

## **Reproductive system of two Mediterranean *Scrophularia* species with large, showy flowers**

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The authors' research is focused on animal pollination, dispersal, systematics, evolution and phylogeography in western Mediterranean and Macaronesian plants, especially *Scrophularia*.

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*Contribution*: she conducted fieldwork, performed the statistical analyses and wrote the manuscript.

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*Contribution*: she directed the research, participated in the fieldwork, formulated the idea of the study and wrote the manuscript.

## **Reproductive system of two Mediterranean *Scrophularia* species with large, showy flowers**

Reproductive biology studies help us to understand how species' reproductive characters contribute to reproductive success and thus population survival but cost considerable time and effort to conduct under field conditions. We investigated the reproductive system of natural populations of *Scrophularia sambucifolia* and *S. grandiflora*, two western Mediterranean endemics with large, colorful flowers that undergo mixed bird–insect pollination. We evaluated: (1) the importance of pollinators in the sexual reproduction of the two species, determining levels of fruit and seed set; (2) the species' compatibility systems (self-compatible or self-incompatible); (3) the role of protogyny in their mating systems; and (4) the occurrence of unequal seed set along the inflorescence. A spontaneous self-pollination test demonstrated that floral protogyny minimizes autogamy and that pollinators are needed to ensure sexual reproduction. A hand-geitonogamous pollination test revealed that both species (especially *S. grandiflora*) are self-compatible and that the potential for geitonogamy extends throughout the flowering period but decreases as inflorescences age. Finally, seed set was similar in all whorls of the inflorescence of both species, possibly reflecting inflorescence architecture.

Keywords: fruit set, geitonogamy, inflorescence size, protogyny, seed set, self-compatibility, *Scrophularia sambucifolia*, *Scrophularia grandiflora*.

### **Introduction**

For a plant to be able to reproduce sexually, pollination is required. The transfer of pollen can be done by abiotic or biotic vectors, or by itself (self-pollination). Most angiosperms are pollinated with the help of animals, of which insects are the most important group (Proctor et al., 1996).

The form of fertilization that occurs after effective pollination depends on the type of transferred pollen, namely, autogamy or geitonogamy with the plant's own pollen or xenogamy with xenogamous pollen. In species exhibiting numerous simultaneously open flowers per plant, fertilization by autogamy or geitonogamy can

have deleterious consequences because of inbreeding depression and/or loss of pollen exported to other individuals (Barrett 2003). Plants show several strategies to avoid or decrease the self-pollination, among them, herkogamy (spatial separation of anthers and stigmas) and dichogamy (temporal separation of floral sexual organs), mechanisms that can prevent such negative effects (Barrett 2003; Routley and Husband 2006; Rosselló-Graell et al. 2007). Two types of dichogamy exist: protandry (the stamens mature before the carpels) and protogyny (the carpels mature before the stamens).

In relation to dichogamy, its effectiveness can be judged by analyzing its role from the perspective of the complete inflorescence (Harder et al. 2004; Harder and Prusinkiewicz 2013). Floral dichogamy does not always reduce the self-fertilization of flowers (e.g. *Aquilegia canadensis*; Griffin, Mavraganis, and Eckert 2000). In these cases, dichogamy can be interpreted as an adaptation to ensure reproductive success when cross-pollination fails (delayed self-pollination according to Lloyd and Schoen 1992). Other factors involved in reproductive success include pollen availability (Burd 1994; Knight et al. 2005), which may vary within plants, between populations and between years (Knight et al. 2005), resource availability, genetic characteristics of individuals (e.g. genetically impoverished populations; Wilcock and Neiland 2002; Mu et al. 2018) or intrinsic architectural limitations of the inflorescence (Diggle 1995, 1997).

Floral design (structure, color, scent and rewards) and inflorescence architecture are key determinants of successful plant sexual reproduction as they strongly contribute to effective pollination. Pollination effectiveness largely depends on the type and foraging behavior of the visiting pollinator as well as the number of flowers simultaneously in anthesis and their arrangement in inflorescences (Barrett 2003).

Along with these inherent aspects of the plant and pollinator, factors such as population

characteristics should be considered as well. For example, pollination may be limited if populations are fragmented (Knight et al. 2005) or subjected to adverse environmental factors (i.e. low temperatures or the presence of wind or rain) that can reduce or prevent pollinator movement (Pacini and Franchi 1984; Kühn et al. 2006).

In the present study, we focused on the reproduction of natural populations of two *Scrophularia* species, *S. sambucifolia* (Iberian–North African endemic) and *S. grandiflora* (endemic to central-western Portugal). Species in this genus have racemose or paniculate inflorescences formed by dichasial cymes of hermaphroditic, pentamerous and zygomorphic (rarely subactinomorphic; Ortega-Olivencia and Devesa 1993a) flowers, which exhibit protogyny. As a result of the unequal maturation times of the sexual organs, the flowers are functionally unisexual, with the male function normally more long-lived due to the sequential maturation of stamens (Ortega-Olivencia and Devesa 1993b).

Although protogyny prevents or reduces self-pollination (Faegri and van der Pijl 1979; review in Bertin and Newman [1993]), its efficacy has been questioned in some *Scrophularia* species (e.g. *S. nodosa* and *S. peregrina*; Müller 1883; Shaw 1962; Ortega-Olivencia and Devesa 1993c) because the stigma remains receptive in the absence of pollinators until the stamens unfold and deposit some of their pollen. In any case, protogyny favors cross-pollination in most studied *Scrophularia* species (Dalgaard 1979; Ortega-Olivencia and Devesa 1993c; Hoffmann and Kwak 2005; Navarro-Pérez et al. 2017), with autogamy, strictly speaking, being of limited importance in the genus. However, most species investigated thus far exhibit self-compatibility (Shaw 1962; Dalgaard 1979; Ortega-Olivencia and Devesa, 1993c; Navarro-Pérez et al. 2017). Pollinators visiting successive flowers on the same inflorescence or different inflorescences on the same individual can thus bring about fertilization with pollen of the

same plant (geitonogamy), which is genetically equivalent to autogamy (Faegri and van der Pijl 1979; Proctor et al. 1996). Such self-compatibility has been observed in American (Shaw 1962), Macaronesian (Dalgaard 1979) and western Mediterranean species (Ortega-Olivencia and Devesa 1993c; Navarro-Pérez et al. 2017). The only experimentally documented case of self-incompatibility is that of *S. fontqueri* (endemic to the Rif, northern Morocco), where it has been shown to be gametophytic (Ortega-Olivencia and Devesa 1998).

Both *S. sambucifolia* and *S. grandiflora* are facultatively xenogamous according to the pollen/ovule ratio (P/O) established by Cruden (1977) (Ortega-Olivencia and Devesa 1993c) and have a mixed pollination system involving insects and passerine birds (Ortega-Olivencia et al. 2012). These two species have the greatest production of nectar, the main reward offered to their pollinators, of any of the Iberian-Balearic species (Ortega-Olivencia and Devesa 1993b) and probably the entire genus. This mixed pollination system evolved from a wasp pollination system (Navarro-Pérez et al. 2013) that is widespread in *Scrophularia*.

In regards to reproduction, no studies have been carried out to explicitly demonstrate whether *S. sambucifolia* and *S. grandiflora* are self-compatible or self-incompatible. Spontaneous self-pollination experiments on plants grown in an experimental garden have indicated that both species need pollinators for sexual reproduction (Ortega-Olivencia and Devesa 1993c).

Given the above background, our main objective in the present study was to understand the reproductive system of natural populations of both species, which are western Mediterranean endemics noted for their large, showy flowers. Our specific goals were as follows: 1) to evaluate the importance of pollinators in the reproduction of *S. sambucifolia* and *S. sambucifolia*; 2) to determine whether these species are self-

incompatible or self-compatible, and, if the latter case, the potential for geitonogamy; 3) to understand the importance of protogyny in the possible reduction of self-fertilization in these species and 4) to check for the existence of unequal seed production along the inflorescence. We sought to determine whether seed number per fruit decreased with position (from the base to the apex of the inflorescence) in these species because the distribution of viable fruits and seeds within plants is rarely uniform (Diggle 1995).

## **Material and methods**

### ***Species studied***

*Scrophularia sambucifolia* and *S. grandiflora* are perennial, rhizomatous herbs with large columnar stems (up to 180 cm in *S. sambucifolia* and 190 cm in *S. grandiflora*). The plants have pinnatisect leaves, and the flowers are arranged in mostly opposed whorls of composed dichasial cymes (rarely simple) forming erect inflorescences. The cymes are subsessile (peduncles up to 5[11] mm) with as many as 10 flowers. The corolla is bilabiate, large—(10)12–19.5 mm—and very strikingly colored (Fig. 1). The bicarpellate gynoecium consists of a long style and a slightly bilobulate stigma at maturity. The androecium comprises four fertile stamens and a fifth sterile one, the purple staminode, which is obovate in *S. grandiflora* and oblong to suborbicular in *S. sambucifolia*. The fruit is an ovoid, septicial capsule up to 13 × 11 mm, lignified at maturity and containing numerous small (0.8–1.3 × [0.4]0.5–0.8 mm), blackish or brown seeds (Ortega-Olivencia and Devesa 1993a; Ortega-Olivencia 2009).

No studies have been carried out on the dispersion of *S. grandiflora* and *S. sambucifolia* diaspores, although presumably seeds are expelled from capsules when fruiting branches are shaken by the wind and animals (semachory), similar to other species in the genus (e.g. *S. canina*; Rodríguez-Riaño et al. 2017; Rodríguez-Riaño et al.

accepted article). Seed germination occurs in autumn. Flowering is in spring (March to May) (Ortega-Olivencia and Devesa 1993a; Ortega-Olivencia 2009) but can be as early as February (in *S. sambucifolia*) or delayed until July (in *S. grandiflora*). Fructification occurs approximately 1 month after flowering.

The main difference between the two species is the indument—glabrous in *S. sambucifolia* and pubescent-glandulous in *S. grandiflora*—which affects the pubescence of the vegetative parts and calyx. Likewise, the corolla, although externally reddish in both species, can be orange-reddish in *S. sambucifolia* and fleshy pink in *S. grandiflora*.

*Scrophularia sambucifolia*, which is distributed throughout the southwestern Iberian Peninsula and northwestern Africa, prefers deep soils of a marly or limestone nature and is found along roadsides, river banks and streams from sea level to 1300 m. *Scrophularia grandiflora* is endemic to west-central Portugal and is located in more or less shady humid places, walls, grassy slopes, roadsides and river banks between 400–500(937) m (Ortega-Olivencia and Devesa 1993a; Ortega-Olivencia 2009; Flora-on 2018).

### ***Populations studied***

Two populations of each species were studied for 2 years. The populations corresponding to *S. sambucifolia* are located in Andalusia (southern Spain), while those of *S. grandiflora* are present in the district of Coimbra within the councils of Penela, Soure, and Condeixa-a-Nova (west-central Portugal) (Fig. 2). The first *S. sambucifolia* population (hereinafter known as Morón) is located in the southern part of the province of Seville and the second population (hereinafter El Gastor), situated approximately 58 km from the previous one, is located in northern Cádiz Province. The first *S. grandiflora* population (hereinafter Rabaçal) is located in Rabaçal (Penela Council), the second population (hereinafter Pombalinho) is situated 2.8 km from the Rabaçal

population near Pombalinho (Soure Council). During the second year of the study, this population had to be replaced by another one with a similar ecology. This third population (hereinafter Zambujal) is 2.6 km from Rabaçal (for detailed information on the populations studied, see the supplemental online material).

### ***Reproductive system***

Fieldwork was carried out during 2010 and 2011, with populations visited weekly during flowering and every 2 weeks during fructification.

Two different pollination tests, hand-geitonogamous pollination (HGP) and spontaneous self-pollination (SSP), were performed, and the results were compared with a third group of control plants (C) subjected to natural pollination. For this purpose, we selected 30 different individuals per population and species and randomly divided them into 10 individuals per treatment (HGP, SSP and C). In the HGP treatment, up to 100 flowers in the female phase (i.e. newly opened flowers) per inflorescence were hand-pollinated, with sufficient pollen deposited onto the stigma from other flowers of the same individual. The flower calyxes and pedicels were then marked with plastic paint, and the inflorescences were covered with white nylon bags. In SSP individuals, the flowers were not manipulated, and the inflorescences were isolated from pollinators as in the previous treatment. The flowers were kept bagged until the last flower wilted.

Fruit set (the ratio of the number of fruits to the number of flowers per individual), and seed set per fruit (ratio of the number of seeds to the number of ovules, S/O ratio), were determined as follows. Most mature fruits were collected by cutting and transporting the whole inflorescence to the laboratory, but those that ripened before the majority were collected before dehiscence and conserved in opaque envelopes. Later, flowers, mature fruits and seeds were counted in the laboratory. The total number



of flowers counted included mature fruits and aborted flowers, the latter identified from the calyxes and/or floral pedicels that usually remained attached to the inflorescence. Viable (well-formed) seeds per fruit were counted under binocular magnification. For HGP and SSP plants, seed set was calculated using all obtained mature fruits; given the high fruit set of C individuals, the calculation was performed for this group using only 10 randomly selected mature fruits (four, three and three fruits from lower, middle and upper whorls [LW, MW and UW], respectively). Because ovules could not be counted in mature fruits, the number of ovules per ovary was counted using 15 randomly selected flower buds of each population under an optical microscope.

To obtain information on the probability of geitonogamy per population and species, 10 individuals per population and year were randomly selected during 2009 and 2010 flowering periods (usually once a week or every 10–12 days). The percentage of flowers in female and male phases per inflorescence was calculated in these individuals. The absolute difference between the percentage of flowers in male and female phases (henceforth D) was then calculated, with a value of 0 corresponding to total geitonogamy and a value of 100 to its absence. The inflorescences were divided into two types: “large” when the total number of flowers was higher than the mean value of flowers per inflorescence and “small” when it was lower than the mean value.

### ***Spatial variation in seed production in the inflorescence***

During 2010 and 2011, the production of seeds per fruit at different inflorescence positions was monitored in 10 individuals of each population under natural pollination. Seeds from four randomly selected fruits in the LW and three fruits each in the MW and UW of each inflorescence were counted as detailed above. We also used seed set data obtained in 2009 from Morón and El Gator populations of *S. sambucifolia* and Rabaçal and Pombalinho populations of *S. grandiflora*, which consisted of data from six

capsules (two each from LWs, MWs and UWs) from each of 10 individuals. The S/O ratio based on the number of ovules determined above was then calculated.

### ***Statistical analysis***

All analyses were performed using the statistical package SPSS version 19. Normality of variables was checked using the non-parametric Kolmogorov-Smirnov test with Lilliefors correction, and homoscedasticity was assessed with Levene's test.

Fruit set was analyzed using a generalized linear model (GZLM) fitted to a negative binomial distribution with a logarithmic link function, with the number of mature fruits used as the dependent variable and the logarithm of the number of flowers treated as the offset variable. After square-root normalization, the S/O ratio was analyzed by one-way analysis of variance (ANOVA), and post-hoc comparisons were made using Tukey's HSD test. In both tests, fruit and seed set, treatment, population and year were used as the principal factors. To analyze seed set spatial variation in the inflorescence, we used the same model as the one described above for seed set, but with whorl, population and year used as the principal factors. In addition, we used global data to compare and check the behavior of fruit and seed set under different treatments between species. As for each species separately, we used a GZLM for fruit set and a one-way ANOVA for seed set, with species and treatments as the principal factors.

The absolute difference (D) between male and female flower phases of each species was analyzed by one-way ANOVA, with inflorescence type used as the principal factor.

## Results

### *Reproductive system*

In both species, fruit set varied among the three treatments (Table 1). Compared with the experimental treatments, control plants exhibited higher fruit set in all populations during all study years (Fig. 3). In plants subjected to the SSP test, the percentage of fruit set never exceeded 5%. In *S. sambucifolia*, the highest value (1.7%) was observed in the El Gastor population in 2011, while the highest percentage in *S. grandiflora* (4.8%) was obtained in the Rabaçal population during the same year (Fig. 3). Consequently, rates of autogamy *s. str.* were very low. Hand geitonogamy increased fruit set considerably, reaching 28.1% in *S. sambucifolia* (El Gastor, 2011) and 44.3% in *S. grandiflora* (Zambujal population) (Fig. 3). Population by treatment and year by treatment interactions were not significant (Table 1). This means that the reproductive strategy of the species under different treatments (C > HGP > SSP) was maintained across years and study populations.

In general, the highest fruit set percentages were obtained in 2011, both in C and in the two tests (HGP and SSP) (Fig. 3). Year, however, was only significant for *S. sambucifolia* (Table 1).

Of the two species, *S. grandiflora* had higher fruit set averaging 3.1% (SSP), 31.8% (HGP) and 61.4% (C) vs. 0.9%, 13.4% and 46.2%, respectively, in *S. sambucifolia*. Although the differences between the two species were significant ( $\chi^2$ Wald = 28.117; d.f. = 1;  $P$  = 0.000), the species–treatment interaction was not ( $\chi^2$ Wald = 5.206; d.f. = 2;  $P$  = 0.074), meaning both species exhibit the same reproductive strategy (C > HGP > SSP).

The highest seed set occurred in control plants in both species, except for *S. grandiflora* in the Zambujal population during 2011 (Fig. 4). Seed set in C was thus

significantly higher than that obtained after HGP ( $P = 0.000$ ) and SSP ( $P = 0.000$ ) in either species (Tukey's post-hoc HSD), with seed set after HGP in turn significantly higher than that after SSP (*S. sambucifolia*:  $P = 0.023$ ; *S. grandiflora*:  $P = 0.000$ ; Tukey's post-hoc HSD). This trend ( $C > HGP > SSP$ ) was not always followed, as shown by the significant year- and population-treatment interactions (Table 2). In sum, both species are self-compatible.

Seed set in *Scrophularia grandiflora* surpassed seed set in *S. sambucifolia* ( $F = 17.60$ ; d.f. = 1;  $P = 0.000$ ) in both the control and experimental treatments. Although both species followed the same trend ( $C > HGP > SSP$ ), the species-treatment interaction exhibited significant differences ( $F = 5.95$ ; d.f. = 2;  $P = 0.003$ ) because there was a smaller difference in *S. grandiflora* in the proportion of seeds in the control compared with those formed under the two treatments.

In regard to potential geitonogamy per inflorescence, this type of reproduction was found to play an important role in the sexual reproduction of both species according to calculated D values (Table 3). The two sexual phases did not occur in the same proportion in each inflorescence; in general, more flowers were present in the male than the female phase (results not shown).

The mean total number of open flowers per inflorescence during the flowering peak was  $16 (\pm 11.21)$  in *S. sambucifolia* and  $22.5 (\pm 15.48)$  in *S. grandiflora*. The D value was similar for both types of inflorescences (large and small) in the two species (*S. sambucifolia*: small inflorescences,  $D = 55.4 \pm 30.9$ ,  $n = 33$  vs. large inflorescences,  $D = 57.6 \pm 27.3$ ,  $n = 17$ ,  $F = 0.063$ , d.f. = 1,  $P = 0.802$ ; *S. grandiflora*: small inflorescences,  $D = 45.4 \pm 29.8$ ,  $n = 27$  vs. large inflorescences,  $D = 47.2 \pm 22.9$ ,  $n = 21$ ,  $F = 0.051$ , d.f. = 1,  $P = 0.823$ ). When the entire flowering period was considered, the

absolute difference in both species for small and large inflorescences followed a similar pattern (data not shown).

### ***Spatial variation in seed production in the inflorescence***

Seed set did not differ with inflorescence position (LW, MW, or UW) in either species under natural pollination (Fig. 5, Table 4). Similarly, no significant difference in seed set among different positions was observed between populations of each species, whereas differences were observed during one studied year in *S. sambucifolia* (Table 4). More specifically, UW seed set was significantly higher than LW seed set in 2011, while MW seed set was intermediate between UW and LW and not significantly different from either (Tukey's HSD).

*Scrophularia grandiflora* had a significantly higher seed production per whorl than *S. sambucifolia* ( $F = 4.77$ ; d.f. = 1;  $P = 0.029$ ). Nevertheless, the overall trend in seed production of the two species with respect to whorl position (LW, MW and UW), was not significantly different ( $F = 0.55$ ; d.f. = 2;  $P = 0.576$ ).

### **Discussion**

Dichogamy is widespread, occurring in at least 144 families of angiosperms, including Scrophulariaceae (Bertin and Newman 1993). According to our results, dichogamy, specifically protogyny, hinders self-fertilization in *S. sambucifolia* and *S. grandiflora*. Indeed, this mechanism prevents autogamy in the strict sense, as the fructification of flowers after spontaneous self-pollination was null or practically null.

Protogyny has been found to be an effective barrier to self-fertilization in most *Scrophularia* species studied thus far, including western Mediterranean species (Ortega-Olivencia and Devesa 1993c, 1998; Navarro-Pérez et al. 2017) and many Macaronesian (Dalgaard 1979) and North American (Shaw 1962) ones. In regard to the Macaronesian

species, Dalgaard (1979) has provided two possible reasons for the effectiveness of protogyny: (1) in most species, the style greatly exceeds the length of the stamens, thereby preventing pollen from contacting the stigma (technically, herkogamy rather than protogyny) and (2) the style becomes more or less deflexed when the first stamen arises from the bottom of the corolla. Concerning the first point, we note that the two species in our study behave unequally. In *S. sambucifolia*, the situation is the same as that of the Macaronesian species. In *S. grandiflora*, however, the style–stamen length relationship varies within populations and even individuals; more specifically, the style is normally longer than the stamens, but sometimes the stigma and anthers are at the same level (pers. observ.). Dichogamy accompanied by herkogamy has already been reported in some other taxa (e.g. *Myosotis*, Robertson and Lloyd 1991; *Cabomba aquatica*, Silva and Leite 2011), but only in one *Scrophularia* species (*S. trifoliata*; Navarro-Pérez et al. 2017). Regarding the second point, the style generally begins its curvature at the lower lip of the corolla (Fig. 1) when the first stamen unfolds; if the stigma has been pollinated, then rapidly withers away. In other species, such as *S. peregrina*, *S. arguta* and *S. nodosa* (Shaw 1962; Dalgaard 1979; Ortega-Olivencia and Devesa 1993c), protogyny is not completely effective, as the style does not always curve downwards; as a consequence, the stigma ends up at the same level as the open anthers, thereby promoting self-pollination.

In addition to these findings, we found practically no fruit production after spontaneous self-pollination, clearly demonstrating how important pollinators are for sexual reproduction. The pollinators in this case correspond to two combined groups—hymenopterans and passerine birds (Ortega-Olivencia et al. 2012)—which are mostly searching for nectar, the main reward (Rodríguez-Riaño et al. 2014). Similar results

have recently been obtained in *S. trifoliata*, a Tyrrhenian Island species that, like *S. sambucifolia* and *S. grandiflora*, has large, showy flowers (Navarro-Pérez et al. 2017).

The hand geitonogamy treatment demonstrated self-compatibility in both species. The level of self-compatibility varied between different years and populations, a phenomenon commonly observed in many species (Charlesworth and Yang 1998; Busch 2005). Fructification percentages reached approximately 50% in some cases, especially in *S. grandiflora*, which had significantly higher values than *S. sambucifolia*. These results are additional evidence that self-compatibility is widespread in the genus (Shaw 1962; Dalgaard 1979; Ortega-Olivencia and Devesa 1993c), as only one case of self-incompatibility is known to date (i.e. *S. fontqueri*, Ortega-Olivencia and Devesa 1998). This self-compatibility would favor geitonogamy, as numerous flowers in both sexual phases are open along the inflorescence (asynchronous protogyny), and, as a rule, most plants have several mature inflorescences per individual at the same time. Although intra-floral protogyny is an excellent barrier to self-fertilization, the existence of asynchronous protogyny does not therefore prevent geitonogamy, as already demonstrated in other species of the genus (Dalgaard 1979; Ortega-Olivencia and Devesa 1993c; Navarro-Pérez et al. 2017).

Another factor, however, is the actual geitonogamy potential of the two studied species. When we monitored the percentage of female-phase flowers relative to those in the male phase during the flowering period, we observed values intermediate between no overlap in male- and female-phase flowers (completely preventing geitonogamy) and complete overlap (equal proportions, maximizing the likelihood of geitonogamy). This intermediate situation could be due to the different durations of the two sexual stages: the male stage lasts approximately four times longer on average than the female (Ortega-Olivencia and Devesa 1993b), which translates into a higher proportion of male

flowers along the inflorescence. For the same reason and given the floral protogyny of these species, the probability of geitonogamy decreases with inflorescence age, as it leads to an increase in the difference between male and female flowers as the flowering period progresses. In older or more mature plants, the oldest flowers in inflorescences are thus usually in the male phase.

Geitonogamy levels are also influenced by the number of visits (Robertson 1992) and the behavior of visiting pollinators (Devaux, Lepers and Porcher 2014). A previous study of *S. sambucifolia* and *S. grandiflora* found that pollinating insects visiting an inflorescence moved more frequently between flowers of the same whorl (horizontal movement) than between flowers of different whorls (vertical movement) (Valtueña et al. 2013). The higher frequency of horizontal movements was related to the short distance between the flowers of the same cyme due to their short pedicels; thus, rather than flying between flowers, the insects often tended to walk along the cyme. The relatively smaller number of vertical movements can also be attributed to the large amount of nectar produced per flower per day (Ortega-Olivencia and Devesa 1993b; Rodríguez-Riaño et al. 2014), as pollinators seeking to obtain maximum resources would be satiated by visiting flowers from only a few whorls. In addition, the probability of a visit is the same in all open flowers of the inflorescence because there is no sexual bias (i.e. both sexual phases, female and male, produce similar volumes of nectar per day; Rodríguez-Riaño et al. 2014). The characteristics that increase pollinator attraction (a high number of large, showy, nectariferous flowers) therefore also encourage numerous sequential visits. These multiple visits increase the level of geitonogamy to the detriment of xenogamy by reducing the fraction of pollen exportable to other individuals (Klinkhamer and de Jong 1993).



Ortega-Olivencia and Devesa (1998) found that the probability of geitonogamy is high in *S. fontqueri* but not actually realized because of self-incompatibility. In the two species in the present study, plant reproductive success is limited not only by the negative effects of geitonogamy, but also by the stigma clogging with the plant's own pollen, with this wasted pollen no longer destined for xenogamy (Rathcke 1983; Shore and Barrett 1984).

The negative effects of geitonogamy tend to become larger as the size of the inflorescence increases (Robertson 1992; Karron and Mitchell 2012). In the two species studied here, by contrast, the size of the inflorescence had no influence on geitonogamy potential. However, caution should be taken when interpreting these results, because we artificially classified the inflorescences as small and large without taking into account visits by pollinators.

The species in this study can be considered to be facultatively xenogamous given their null fruit set after SSP as well as their intermediate levels of fructification after manual geitonogamy and high levels after natural pollination, a breeding system already inferred by the P/O ratio (Ortega-Olivencia and Devesa 1993c). Regarding SSP, other xenogamous and protogynous species differ from the species studied here. For example, the protogynous species *Sanguinaria canadensis* (Lyon 1992) exhibits higher autogamy rates than ours, which implies that dichogamy is less effective in that species.

In regard to the seed set in C, which was low and rarely exceeded 60% (ca. 52.3% in *S. sambucifolia* and ca. 55.3% in *S. grandiflora*), these values are intermediate between those indicated for self-pollinated perennials ( $62.1 \pm 16.7$ ) and cross-pollinated ones ( $49.1 \pm 20.3$ ) (Wiens 1984). Although Wiens (1984) has pointed out that the S/O ratio is independent of mating system, xenogamous plants tend to have higher levels of abortion than self-pollinated plants. Furthermore, only a certain fraction of ovules

generally become seeds in angiosperms (Bawa and Buckley 1989), which is attributable to multiple reasons (Stephenson 1981; Wiens 1984; Lee 1988; Charlesworth 1989; Wilcock and Neiland 2002).

The lower percentage of fruit and seed set after HGP compared with the control is probably not due to pollen or pollinator limitations. Although various adverse weather conditions (rain, wind or cold temperatures) lower or completely inhibit the activity of pollinating insects, passerine birds are not so conditioned by such factors (Ortega-Olivencia et al. 2005). The more likely cause is pollen identity (own pollen) which is probably responsible for the early inbreeding depression observed in these species in form of lower seed set from geitonogamous selfed flowers (see Fig. 4). Some authors have pointed out that delayed self-pollination after allowing opportunities for outcrossing, i.e. pollinator visits, provides reproductive assurance in species with hermaphroditic flowers (Goodwillie et al. 2005). This phenomenon occurs in *Passiflora edulis*, a self-incompatible species in its native habitat that behaves as self-compatible in non-native habitats because pollination is limited by the absence of pollinators (Shivanna 2012). In *Collinsia verna* (Kalisz and Vogler 2003; Kalisz, Vogler and Hanley 2004) and *Butea monosperma* (Tandon, Shivanna and Ram 2003), weak self-incompatibility provides flexibility towards autogamy and geitonogamy, which is useful when xenogamy cannot be guaranteed.

Finally, seed set was similar among different whorls of a given inflorescence, possibly because of the inflorescence architecture, as indicated in other species (e.g. *Lupinus luteus*; Lee 1988 and references; see revision in Diggle 1995). Flowers in *S. sambucifolia* and *S. grandiflora* open in acropetal order. Because inflorescences are not simple racemes of flowers but are instead racemes of dichasia, however, new flowers are always formed in different whorls (see Fig. 3D–F in Ortega-Olivencia and Devesa

1993b). Nevertheless, the marginal differences observed between years in *S. sambucifolia*, in which a significantly higher production was seen in the upper whorls compared with the lower ones during 2011, could be due to the different annual environmental conditions experienced during these flowering periods and stochastic pollination environments (Knight et al. 2005) as well as unequal resource allocation. The lower seed set along the inflorescence of *S. sambucifolia* compared with *S. grandiflora*, which favors a balance between whorls, is probably influenced by the variable herkogamy indicated above and corroborated by differences found in the analyses of the species  $\times$  treatment interaction.

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Table 1. Generalized linear model (GZLM) fitted to a negative binomial distribution with a logarithmic link function to analyze the effect of treatment (hand-geitonogamous pollination, spontaneous self-pollination and control), population (*Scrophularia sambucifolia*: Morón and El Gastor; *S. grandiflora*: Rabaçal and Pombalinho/Zambujal) and year (2010 and 2011) on fruit set (fruit/flower ratio). Significant results are shown in bold.

Table 2. One-way analysis of variance of the effect of treatment (geitonogamous pollination, spontaneous self-pollination and control), population (*Scrophularia sambucifolia*: Morón and El Gastor; *S. grandiflora*: Rabaçal and Pombalinho/Zambujal) and year (2010 and 2011) on seed set (seed/ovule ratio). Significant results are shown in bold.

Table 3. Absolute difference (D; mean  $\pm$  s.d.) between the percentage of male-phase and female-phase flowers of 10 randomly selected individuals from each phenological period (year).

Table 4. One-way analysis of variance of the effect of whorl (lower, middle and upper), population (*Scrophularia sambucifolia*: Morón and El Gastor; *S. grandiflora*: Rabaçal and Pombalinho/Zambujal) and year (2009, 2010 and 2011) on seed set (seed/ovule ratio) variation along the inflorescence. Significant results are shown in bold.

Figure 1. Flowers of the two *Scrophularia* species investigated in this study. (A) Female-phase flower of *S. sambucifolia* and (B) male-phase flower of *S. grandiflora*.

Figure 2. Locations of the studied *Scrophularia sambucifolia* (green squares) and *Scrophularia grandiflora* (red points) populations.

Figure 3. Percentage of fruit set (mean  $\pm$  s.d.) of two *Scrophularia* species after natural pollination (control) and two different pollination treatments (SSP, spontaneous self-pollination; HGP, hand-geitonogamous pollination). Populations are abbreviated as follows: Pomb, Pombalinho; Zamb, Zambujal.

Figure 4. Seed set (seed/ovule ratio; mean  $\pm$  s.d.) of two *Scrophularia* species after natural pollination (control) and two different pollination treatments (SSP, spontaneous self-pollination; HGP, hand-geitonogamous pollination). Populations are abbreviated as follows: Pomb, Pombalinho; Zamb, Zambujal.

Figure 5. Seed set (seed/ovule ratio; mean  $\pm$  s.d.) per inflorescence whorls of two *Scrophularia* species after natural pollination. LW, lower whorls; MW, middle whorls; UW, upper whorls; Pom, Pombalinho; Zam, Zambujal.