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ARTICLE



# Disentangling the role of herkogamy, dichogamy and pollinators in plant reproductive assurance

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## ABSTRACT

**Background:** Autonomous selfing can be favoured by reduced floral herkogamy, dichogamy and pollinator visitation. Autonomous selfing diminishes as pollinator abundance increases; however, the ways pollinators contribute to such result have not been tested. Pollinators can reduce the occurrence of autonomous selfing by two not mutually exclusive mechanisms: successful pollen deposition on stigmas and pollen removal from anthers.

**Aims:** We tested the role of herkogamy and protandry on autonomous selfing and the role of pollinators to reduce it via pollen removal.

**Methods:** We studied *Lepechinia floribunda* (Lamiaceae) in two natural populations in the Chaco Serrano forest, Argentina. Pollination treatments were conducted to test if floral herkogamy and pollen removal by one pollinator visitation decreased seed set by autonomous selfing.

**Results:** Seed set in pollinator exclusion and emasculation treatments were higher in flowers with lack of herkogamy and selfing increased seed set only in these flowers. Pollen removal during the male phase decreased by 72% the probability of autonomous self-pollination.

**Conclusions:** Flowers with lack of herkogamy are reproductively more advantageous than flowers with approach herkogamy independently of pollinator abundance. We demonstrated for the first time that only one pollinator visitation during the male phase can strongly decrease autonomous selfing by pollen removal.

## ARTICLE HISTORY

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## KEYWORDS

Autonomous self-pollination; autonomous selfing rate; dichogamy; herkogamy; *Lepechinia*; protandry; reproductive assurance

## Introduction

In animal-pollinated hermaphrodite flowers, for successful pollination to occur, the pollen and stigmas must be presented in approximately the same position within a flower, so that the same part of a pollinator's body successively contacts both, pollen and stigma. However, if pollen and stigmas are placed at the same location and are simultaneously mature in a flower, then they are likely to result in self-pollen deposition, decreasing opportunities for pollen exportation and cross-pollen deposition (Lloyd and Webb 1986). Plant species have evolved ingenious floral morphologies and phenological strategies that enable them to deal with such conflicts. Variations in herkogamy and dichogamy (spatial and temporal separation between pollen presentation and stigma receptivity, respectively) modify pollen exportation and selfing rate (Barrett 2002). Herkogamy (including heterostyly and enantiostyly) and dichogamy can increase not only pollen removal, exportation and outcross siring success (e.g. *Chamerion angustifolium*, Routley

and Husband 2003; *Passiflora incarnata*, Dai and Galloway 2011; *Polemonium brandegeei*, Kulbaba and Worley 2014), but also decrease selfing rate as they may decrease autonomous and facilitated selfing (e.g. *Clarkia tembloriensis*, Holtsford and Ellstrand 1992; *Aquilegia caerulea*, Brunet and Eckert 1998; *Collinsia verna*, Kalisz et al. 1999; *Aquilegia canadensis*, Herlihy and Eckert 2007; *Blackstonia perfoliata*, Brys et al. 2013).

When the availability of pollinators is unpredictable, individuals of self-compatible plant species with lack of herkogamy or dichogamy can take advantage of the best of both worlds; i.e. to reproduce via outcrossing when animal pollinators are abundant and via autonomous selfing when they are absent (Charlesworth and Charlesworth 1987; Lloyd and Schoen 1992; Kalisz et al. 2004; but see Vaughton and Ramsey 2010). It has been shown for several plant species that lack of herkogamy (spatial proximity between anthers and stigma) or reduction in dichogamy (low temporal separation between pollen exposure and stigma receptivity)

increase the potential for autonomous self-pollination within a flower; thus, providing reproductive assurance when pollinators are scarce or absent (e.g. Brunet and Eckert 1998; Kalisz et al. 1999; Moeller and Geber 2005; Moeller 2006; Herlihy and Eckert 2007; Vos et al. 2012; Brys et al. 2013; Love et al. 2016). Spatial proximity among anthers and stigma but temporal separation between male and female functions allows autonomous pollen deposition and timely fertilisation when fertile organs become mature as has been shown, for example, in *A. caerulea* and *Collinsia heterophylla* (Brunet and Eckert 1998; Lankinen and Kiboi 2007). Similarly, temporal overlapping between pollen and stigma maturation, but with spatial separation, allows autonomous selfing (hereafter AS) when movement of floral parts allow fertile organs come into contact at some time during the lifetime of a flower (e.g. *A. canadensis*, Eckert and Schaefer 1998; *C. verna*, Kalisz et al. 1999; *Kosteletzkya virginica*, Ruan et al. 2008; *Gesneria citrina*, Chen et al. 2009). Moreover, spatial and temporal proximity between sexual functions can also favour pollination when pollinators are inefficient or non-specialised as a single visit is needed to carry out facilitated self-pollination within the flower (Anderson et al. 2003).

It has been postulated that facilitated selfing, for it requires pollinators, does not provide reproductive assurance when it is most needed; i.e. when pollinators are absent (Lloyd 1992). However, when conditions for outcrossing are limited, facilitated selfing can increase seed set (Vaughton and Ramsey 2010). It has been found for two species of *Roridula* that when flying pollinators that contribute to outcrossing were nearly absent, non-flying pollinators increased seed production through facilitated selfing (Anderson et al. 2003). Thus, when pollinators are scarce, absent, or the available ones are unable to move pollen among flowers, selfing can increase seed production. Such increase can be quantified as the proportional difference in seed set between flowers capable of selfing (intact flowers) and those for which capacity of selfing has been eliminated by removing anthers before they expose pollen (emasculated flowers, Eckert and Schaefer 1998). Accordingly, a higher seed set in intact flowers than in emasculated ones indicates that either autonomous or facilitated modes of selfing are taking place. Alternatively, when seed set of intact and emasculated flowers do not differ; it may be deduced that pollinators undertake most pollinations by moving pollen among flowers (Kalisz et al. 2004; Brys et al. 2011). Given that the

two modes of selfing usually occur over the same time interval, they are difficult to distinguish from one another and, therefore, they have been rarely quantified separately (e.g. Schoen and Lloyd 1992; Brunet and Eckert 1998; but see Anderson et al. 2003; Vaughton and Ramsey 2010).

When pollinators are abundant selfing is usually relatively low (e.g. Kalisz and Vogler 2003; Kalisz et al. 2004; Moeller 2006; but see Anderson et al. 2003), but such result can be the consequence of two non-mutually exclusive mechanisms. On the one hand, pollinators can successfully deposit pollen (self or cross) on stigmas, which otherwise would be occupied by self-pollen. On the other hand, pollinators can carry pollen on their bodies and thus remove pollen that otherwise would be available for selfing. Although these ideas have been discussed and modelled in classical studies of plant reproduction (e.g. Lloyd and Webb 1986; Lloyd and Schoen 1992) and inferred by studies of plant mating system (Eckert et al. 2010), there have been no studies that empirically evaluated the role of pollinators to reduce the likelihood of AS by pollen removal. Indeed, most studies have focused on the other side of this interaction; i.e. the level to which pollinators increase pollen removal and exportation (Routley and Husband 2003; Dai and Galloway 2011; Kulbaba and Worley 2014) and, thus, seed production. Increased pollen exportation may in turn decrease selfing (e.g. Karron et al. 1997; Brunet and Eckert 1998; Motten and Stone 2000; Jersáková and Johnson 2007; but see Vaughton and Ramsey 2010). However, whether the quantity of pollen removed is enough to decrease the chances of AS remains an open question. Answering these questions would lead to a better understanding of plant mating systems. In protandrous species, if pollinators remove most of the available pollen during the male phase (protandry), then autonomous and even facilitated selfing may be completely precluded. Thus, contrary to what has traditionally been proposed, protandry could be as efficient as protogyny in avoiding selfing within flowers (Lloyd and Webb 1986).

The objective of this study is to test the role of herkogamy, protandry and pollinators on AS, by testing whether pollinators reduce the probability of autonomous self-pollination via pollen removal. We chose *Lepechinia floribunda* (Benth.) Epling (Lamiaceae) as our study species because it presents bifid stigmas, incomplete protandry, autonomous self-fertilisation and variable levels of herkogamy among the flowers of an individual. Some flowers have the stigma at the same level as

the anthers (lack of herkogamy) and others have the stigma above them (approach herkogamy) (Roldán 2016). Bifid stigmas are closed when not receptive; thus, precluding pollen deposition during unreceptive stages (Lankinen and Kiboi 2007). When the flower opens, pollen is exposed and pollinators can only remove pollen while searching for nectar, without any possibilities of depositing pollen on the stigma (Camina 2018). As pollen removal increases with successive visits during the male phase the probability of subsequent selfing within a flower (autonomous and pollinator-facilitated) may decrease and eventually be completely precluded if all pollen has already been removed before the stigma became receptive. On the contrary, if a flower has not been visited during the male phase, it conserves all the pollen and offers a large potential for self-pollination on the stigma opening and becoming receptive (Lloyd and Schoen 1992; Mallick 2001).

We hypothesised that selfing within a flower (autonomous and facilitated) is more likely to take place in flowers with lack of herkogamy than in flowers with approach herkogamy. However, if pollinator visitations occurred during the male floral stage they would favour pollen removal, and it would be expected that the autonomous component of selfing decreased. To test whether pollinators affected the probability of AS we compared the seed set of bagged flowers where the potential for AS was maximum (i.e. potential autonomous selfing), with the seed set of flowers that received one visitation during male phase, which were bagged after visitation (i.e. actual autonomous selfing). In these two pollination treatments seed set was due to autonomous self-pollination, and selfing facilitated by pollinators was completely avoided. Thus, if pollinators decreased the probabilities of AS by pollen removal, the seed set of visited and bagged flowers should be lower than the seed set of non-visited bagged flowers.

## Materials and methods

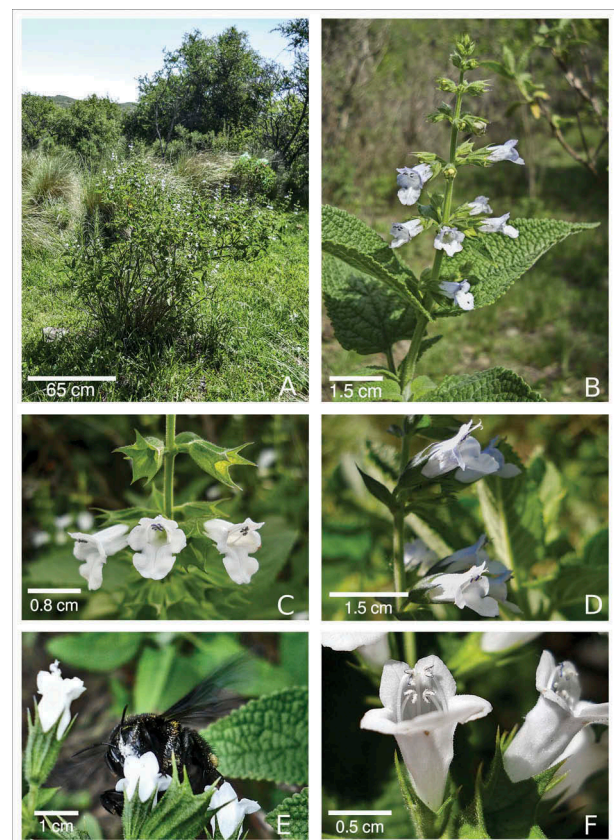
### Study site

The two study sites were in the subtropical dry forest in the Chaco Serrano biogeographical district (within the Gran Chaco region): La Quebrada (LQ, 31°9'11,29'' S; 64°20'28,85'' W) and Los Manantiales (LM, 31°9'40,34'' S; 64°21'03,67'' W) in Córdoba Province, Argentina. The sites were separated by

1.3 km and located between 800 and 1200 m above sea level, sharing similar plant species composition and environmental conditions (Giorgis et al. 2011). The characteristic vegetation is low and open woodland with three strata: arboreal, shrub and herbaceous (Cabido et al. 1991). Rainfall (944 mm annual) is concentrated in the warm season (October–April) and minimum and maximum mean temperatures range between 10°C and 26°C, respectively.

### Study species

*L. floribunda* is a native shrub species (Figure 1) distributed in Bolivia, Perú and Argentina (Epling 1938). It has perfect tubular white flowers (1.5 cm long) grouped in terminal inflorescences (Figure 1). Most flowers open in the morning and the stigma becomes receptive 6 h after floral anthesis. After the stigma becoming receptive it begins the overlapping period between pollen exposure and stigmatic receptivity, which lasts, on average, 6 h (Camina 2018). Flowers, during their



**Figure 1.** Habitat, floral traits and the main pollinator of *Lepechinia floribunda*, Chaco serrano forest, Argentina (A) An individual of *L. floribunda* in its natural habitat. (B) Inflorescence. (C) Flowers with lack of herkogamy: stigma at the same height of anthers. (D) Flowers with approach herkogamy: stigma above the anthers. (E) *Bombus* sp. with pollen of *L. floribunda* (white) on its clipeus. (F) Flower showing exposed pollen in the anthers.



one day lifetime, offer nectar and pollen as reward and each one contains four ovules (Epling 1938). The level of herkogamy is variable among flowers of an individual with the length of the style presenting continuous variation (Figure 1). We considered two levels of herkogamy: (1) lack of herkogamy (hereafter LH) where the stigma is at the same level of the anthers and both are at the same level of the corolla and (2) approach herkogamy (hereafter AH) where the stigma is above the anthers and only the stigma is exerted (Figure 2). Once the flower opens the length of the style is fixed, i.e. it does not change throughout the lifetime of the flower. There is no association between the level of herkogamy and the position of the flowers in the inflorescence neither in the position of the inflorescence on the plant (L. Ashworth, pers. obs.). Given the lack of pattern in the expression of herkogamy, the herkogamy level of each flower can only be determined when the corolla is open, so during our experiment we first determined the herkogamy level and then tagged the flowers accordingly. This species does not set seeds by apomixis and the most frequent pollinators are bumblebees and carpenter bees and much less frequently small bees and hummingbirds (Camina 2018). All pollinators search for nectar throughout the life of the flower, thus pollen

removal during the male phase is feasible. The only pollinators than collect pollen intentionally are small bees; other pollinators do not collect pollen intentionally (Camina 2018). Bumblebees embrace the corolla and move down into the flower when searching for nectar, which is found at the bottom of the corolla tube. Because the floral tube is narrow bumblebees can contact anthers and stigmas only with their clipeous (Figure 1). Pollen is very exposed in the anthers, therefore, it can be easily deposited on the body of floral visitors (Figure 1).

### Sampling design

We tagged 13 individual plants per study site. The selected plants were large in order to have a sufficient number of flowers for the four pollination treatments. However, not all marked plants had enough flowers at both levels of herkogamy (LH and AH) to conduct the four pollination treatments (some plants had few flowers with AH); thus, we used 11 plants in LM and six in LQ; total 17 plants). Plants were separated among them by at least 10 m. During the flowering peak of *L. floribunda* (from mid-November to mid-December) we visited each study site once a week. Each day we applied the four pollination treatments described below to all plants. At the end of December, when seeds were mature we quantified seed set per flowers.

### Pollination treatments

**Pollinator exclusion.** To quantify the probability of AS in the absence of pollinators we carried out a pollinator exclusion treatment, where two to four inflorescences with unopened flowers per plant were bagged with tulle. As the flowers began to open within the bag, individual flowers were marked with two different colour threads to identify LH flowers ( $n = 10\text{--}15$  flowers per plant) and AH flowers ( $n = 10\text{--}15$  flowers per plant, see total  $n$  of flowers in Table 1). Once the flowers were tagged, the inflorescences were bagged again to preclude pollinator visitation. To avoid self-pollination during manipulation of bags, only flowers in male phase (closed stigma) were tagged. Bags were left closed until seed maturation. To evaluate if AS in the absence of pollinators differed between flowers with different level of herkogamy, seed set in the pollinator exclusion treatment was compared between LH and AH flowers by means of a generalised linear mixed model with binomial distribution of errors since the response variable seed set is a proportion of seeds/



**Figure 2.** Flowers of *Lepechinia floribunda*. (A) Lack of herkogamy: stigma at the same height of anthers. (B) Approach herkogamy: stigma above the anthers.

**Table 1.** Mean  $\pm$  SD of seed set per pollination treatment and herkogamy level in *Lepechinia floribunda*. N: number of plants,  $n$ : number of flowers. Overall means were obtained by averaging population means from sites Los Manantiales and La Quebrada, Chaco serrano forest, Argentina. ND: no data.

Pollination treatment	Lack of herkogamy			Approach herkogamy		
	Overall mean $\pm$ SD	Los Manantiales (N = 11)	La Quebrada (N = 6)	Overall mean $\pm$ SD	Los Manantiales (N = 11)	La Quebrada (N = 6)
Pollinator exclusion	0.57 $\pm$ 0.02	0.59 $\pm$ 0.19 (n = 150)	0.56 $\pm$ 0.14 (n = 90)	0.30 $\pm$ 0.11	0.38 $\pm$ 0.32 (n = 115)	0.22 $\pm$ 0.29 (n = 68)
Open pollination	0.73 $\pm$ 0.13	0.82 $\pm$ 0.12 (n = 188)	0.64 $\pm$ 0.19 (n = 115)	0.49 $\pm$ 0.13	0.58 $\pm$ 0.18 (n = 170)	0.40 $\pm$ 0.11 (n = 103)
Emasculation	0.58 $\pm$ 0.28	0.78 $\pm$ 0.19 (n = 142)	0.38 $\pm$ 0.16 (n = 111)	0.48 $\pm$ 0.17	0.61 $\pm$ 0.27 (n = 140)	0.36 $\pm$ 0.26 (n = 85)
Single visit	0.16 $\pm$ 0.007	0.17 $\pm$ 0.20 (n = 57)	0.16 $\pm$ 0.21 (n = 42)	ND	ND	ND

ovules per flower (*glmer* function from the *lme4* package, Bates et al. 2015). Herkogamy was the fixed factor and site and individual the random factors, with individual plants nested within site and flowers nested in individuals.

*Emasculation vs. open pollination.* To quantify the probability of selfing within a flower (autonomous and pollinator-facilitated) we carried out a comparison of emasculation vs. open pollination. In the emasculation treatment, after flower opening and before the aperture of the stigma, we manually removed the anthers of LH flowers ( $n = 12$ – $20$  flowers per plant) and AH flowers ( $n = 12$ – $20$  flowers per plant), which were left exposed to pollinators. In the open pollination treatment, intact LH flowers ( $n = 15$ – $20$  flowers per plant) and AH flowers ( $n = 15$ – $20$  flowers per plant) were marked and left exposed to pollinators. We analysed the effect of pollination treatment as a function of herkogamy (Lack and Approach) on seed set using a bifactorial generalised linear mixed model with binomial distribution of errors (*glmer* function from the *lme4* package, Bates et al. 2015). Pollination treatment and herkogamy were considered fixed factors and site and plant random factors, with individual plants nested within site and flower nested in individuals. The interaction between the two fixed factors was tested in this model (Table 2). After a significant interaction between factors, post hoc contrasts were tested using *lsmeans* package (Lenth 2016). The magnitude of reproductive assurance by selfing was estimated by averaging mean values of seed set across the two populations for LH and AH flowers

separately as:  $1 - (n_e/n_i)$ , where  $n_e$  is the overall mean seed set of emasculated flowers and  $n_i$  is the overall mean seed set of intact flowers. Population and overall means used for this calculation are shown in Table 1.

*Single pollinator visitation vs. pollinator exclusion.* To evaluate if pollen removal during floral male phase decreased the probability of AS, we marked buds at the beginning of the anthesis and observed them until the corolla was fully opened. Once flowers were opened we applied a single-visit treatment where recently open flowers were observed during the male phase until one pollinator visitation was registered ( $n =$  five to seven flowers per plant). In these flowers pollen was exposed but the stigmas were closed and, therefore, unable to receive pollen. After one pollinator visit, flowers were marked and bagged with tulle until seed maturation. Due to the labour intensity of this experiment, it was carried out using LH flowers only. To test if a single visit by pollinators decreased the probability of AS, seed set of this treatment was compared with seed set of LH flowers in the pollinator exclusion treatment. Seed set of single visit and seed set of pollinator exclusion treatments were exclusively due to autonomous self-pollination. Seed set was compared by using a generalised linear mixed model with binomial distribution of errors (*glmer* function from the *lme4* package, Bates et al. 2015). Pollination treatment was the fixed factor and site and plant the random factors, with individual plants nested within site and flower nested in individual. The magnitude of AS reduction due to one pollinator visitation in male phase was estimated by averaging population means as:  $1 - (n_s/n_{pe})$ , where  $n_s$  is the overall mean seed set of single-visited flowers and  $n_{pe}$  is the overall mean seed set of pollinator exclusion flowers, using the subset of LH flowers (Table 1). To quantify the AS in the absence of pollinators we estimated the mean magnitude of the potential autonomous selfing rate by averaging mean values of seed set across the two

**Table 2.** Model description and significance of fixed effects of herkogamy, pollination and its interaction on seed set in *Lepechinia floribunda*.  $\chi^2$ ,  $P$  and df values obtained by likelihood ratio test.

Model	Model description	df	$\chi^2$	$P$
A	Seed set ~ herkogamy	1	74.13	< 0.001
B	Seed set ~ pollination: herkogamy	1	7.78	< 0.01
C	Seed set ~ pollination	1	60.84	< 0.001

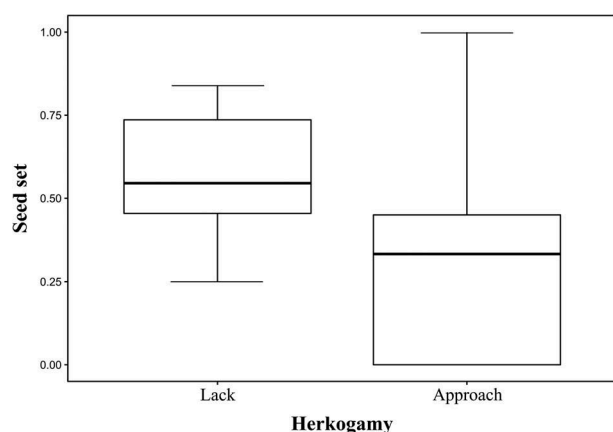
population for LH and AH flowers separately: overall mean seed set of pollinator exclusion flowers/overall mean seed set of open flowers (Fenster and Martén-Rodríguez 2007). Similarly, to quantify the magnitude of AS in LH flowers when pollinators were present and visited them, we estimated the mean magnitude of actual autonomous selfing rate by averaging population means as: overall mean seed set of single visited flowers/overall mean seed set of open flowers (Table 1).

The significance of fixed factors and their interactions were assessed by likelihood ratio test (*L ratio*), comparing two models, one with the fixed effect or interaction and the other one without it. All analyses were made in the R environment (R core team 2017, version 3.4.0).

## Results

### Autonomous selfing in the absence of pollinators

Seed set from the pollinator exclusion treatment differed between flowers with different levels of herkogamy (model A in Table 2). As expected, in the absence of pollinators, LH flowers set more seeds than AH flowers (Figure 3). With regards to the random factors, individual plants within each site explained nearly all variation (99.9%), whereas variation among sites was nil (0.001%). The mean of potential autonomous selfing rates per LH and AH flowers was 0.79 and 0.61, respectively.



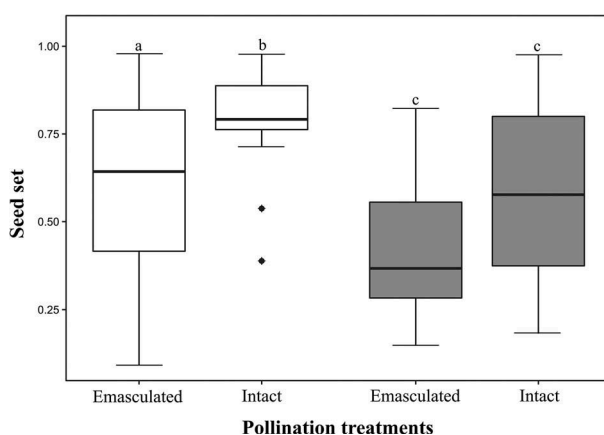
**Figure 3.** Seed set from the pollinator exclusion treatment according to the floral herkogamy level in *Lepechinia floribunda*. Lack of herkogamy: stigma at the same height of anthers. Approach herkogamy: stigma above the anthers. The bottom of each box is the 25th percentile and the top is the 75th percentile, horizontal lines inside the boxes correspond to the median. The vertical lines outwards from boxes represent 95% confidence interval of the median.

### Selfing rate

The seed set of intact flowers was higher than that of emasculated ones (model B in Table 2) in LH flowers (*Z ratio* = -5.4,  $P < 0.001$ ), but not in AH flowers (*Z ratio* = -1.284,  $P > 0.98$ , Figure 4). Thus, self-pollination increased seed set only in LH flowers and the mean magnitude difference was about 21%. Moreover, seed set of emasculated LH flowers was higher than that of emasculated AH flowers (*Z ratio* = 5.616,  $P < 0.001$ ). Similarly, intact LH flowers had higher seed set than intact AH flowers (*Z ratio* = 10.735,  $P < 0.001$ , Figure 4). The percentage of the model variance explained by the variability among sites and among plants within site (random factors) was 56% and 44%, respectively.

### Autonomous selfing in the presence of pollinators

The most common floral visitors were *Bombus* sp. (Figure 1), *Xylocopa* sp., *Apis mellifera* and *Chlorostilbon* sp. In the single visit treatment only one floral visitation by *Bombus* sp. was allowed. We restricted this treatment to *Bombus* sp. because it was the most frequent visitor, and also because working with a single species decreased the variability in pollen removal related to body size and foraging behaviour of different species. Seed set from the pollinator exclusion treatment was higher than seed set from single visit treatment where one pollinator visitation was allowed during male phase (model C Table 2; Figure 5). These results show



**Figure 4.** Seed set from Emasculated and Intact flowers in *Lepechinia floribunda*. Lack of herkogamy: stigma at the same height the anthers (white boxplots); Approach herkogamy: stigma above the anthers (grey boxplots). The bottom of each box is the 25th percentile and the top is the 75th percentile, horizontal lines inside the boxes correspond to the median. The vertical lines outwards from boxes represent 95% confidence interval of the median. Different letters above the boxes show significant differences among treatments after post hoc contrasts. Black dots are outliers.

that pollinator visitation decreased the likelihood of AS in LH flowers. The mean seed set by AS after one pollinator visitation was 0.16, a decrease in seed set of 72% compared with the pollinator exclusion treatment (Table 1). The percentage of the model variance explained by variability between sites and among plants within site (random factors) was 0.005% and 99.9%, respectively. The mean magnitude of actual autonomous selfing rate for LH flowers was 0.23, which is more than three times lower than the potential autonomous selfing rate.

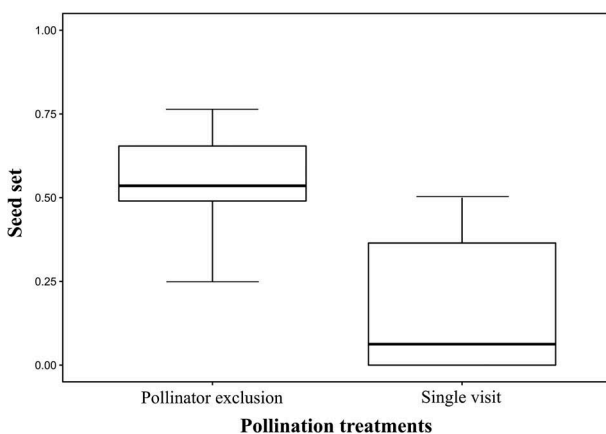
## Discussion

The ability of plants to set seeds via AS is a key trait that allows them to reproduce sexually when pollinators are not available. Here, we found that in *L. floribunda* the probability of AS was high, especially so in LH flowers. However, the potential to achieve high seed set without pollinator visitation does not mean that autonomous autogamy occurs in natural conditions. The pollination treatments conducted here enabled to quantify specifically the autonomous component of selfing, and to separate it from the facilitated one. Indeed, we demonstrated that while the potential autonomous selfing rate in *L. floribunda* was 79%, the actual autonomous selfing-rate realised was 23%, and such strong decrease was attributable to pollen removal after a single pollinator visitation. These results

show that AS can be drastically diminished even when flowers do not receive many visits.

Intact flowers exposed to open pollination had significantly higher seed set than emasculated ones, depending on the level of herkogamy. An increase in seed set by selfing was observed only in LH flowers. Similar results have been found for plant species from different plant families, suggesting that the lack of herkogamy can help reproductive assurance in the absence of pollinators (Brunet and Eckert 1998; Kalisz et al. 1999; Moeller and Geber 2005; Moeller 2006; Herlihy and Eckert 2007; Vos et al. 2012; Brys et al. 2013; Love et al. 2016). Interestingly, LH flowers either emasculated or pollinator excluded had also higher seed set than AH flowers. Overall, these results show that LH flowers in *L. floribunda* would be reproductively more advantageous than AH flowers in any pollination scenario: when pollinators are scarce or absent and also when pollinators are the unique vector for setting seeds (i.e. emasculation treatment).

Variations in seed set from emasculated flowers with different levels of herkogamy suggest that the relative position of the stigma within the flower affects the efficiency of pollinators for pollen deposition. The match between floral traits and the body size of bumblebees, would be higher in LH flowers (non-exerted stigmas), as evidenced by the higher seed set of these flowers in the emasculation treatment. When bumblebees search for nectar, they introduce part of their head into the floral tube and pollen is deposited in the clipeous. Due to this mechanical fit, bumblebees probably contact more effectively non-exerted (LH flowers) than exerted stigmas (AH). Indeed, we observed that when bumblebees sucked nectar, exerted stigmas stayed above their back with low possibilities of contacting their bodies. However, given that the relative position of the anthers does not vary with herkogamy, bumblebees can contact effectively anthers in both types of flowers. This fact suggests that AH flowers may function better as pollen donors because of the female inaccuracy, i.e. the position of the stigmatic surface does not match the location of pollen on pollinators (Armbruster et al. 2009). Thus, AH flowers may function mainly to increase pollen availability and floral display and thus pollinator attraction. Such increased pollinator attraction may, in turn, increase the likelihood of xenogamy, by favouring the movement of pollen among plants, but also of geitonogamy if the foraging of pollinators is restricted mainly to flowers within a plant.



**Figure 5.** Seed set from the pollinator exclusion treatment and single visit treatment in *Lepechinia floribunda*. In pollinator exclusion, pollinators were excluded throughout all the flower lifetime. In single visit, a single pollinator visitation was allowed during male stage and after that, flowers were bagged. The bottom of each box is the 25th percentile and the top is the 75th percentile, horizontal lines inside the boxes correspond to the median. The vertical lines outwards from boxes represent 95% confidence interval of the median.



In *L. floribunda*, protandry is not completely synchronised among flowers of an individual plant. Most flowers open early in the morning and these flowers are synchronised in their floral stages. However, a smaller number of flowers open at different times of the day, so it is likely to be found simultaneously open flowers in male and female stage (desynchronised protandry) within a plant. This desynchronisation may allow geitonogamous pollinations, i.e. pollen transfer among flowers of the same plant. However, in the morning most flowers have pollen exposed and stigmas closed; thus, pollen removed from the anthers has low chances of being immediately deposited, but it can be accumulated on the pollinator's body. As more protandric flowers were visited by an individual pollinator, pollen carry-over and diversity of pollen donors would increase. Such cumulated diversity of pollen donors on the body of pollinators would increase the likelihood of stigmas receiving a mix of pollen when opened, thereby reducing the likelihood of geitonogamy (Morris et al. 1994). These mechanistic explanations suggest that protandry could be an effective strategy to reduce geitonogamy in *L. floribunda*, but genetic studies are needed to corroborate this hypothesis.

Most studies on plant reproductive assurance show that when the abundance of pollinators increase, the reproductive assurance by selfing decrease (e.g. Kalisz and Vogler 2003; Kalisz et al. 2004). In our study, the level of reproductive assurance was about 21%, which is relatively low compared to that found in other plant species (Fenster and Martén-Rodríguez 2007). Such low magnitude of selfing in *L. floribunda* may be the consequence of two processes mediated by pollinators. We found that pollinators were not only efficient in depositing pollen on stigmas, as evidenced by the high seed set of emasculated flowers, but also in removing pollen from anthers in the male phase, thus largely decreasing autonomous selfing rate.

We found a strong reduction (72%) in autonomous seed set after a single pollinator visitation. As the flowers of *L. floribunda* can receive more than one visit during male phase, then the probability of autonomous and even facilitated selfing can be lowered down to zero if pollinators completely remove pollen from anthers during this floral stage. In that case, intact flowers would have no possibility of AS, as they would be functionally similar to emasculated ones. If pollinators depleted pollen from the anthers before the stigma becomes receptive, an incomplete protandry physiologically programmed could

function as a complete protandry where there is no overlap between sexual functions within a flower. Assuming that pollen removal occurred in a similar rate in flowers with different herkogamy levels, and AS is less likely in AH flowers, it would be expected that selfing was more readily reduced in AH flowers as found here. In this regard, genetic studies where higher outcrossing rates were found in herkogamous flowers (Brunet and Eckert 1998; Barrett 2003) support these explanations. In *L. floribunda*, herkogamy do not only decreases selfing rate but also pollen import. Thus, although herkogamy could preclude selfing, the absolute quantity of seeds produced by imported pollen from other flowers (see emasculation treatment) would be also lower in AH flowers. Each AH and LH flower produces about 1.5 and 2.5 seeds by imported pollen, respectively. Although such difference could seem minimal, when extrapolated to all the flowers of a plant it is evident that herkogamy have a negative effect on female fitness.

Instead, dichogamy may be reproductively more advantageous than herkogamy in *L. floribunda*. We demonstrate for the first time that protandry, although incomplete, is an efficient mechanism to reduce the frequency of AS even in absence of herkogamy and low frequency of floral visits. Thus, incomplete protandry not only reduces AS when pollinators are present but also allows it when they are absent. We did not control for variations in protandry, however, given the close proximity between anther and stigma in LH flowers, lack of protandry would likely result in sexual interference, where pollen removal in flowers of *L. floribunda* interferes with pollen deposition, resulting in gamete wastage (Barrett 2002; Routley and Husband 2003).

## Conclusions

The combination of protandry and lack of herkogamy seems to work well in *L. floribunda*, where the conflict of interests between male and female functions would have been resolved by placing fertile organs in the same place within the flower but maturing them at different times. A recent study conducted in the protogynous *Delachampia scandens* has shown a similar strategy, reduced herkogamy that facilitates AS does not preclude outcrossing when the pollination service is reliable (Opedal et al. 2016). These findings suggest that the floral strategy of spatial proximity but temporal separation in sexual functions may be more common and reproductively successful than previously thought. This strategy guarantees sexual reproduction under any pollination scenario and also has the potential to

promote outcrossing under pollinator scarcity. Our findings highlight the importance to disentangling the different ways that pollinators and floral traits interact with each other and thus determine temporal and spatial variation in plant mating system.

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