juvenile F1 traits. *Hypochaeris radicata* expressed outbreeding depression in the F1 generation (Becker et al., 2006). Gallow & Etterson (2005) found outbreeding depression was strongest in the juvenile traits of the F1 generation. It is possible that the juvenile traits were maladaptive, as found in *Gentianella germanica* (Fisher & Mattheis, 1997) where outbreeding depression was detected in the F1 generation. They concluded that the inter-population crosses caused the progeny to become maladapted to the local environment, which was unlikely to occur in this experiment as these species as these crosses were within a population and were grown in a benign environment. Thus, this suggests that the outbreeding depression has a genetic basis, rather than a maladapation basis, and that it is severe as is has been detected in the F1 generation. It would be expected that an F2 generation would exhibit increased outbreeding depression like in *Anchusa crispa*, Quilichi (2001) identified earlier expression and more severe outbreeding depression in the F2 generation than witnessed in the F1 generation. However, it is not possible to predict the long-term consequences of continued cross-pollination.

In *D. scandens*, the pollen origin had a significant effect on seed weight while controlling for the position of the seed (Table 3). It indicates that the cross-pollination on the central female produced heavier seeds than the cross-pollination on the-peripheral and heavier seeds than the self-pollination on the central female. This could be in part due to the central female having preferential access to resources like photosynthates as an alternative the reduction in the number of seeds produced by cross-pollination could have allowed more resources to be available to the seeds in production. On the other hand it is likely that given the lack of significance of pollen origin on seed weight in both species, that both theories are occurring; a reduction in seed numbers coupled with a preferential access to resources.

One of the aims of this project was to compare the results obtained in the two species. Given the similarities between the floral morphology and the early stages of development it is possible that the later stages would respond in a similar manner in *D. scandens* as seen in *D. heteromorpha*. The female flowers have been shown to respond differently based on their position within the blossom to pollen origin in *D. scandens*, as seen in weight of the seed and probability of seed germination. It would be expected that this process has occurred in *D. heteromorpha* given the similarity in blossom morphology and developmental of seeds. Another important consideration is the composition of the pollen load that was conducted in *D. scandens.* Schemske and Pautler (1984) found in *Costus allenii* that three-pollen source crosses resulted in higher offspring quality compared to five-pollen crosses. It was suggested that outbreeding depression could be a factor as sampling of pollen had to be from further away; yet, pollen-pollen interaction could be a factor. Nemeth & Smith-Huerta (2002) and Cruzan (1990) found evidence of pollen inhibition with two pollen donors, especially with increasing contact between the two pollen sources. Despite the interaction, the most compatible pollen had the greatest chance of fertilisation (Lankinen & Skogsmyr, 2001). The difference in the pollen composition between *D. scandens* and *D. heteromorp*ha could be important as Schemske and Pautler (1984) reported in *Costus allenii* that multiple donor crosses were more successful than single donor crosses. However, Lee and Bazzaz (1982) found no difference between multiple and single donor crosses in *Cassia*, suggesting that some species or populations were more sensitive to the pollen origin. It is unknown if the three paternal donors are causing an increase in fitness or if there could be pollen inhibition between the three predefined pollen sources. This would need further investigation.

A flaw with this experiment is that it did not include any reproductive traits in the analysis; reproductive traits express a great deal of the genetic material (Husband & Schemske, 1996). Additionally, the inclusion of post-fertilisation traits in the *D. scandens* anaylsis.

Effect of pollen competition

Comparison between *D. heteromorpha* and *D. scandens*

Prediction two stated that offspring resulting from higher pollen competition will display increased fitness. It was found that between *D. scandens* and *D. heteromorpha* that prediction two could not be rejected or upheld. The results suggest that the high pollen load could be advantageous as it increased the seed production in both species (Table 1 & 3), and seeds resulting from higher pollen competition showed an increased proportion of seed germinating in *D. heteromorpha* (Table 1). On the other hand, in *D. heteromorpha*, the growth rate on day 74 and the number of leaves on day 88 both indicate a superior outcome from the cross-low pollination treatment (Table 2). The growth rate on day 74 suggest what pollen competition has not occurred in the cross-pollination treatment, or is mild and thus not detected. Yet, the results of the number of leaves on day 88 suggests that the high pollen load has been disadvantageous to the cross-pollination treatment. The inbreeding depression co-efficient values suggests that in the early traits high pollen load has brought a benefit ($\delta = 0.44$), but then in the later traits shows a reduction fitness ($\delta = - 0.67$). In *D. scandens*, the inbreeding depression co-efficient between the pollen loads suggests the high pollen load is beneficial to seed production and weigh (see Table 6) but not to germination and the radicle growth rate. However, pollen competition was only statistically significant for the number of seeds produced (Table 1). Overall, the inbreeding coefficient suggests that the medium pollen load has progeny with the highest fitness ($\delta_{\text{low-medium}} = 0.15$, $\delta_{\text{medium-high}} = - 0.16$, $\delta_{\text{low-high}} = 0.01$, Table 6) and that the high pollen load is disadvantageous. There is evidence that high pollen load was disadvantageous in *D. heteromorpha* and in *D. scandens*, suggesting that there could be an optimal pollen load. The evidence suggests that the medium pollen load in *D. scandans* with ~ 40 pollen grains per stigma was the closest to this optimal compared to the high pollen loads in of ~80 in *D. scandens* and ~150 in *D. heteromorpha*.

Higher seed production has been reported in other studies (e.g. Quesada et al, 1993, 1996, 2001; Armbruster & Rogers, 2004), showing that high pollen loads have a positive effect on female fitness (Esser, 1953; cited in Björkman, 1995, Ter-avanesian, 1978 cited in Björkman, 1995, and higher germination rates have been reported (Quesada et al., 1996). The results also indicate that the cross-pollinated offspring experienced the greatest benefit from pollen competition, supporting the views from Husband & Schemske (1996) and Armbruster & Rogers (2004). However, there were also negative effects in cross-pollinated offspring in *D. heteromorpha*. The negative side effects of a large pollen load could cause stigma clogging or pollen-pollen interference could occur (Cruzan, 1986; Cruzan 1990; Nemeth & Smith-Huerta, 2002, Varis et al., 2010). Work by Parra-Tabla & Bullock (2005) on *Ipomoea wolcottiana* showed that large quantities of pollen on the stigmas stopped the development of pollen tubes. Nevertheless, no direct evidence for possible effects of pollen-pollen interaction exists (Pasonen & Käpylä, 1998), it is only theorised (Lankin & Skoysmyr, 2002). If pollen clogging were occurring, the optimal of ~40 grain shown in *D. scandens* could explain why ~80 and ~150 pollen grain reduced fitness. In vitro pollen germination experiments at various pollen loads could identify an optimal number of pollen grains needed.

In *D. scandens*, the inclusion of the female flowers in the analysis demonstrated it to be an important factor for seed production and seed weight with pollen load. The results revealed that the high pollen load increased seed production and seed weight at the central female, irrespective of the pollen origin. This continues to indicate that the central female flower has preferential access to resources compared to the peripheral females.

Comparisons between populations

Prediction two stated that offspring resulting from higher pollen competition will display increased fitness. Prediction five, pollen competition will be of greatest benefit to populations with the largest ASD. Prediction two cannot be upheld as pollen competition was only seen to occur in Chamela at the central female which produced more and heavier seeds. However, the inbreeding depression estimates were somewhat limited because it was not possible to obtain fitness estimates on later traits or perform cross pollinations with genetically different pollen. However, the lack of pollen competition suggests that all populations are used to inbreeding and that increased pollen load does not bring detectable benefits for self-pollination. Chamela produced the fewest seeds so a higher pollen load could be reducing pollen limitation and it is possible that this is why it was detectable. Prediction five must be rejected for self-pollination as the only detectable pollen competition was located within the predominantly selfing population which had the smallest ASD. Future work should include progeny from cross-pollination and the inclusion of subsequent growth and reproductive traits of the progeny would affect fitness.

ASD population comparison

Prediction three stated that populations would respond differently to pollination experiments due to their difference in mating systems as predicted by ASD. The initial differences between the populations of *D. scandens* is that Chamela, the predominant selfer has the smallest ASD, lightest seeds and shortest maturing time for the fruits (Figure 6) when compared to the outcrossers and the other selfer popualtion. La Mancha, the selfer, has a larger ASD, heavier seeds (which were lighter than those of the outcrossers) and a maturing time the same as the outcrossers. These findings fit with the expectation that the evolution of small blossoms is associated with the independent appearance of both a shorter duration of floral development and a rapid floral growth (Fenster et al., 1995). This could explain why Chamela presents both small ASD and rapid fruit development, but La Mancha presents a small ASD and a longer fruit development. This might indicate that La Mancha has recently evolved to increase self pollination. The evidence also presented confirms the theory that a reduced ASD increased self-pollination and in turn the seed production. It was found that there was also a positional effect that the central female received more pollen and this translated into seed production.

Prediction four stated that ‘selfer’ populations (Chamela and La Mancha), that show reduced ASD, would experience less inbreeding depression. However, the selfers did not fit the prediction; the predominant selfer, Chamela, had the lowest germination rate of all the populations. In contrast, the ‘selfer’, La Mancha, showed the highest germination rate. The results indicate that the ‘predominant selfer’ responds differently to the ‘selfer’, suggesting there is a difference between them, despite the high selfing rates. If seed weight determined the level of germination, La Mancha would not have achieved such a higher level of germination. This suggests that Chamela has become highly inbred due to the predominance of selfing, and La Mancha has purged some of the lethal and semi-lethal alleles and so it experiences increased fitness exhibited as more germination, although the outcrosser behaved as expected: they suffered from some inbreeding depression measured by their germination rate (compared to La Mancha). These results suggest that the predominant selfing populations have become highly inbred and fits with current consensus that predominant selfers are likely to go extinct (Igic & Busch, 2013)

The heat stress (about 50°C for 1 day) during germination increased mortality across all populations, to 10% germination, when compared to the germination rate of *D. heteromorpha*, 30%. The smaller seeds produced by the ‘selfer’ populations could be more susceptible to the heat stress. However, the disparity between the germination rates between Chamela and La Mancha indicated that the stress could have allowed the expression of inbreeding depression (Armbruster & Reed, 2005) and increased mortality in the less fit seeds. Additionally, there is minimal damage to the embryos from both the process of scarification and potential post-fungal infection. However if any did occur it would be expected that the smaller seeds would be most affected, but the high germination rate of La Mancha discounts this.

The female position within the blossom explained a large proportion of the variation observed in the fitness estimates. In Chamela, seeds produced by the central female had a better chance of germination. In La Mancha, there was a trend towards greater seed production at the central female. In Maria la Gorda, the central female produced more and heavier seeds. However, seedlings produced by the peripheral females had better radicle growth rates. In Puerto Morelos, seed produced by the central female had a higher probability of germination and a trend towards better radicle growth rate.

Resource allocation in *D. scandens*

It was found that there were potential issues associated with recourse allocation when two pollinations occurred at a similar time on a plant. This was not a focus of the project but it had implication on the methodology that was employed. Since blossoms could not be guaranteed to be independent if multiple pollinations occurred on a plant simultaneously, only one pollination was done at a time on a given plant.

The results indicated that the abortion rate increased when both blossoms received pollinations from the same pollen origin. Seed production in the surviving blossoms also responded to pollen origin, although these effects were detected only in the central female. Self-pollinated blossoms produced more seeds than cross-pollinated blossoms (see discussion above). However, if the neighbouring blossom was self-pollinated, then the central female on the main blossom experienced reduced seed production. The reverse was detected when the neighbouring blossom was cross-pollinated, then the central female produced more seed. Taken together, these results indicate that the maternal plant can initially detect the pollen origin of the blossom and increase abortion if they are of the same pollen origin. This differential abortion suggests that the maternal plant favours genetically different pollen to sire the offspring. The early abortion will save maximal resources (Gurusamy & Bal, 2000). Selective abortion can occur at the seed level within flowers (Marshall & Ellstrand, 1988) and at the fruit level (Nakamura, 1986; Iwata et al., 2012), however it has not been reported between flowers. Increasing pollen diversity within a blossom can increase reproductive success (Schemske & Pautler, 1984; Paschke et al., 2002; Kron & Husband, 2006) through pollen competition between genotypes (Marshall & Ellstrand, 1986) or pollen mentoring (Ganeshaiah & Uma Shaanker, 1988a). From a theoretical perspective, it is reasonable to argue that if there are mechanisms within a blossom to favour pollen diversity, there could also be mechanisms that occur between blossoms. This process could have important implications for the genetic structure of a natural population: it would maintain genetic diversity and prevent random population events such as genetic drift.

Based on the previous results and discussions, the seed production follows what is expected: more seeds in the central females after self-pollination. However, the peripheral females did not respond to pollen origin. The effect of female position on seed production could indicate an early plant wide assessment across blossoms. It is possible that the mechanism that increases abortion (discussed above) could also allow allocation of resources to seeds fertilised by more favourable pollen origin or fruits with more seeds. The central female seems to be the focal point of resource allocation determination by the maternal plant. It is unclear why the peripheral females do not respond to pollen origin.

Radicle growth rate

Radicle growth rate has been demonstrated to be an excellent predictor of future growth (Kennedy & Elle, 2008) and this has been demonstrated in *D. heteromorpha*. However, neither pollen origin nor pollen load were able to explain the variation in the radicle growth rate however, pollen competition has been seen to increase radicle growth rate in *D. scandens* (Armbruster & Rogers, 2004). This suggests that there could be other factors influencing the radicle growth rate. It could be that the radicle growth rate was influenced by resource allocation or superior genetics but this was unclear. There could even be abiotic factors to consider such as the scarification process, the trays they were germinated in (which was randomised between the embryos and within the greenhouse), the water, even temperature and light within the greenhouse. The inclusion of growth rates and reproduction traits could help determine if this initial growth rate continues right up to the reproductive stages. Further investigation into what influences the radical growth rate is needed.

Female position

Dalechampia spathulata, a related species found in the forest understory, a low-light environment, displayed sequential development of the female flowers: there was a predominance of the central female flower to develop first (62% of 29 cases) (Armbruster, 1982). Armbruster (1982) advocated that these trends could be caused by a hormonal suppressant over the other females. The offspring produced by the central female after cross-pollination was heavier than that produced by peripheral females. This would initially support the idea that cross-pollination is favoured over self-pollination, it must be considered in context of the seed number vs. seed weight trade off. Production of fewer seeds allows more resources to be invested into the fertilised ovules. In addition, the total seed weight was not influenced in either species by pollen origin (not shown in the results). In *Mesua farrea* heavier seeds were produced by fewer seeded fruits (Khan et al., 1999). This preferential access to resources by the central female is improving fitness, which has reduced or can mask the expression of inbreeding depression and outbreeding depression in the early traits. Unfortunately, the data for *D. heteromorpha* data does not allow the analysis of the female position in relation to seed production and other fitness estimates. It is likely that the central female is acting in a similar way in *D. heteromorpha* and potentially in all *Dalechampia* species given the dominance of the central female in *D. spatulata* and *D. scandens*.

Conclusion

This project aimed at assessing if two self-compatible species displayed inbreeding depression, and the level to which pollen competition could reduce its negative effects. The comparison between species revealed that the offspring suffer from outbreeding depression since the self-pollination performed better than offspring from cross-pollination in the early traits. However, inbreeding depression was detected for the later traits in *D. heteromorpha*. The morphology of the blossom of *D. heteromorpha* and the study population of *D. scandens* suggests that these species may be highly inbred because they exhibit a reduced ASD and stigmas commonly receive high amounts of self-pollen when the male flowers open. Furthermore, pollen competition increased early trait fitness mostly for cross-pollination, which was expected (Husband & Schemske, 1996; Armbruster & Rogers, 2004). However, the effect of pollen competition was weaker than expected according to previous research (Armbruster & Rogers, 2004; Lankinen & Armbruster, 2007; Madjidian et al, 2012), probably it occurs, but it is mild and hence more difficult to detec. This suggests that perhaps these species may be close to their optimum for pollen competition and therefore they do not respond to pollen competition any longer. Overall, these findings agree that inbred species exhibit less inbreeding depression (Lande & Schemske, 1985; Dole & Ritland, 1993; Goodwille & Knight, 2006).

Comparisons between the four populations of *D. scandens* that exhibit different floral morphology. Populations were classified as ‘predominant selfer’, ‘selfer’ or ‘outcrosser’ based on their ASD. ‘Predominant selfer’ and ‘selfer’ populations displayed small ASD, which suggested that these populations may have had a long inbred history. In contrast, ‘outcrosser’ populations displayed large ASD, indicating that they required visitation from pollinators to receive pollen on the stigmas (Armbrsuter & Rogers, 2004; Perez-Barrales et al., 2013). Seed weight was lower in the ‘predominantly selfer’ then the “selfer” populations compared to the “outcrosser” populations. In addition, germination rate was lowest in the ‘predominant selfer’ but much higher in the ‘selfer’. The germination rate of the ‘outcrosser’ populations fell between the ‘predominant selfer’ and ‘selfer’ populations. Pollen competition was seen to improve the fitness of the population with the smallest ASD, which would have achieved a high level of self-pollination. The lack of strong positive effects of pollen competition in the offspring of the ‘outcrosser’ population, could suggest that these populations self-pollinate to some extent. The variations in germination rate suggest that, if the floral morphology affects self-pollination rates, a certain level of inbreeding may be beneficial to population fitness. In contrast, high levels of inbreeding (predominant selfer) or being fully outcrossed can reduce fitness upon self-pollination.

In addition, this study showed that the central female had dominance over the peripheral females within a blossom. The central female frequently produced greater number of seeds which also were heavier, regardless of treatment. Also, these seeds could have a greater chance of germination. *Dalechampia spathulata*, which exhibits sequential development of the female flowers, has shown that the central female usually develops first (Armbruster et al., 1982). This central female is improving fitness, which could reduced or mask the expression of inbreeding depression and outbreeding in the early traits.

The results from a resource allocation experiment between blossoms with different pollen origins showed that when two blossoms received pollen from the same pollen origin there was an increased likelihood of abortion. This suggests that the maternal plant can detect pollen origin between blossoms and then favour genetic diversity of the blossoms and subsequently the offspring. Literature shows that the maternal plants can favour genetic diversity within a blossom(e.g. Nakamura, 1986; Marshall & Ellstrand, 1988; Iwata et al., 2012). It would seem within reason that this could occur at the plant level. In surviving blossoms, seed production was affected at the central female by the pollen origin. The neighbouring blossom also influenced seed production; self-pollinated blossom reduced seed number despite this cross-pollinated blossom did not. However, the peripheral females did not respond. It was unclear why the peripheral females did not respond.

Future work

This project has provided valuable information on the relationships between floral morphological variation, plant mating systems and their effects in the female fitness. In addition, it has opened up new challenging questions to better understanding the interface between inbreeding, inbreeding depression and strategies to increase genetic variation in the offspring. Future research should aim at assessing if cross-pollination from genetically different individuals benefits offspring fitness compared to offspring from self-pollinated blossom and how this might affect a reproductive traits and second generation. The continued assessment of pollen competition based on the difference between the pollen origin, such as multiple sires present on the stigma. Also, research aimed at assess if too much pollen on a stigma can caused pollen clogging Future research should address the questions raised by which the mechanism of the central female exerts dominance over the peripherals females. The assessment of subsequent growth rates could determine if the advantage shown how long the effects could last. The questions raised by the resource allocation, suggest a strategy to increase genetic variation in the offspring could have lasting impactions on the genetic structure of a population.

Exploration of these questions should continue assessing at both species and population level. The offspring should be assessed, at the early stages of development, later growth stages and finally the assessment of reproductive traits since expression of inbreeding depression can vary.

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