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**Andreia Jorge, João Loureiro & Sílvia
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Plant Systematics and Evolution

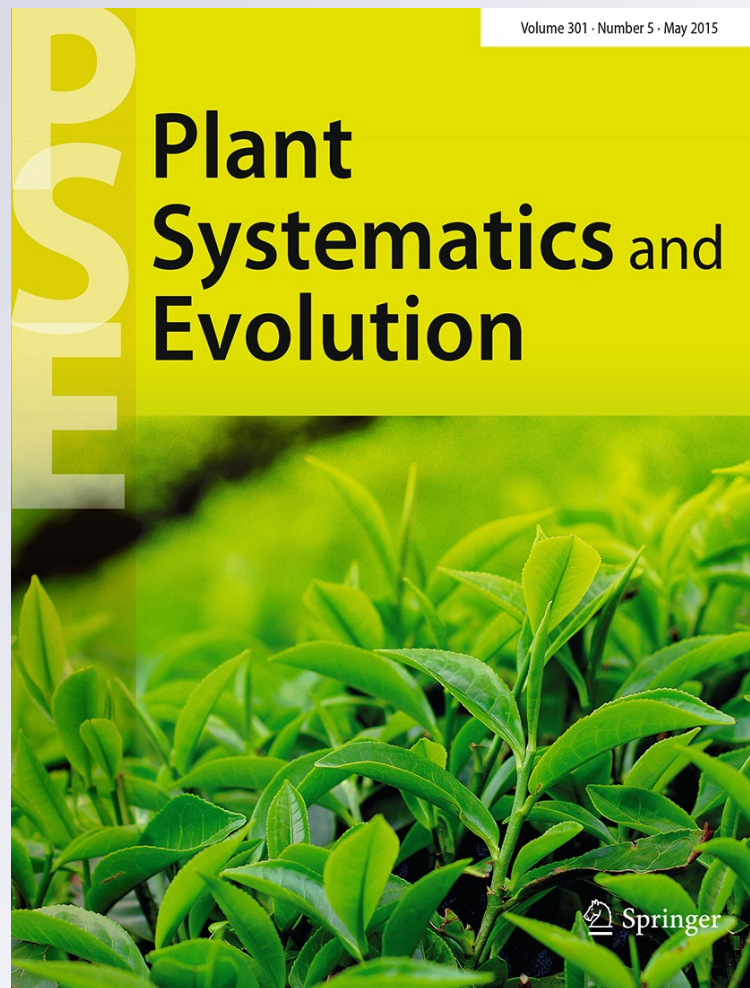
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Flower biology and breeding system of *Salvia sclareoides* Brot. (Lamiaceae)

Andreia Jorge · João Loureiro · Sílvia Castro

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Abstract A remarkable diversity of floral adaptations and reproductive strategies has evolved in flowering plants as a result of natural selection mediated by pollinators. The form and functioning of sexual units determine the patterns of pollen dispersal and receipt and, together with post-pollination mechanisms including incompatibility systems, determine the plant reproductive fitness. Reproductive biology assumes a special importance in rare and endemic species due to restricted population/distribution sizes. *Salvia sclareoides* Brot. (Lamiaceae) is an endemic species of Iberian Peninsula with conservation interest for which little information is available. The aim of this work was to study the flower biology and breeding system of *S. sclareoides* and assess how reproductive traits, pollination services and herbivory affected the reproductive success of this species. One natural population was selected to study flower lifespan, floral rewards and sexual functioning and to determine the reproductive system; additionally, in three natural populations, pollen limitation and herbivory of sexual structures were quantified. *Salvia sclareoides* presents short-lived, nectar rewarding flowers arranged in inflorescences with several flowers simultaneously open. The flowers are protandrous, although the peak of pollen germinability and stigmatic receptivity coincided. Pollination experiments revealed that *S. sclareoides* is self-compatible, having some ability of spontaneous selfing. Still, the presence of pollinators significantly increased the

reproductive success. Also, both quantitative and qualitative pollen limitation was observed. Finally, it was revealed that the reproductive success was also affected by herbivores that damaged significantly the sexual structures. The consequences of the obtained results for the dynamics of *S. sclareoides* populations are discussed.

Keywords Facultative xenogamy · Herbivory on sexual structures · Nectar production · Pollen limitation · Protandry

Introduction

Reproduction is one of the crucial steps determining the abundance and distribution of living organisms. Reproductive strategies determine the quantity and quality of the offspring and, consequently, the movement of the genes in time and space (Barrett 2003, 2010; Charlesworth 2006). The reproductive strategy assumes a special relevance in rare, endemic and/or narrowly distributed species, as it will act in a reduced number of individuals/populations directly determining their fitness and genetic composition, and affecting population dynamics and long-term persistence (Oostermeijer 2003).

A remarkable diversity of floral adaptations and reproductive strategies has evolved in flowering plants as a result of natural selection during interaction with different pollination agents (Harder and Johnson 2009; Barrett 2010; Goodwillie et al. 2010). Although many plants evolved to promote cross-fertilization, mating strategies can vary from obligate outcrossing and mixed mating systems to autonomous selfing. The reproductive strategy depends on the form and function of the sexual units (stamens, pistils, flowers and/or inflorescences) that determine the patterns

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A. Jorge (✉) · J. Loureiro · S. Castro
CFE, Centre for Functional Ecology, Department of Life
Sciences, University of Coimbra, Calçada Martim de Freitas,
3000-456 Coimbra, Portugal
e-mail: andreiajorge.87@gmail.com

of pollen dispersal and receipt, together with post-pollination mechanisms such as, genetically controlled incompatibility systems (Barrett 2003, 2010; Charlesworth 2006). Animal-pollinated plants promote outcrossing by producing visually attractive floral displays and floral rewards or, ultimately, by evolving separated sexes (Barrett 2003; Harder and Johnson 2005; Goodwillie et al. 2010; Fisogni et al. 2011). Selfing can be reduced through mechanisms such as, temporal and spatial separation of male and female functions (dichogamy and herkogamy, respectively) being ultimately prevented through the development of self-incompatibility systems (Lloyd and Webb 1986; Barrett 2003; Charlesworth 2006; Narbona et al. 2011); alternatively, morphological adaptations of the flowers could favor selfing, for example, through spontaneous or delayed selfing (Schoen et al. 1996; Nagi et al. 1999; Goodwillie et al. 2010).

Each reproductive strategy has its benefits and drawbacks (Takebayashi and Morrell 2001; Goodwillie et al. 2005; Wright et al. 2013). Overall, outcrossing gives the opportunity for gene flow within and/or among populations, thus increasing genetic diversity and, potentially, enhancing offspring performance and its potential for adaptation (Franklin-Tong 2008; Morran et al. 2009). However, promoting outcrossing also bears its costs, directly through the energy necessary for producing attractants and rewards, and indirectly due to fluctuations in the abundance of pollinators or in the attraction of herbivores (Pyke 1991; Barrett 1998; Knight et al. 2005; McCall and Irwin 2006). Under unpredictable pollination services or mate availability, selfing can bear some advantages, as it can work as a reproductive assurance mechanism, and it can enable the preservation of well-adapted genotypes (Schoen et al. 1996; Pannell and Barrett 1998; Kalisz and Vogler 2003; Charlesworth 2006); still, it comes with a cost of reduced genetic diversity of the populations, potentially leading to inbreeding depression (Barrett 1998, 2003; Charlesworth 2006). However, this dichotomy in the mating strategy is not always clear and recent studies have shown that mixed mating systems are actually common in nature (Goodwillie et al. 2005).

Salvia L. (tribe Mentheae, Lamiaceae) is the biggest genus of Lamiaceae, being represented by almost 1,000 species distributed in temperate and warm regions of both hemispheres (Claßen-Bockhoff et al. 2003). From a morphological point of view the genus presents a wide diversity of floral traits and pollination syndromes (Barrett et al. 2000; Claßen-Bockhoff et al. 2004; Wester and Claßen-Bockhoff 2006, 2007, 2011; Espino et al. 2012), although the reproductive biology has been studied only in a few species. The available studies have shown that the species studied are mostly self-compatible (e.g., Haque and Ghoshal 1981; ZhenQiao et al. 2009) and protandrous (e.g.,

Navarro 1997), but different scenarios from predominantly outcrossers to predominantly selfers have also been reported (Haque and Ghoshal 1981; Barrett et al. 2000; ZhenQiao et al. 2009; Aximoff and Freitas 2010). *Salvia sclareoides* Brot. is an endemic species from southwest Iberian Peninsula and a key species in specific vegetation communities (*Ulici densi-Thymion sylvestris*; Costa et al. 2000, 2009), being included in several national conservation plans (Anonymous 2000, 2001) (Fig. 1). In the last years, the populations of this species are also being subjected to strong anthropogenic pressures from continuous plant and seed collection (Lopes 2013) as a result of the discovery of medicinal properties (Rauter et al. 2007, 2012; Branco 2010). Despite its endemism and conservation importance, nothing is known about its reproductive biology. In this context, the objectives of this work were to study the flower biology and breeding system of *S. sclareoides* and assess how reproductive traits, pollination services and herbivory affect the reproductive outcome of natural populations. To accomplish these objectives the following features were studied: (1) floral rewards and floral display, traits directly involved in pollinator's attraction; (2) male and female functioning through flower lifespan, to evaluate the occurrence of mechanisms that prevent or open the possibility for selfing and reproductive assurance; (3) breeding system, through controlled hand pollination experiments involving exclusion of pollinators and pollen from different sources; (4) pollen limitation due to limited pollination services; and finally, (5) herbivory of sexual structures and its impact in the final reproductive success of natural populations. The obtained results constitute the first contribution on the reproductive biology of the endemic *S. sclareoides* generating new questions for future research.

Materials and methods

Study species

Salvia sclareoides is a 15–90 cm high, biennial or perennial herb, with leaves mostly arranged in a basal rosette (Fig. 1b). The inflorescence has several verticillasters, each with six flowers (Fig. 1c). The small flowers are typically bilabiate, purple to violet bluish; the upper lip is falcate enclosing the anthers, and the lower lip is trilobed serving as landing platform for insects. The staminal lever mechanism is well-developed with stamens having the connective greater than the filament and with a sterile lower theca; the sterile lower thecae are activated during the probe depositing the pollen from the fertile thecae on the dorso of the pollinator. The style is bifurcated and generally exposed from the upper lip. The fruit is composed by four

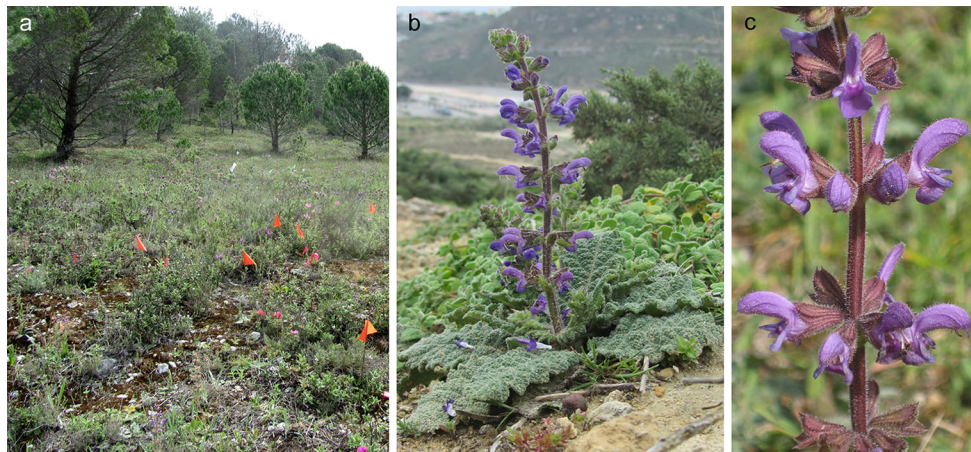


Fig. 1 *Salvia sclareoides*: **a** habitat, Poço das Casas population, **b** plant habit, **c** detail of the inflorescence with bud and open flowers displayed in verticillasters

Table 1 Description of the populations studied

Population	Geographical coordinates	Altitude (m a.s.l.)	Area (m ²)	Population size	Vegetation
Poço das Casas	40°4'35.85"N 8°28'24.54"W	180	270	800	<i>Cistus</i> spp., <i>Lavandula</i> sp., and <i>Thymus</i> sp., few <i>Olea europaea</i> , <i>Pinus pinea</i> , and <i>P. pinaster</i>
Santa Clara	40°12'11.32"N 8°26'51.15"W	75	970	1,500	Gramineae, few <i>O. europaea</i> and <i>P. pinaster</i>
Lôgo de Deus	40°15'51.00"N 8°24'35.01"W	100	50	400	<i>Lavandula</i> sp. and <i>Cistus</i> spp., <i>Eucalyptus globulus</i> , <i>Quercus</i> sp. and <i>P. pinaster</i>

1-seeded brown nutlets (A. Jorge field observations; Coutinho 1939; Sáez 2010). *Salvia sclareoides* grows in forest clearings, roadsides, steep slopes or coastal sites, usually in limestone or loam soils (Hedge 1972; Sáez 2010; Fig. 1a). The plant flowers from mid-March until early-June and fruits mature 4 weeks later (A. Jorge, field observations).

Study populations

The study was performed in the district of Coimbra (Portugal) between the end of March and mid-June of 2012, where three populations were selected: Poço das Casas, Santa Clara and Lôgo de Deus. This region is influenced by a meso-mediterranean climate, characterized by warm and dry summers, and mild and humid winters. The three populations have limestone soil and native Mediterranean vegetation. The population more to the south, Poço das Casas, is at 15 km distance from Santa Clara, the middle population, which distances 6.5 km from the most northerly population, Lôgo de Deus (Table 1).

Flower lifespan, floral rewards and sexual functioning of the flower, as well as, breeding system were investigated in

Santa Clara population. Floral display, pollen limitation and herbivory were studied in the three populations selected.

Flower lifespan and display

To study the flower lifespan, 34 flower buds each belonging to distinct individuals were randomly selected and subsequently labeled in the pedicel with nylon strings. Flowers were then monitored daily until senescence and the following variables were recorded: opening day, availability of pollen in the anthers, stigmatic lobes opening and senescence day. Pollen availability was assessed daily after gently touching the anthers with a microscope slide and observing the presence of pollen grains on the slide under a magnifying glass. Stigmatic lobes were considered open when they started to bifurcate (an indication of stigmatic receptivity; see results).

Floral display, defined as the number of open flowers per plant per day, was characterized at the peak of flowering. Plants were randomly selected across each population ($n_{\text{Poço das Casas}} = 31$; $n_{\text{Santa Clara}} = 38$; $n_{\text{Lôgo de Deus}} = 62$) and the number of open flowers per individual was registered.

Floral rewards

Preliminary field observations revealed that the floral visitors of *S. sclareoides* searched for nectar and/or pollen, and thus, both rewards were quantified. To characterize daily nectar production, 59 flowers belonging to 16 distinct individuals randomly selected (3–9 flowers per individual distributed along 18 verticillasters, each including flowers in different developmental stages; Fig. 1c) was bagged 24 h before nectar quantification. Nectar production was measured with a capillary micropipette and sugar concentration was determined (in w/w, %) with a portable refractometer (0–50°Brix, Eclipse Professional, Bellingham and Stanley Ltd). The amount of sugar produced by each flower was calculated according with Prys-Jones and Corbet (1987).

To characterize the pollen produced per flower, the number of pollen grains was counted in one anther from 10 bud flowers belonging to distinct individuals randomly selected. Each anther was placed in a drop of glycerine 50 % over a microscope slide, opened and squashed beneath a cover slip. All pollen grains were counted under a light microscope (amplification = 100x). The total amount of pollen produced per flower was calculated by multiplying the value obtained by two (corresponding to the number of functional thecae per flower).

Flower sexual functioning

To determine stigmatic receptivity along the flower lifespan, fruit production was assessed in flowers pollinated with xenogamous pollen at different ages. For this, prior to anthesis, flowers were emasculated, bagged and marked with the day of flower opening; all flowers were then hand-pollinated with a fresh mixture of pollen collected in at least five distinct individuals; hand-pollinations were made in flowers with 1 ($n = 17$), 2 ($n = 24$) and 3 ($n = 31$) days old (ages defined according with the results obtained in “Flower lifespan and display”). Flowers were maintained bagged until fruiting stage. Fruit set was recorded when mature. The production of fruits was used as an indicator of stigma receptivity and the percentage of receptive stigmas was calculated for each age class.

To determine pollen germinability along the flower lifespan, the percentage of germinated pollen grains was assessed. For this, two groups of flowers were selected and bagged: one to serve as pollen recipients and the other to act as pollen donors. Pollen from 1 ($n = 17$), 2 ($n = 24$) and 3 ($n = 31$) days old were then used to pollinate the recipient flowers (i.e., flowers with 2–3 days old with open stigmatic lobes). Because the stigmas of *Salvia* are dry, the observation of the pollen tubes followed Dafni et al. (2005) protocol with modifications to reduce the loss of pollen

grains during submersion of the pistils in consecutive solutions. Thus, after 6 h (time necessary for successful pollen germination; A. Jorge preliminary observations), stigmas were collected and placed in a microscope slide with a drop of aniline blue, squashed and left for overnight staining. Preparations were then observed in a fluorescence microscope (Leica DM 4000B) and the total number of pollen grains and the number of germinated pollen grains were recorded.

Breeding system and pollen limitation

To determine the reproductive system of *S. sclareoides* the following treatments were applied: (1) spontaneous autogamy, flowers were bagged with mosquito net to exclude insect interactions; (2) obligated autogamy, flowers were bagged and pollinated with its own pollen; (3) geitonogamy, emasculated flowers were bagged and pollinated with pollen of flowers from the same individual; (4) xenogamy, emasculated flowers were bagged and pollinated with a fresh pollen mixture collected in at least five distinct individuals; (5) supplementary pollination, flowers were pollinated with a fresh pollen mixture collected in at least five distinct individuals and left for open pollination; (6) open pollination, flowers without treatment. Thirty-four individuals were selected and bagged to receive treatments 1–4, one flower per treatment; 34 additional individuals were selected to receive treatments 5 and 6, one flower per treatment. The latter two treatments were also performed in the other two populations to evaluate if the reproductive success of *S. sclareoides* is limited by pollination services in other sites. All flowers treated were located at the first or second verticillaster to avoid differences due to resource allocation patterns among verticillasters (Navarro 1997). When mature, fruit and seed production were recorded and seeds collected for seed weight and seed viability. Seed viability was assessed by cutting the seeds and scored them as full (potentially viable) or empty (aborted).

To characterize the breeding system of *S. sclareoides* several indexes were calculated. Self-compatibility index (SCI) and self-fertilization index (SFI) were calculated following Lloyd and Schoen (1992). SCI is the proportion between the seed set for self-pollinated flowers and the seed set for cross-pollinated flowers. SFI is the proportion between the seed set for spontaneous self-pollination and the seed set for cross-pollinated flowers (Lloyd and Schoen 1992). The selfing rate (S) was calculated according to Charlesworth and Charlesworth (1987) as: $S = (P_x - P_o) / (P_x - P_s)$, where P_x is the seed set of outcrossing pollination, P_o is the seed set of open pollination, and P_s is the seed set of autogamy. Levels of inbreeding depression (δ) were calculated using fruit set, seed set, and seed weight of geitonogamous pollinated flowers (w_g) and of cross-

pollinated flowers (w_x): $\delta = 1 - (w_g/w_x)$ (Charlesworth and Charlesworth 1987). Finally, the total number of pollen grains produced per flower was divided by the number of ovules to calculate the index of Cruden (Cruden 1977), as an indicator of the breeding system.

Herbivory of sexual structures

To assess the impact of herbivory in the loss of reproductive structures of *S. sclareoides*, herbivory was quantified at different stages of the development of the reproductive organs, including flower buds, flowers and fruits. Herbivory of these structures was easily recorded by the presence of eaten and damaged tissues and galls (i.e., abnormal outgrowth in plant tissue caused by parasitic insects) in the fruits and was assessed in the organs of up to 60 plants from each population. The percentage of organs with damage was calculated for each developmental stage.

Reproductive success

The reproductive success of each population was calculated considering the rates of herbivory obtained above, as well as, the natural reproductive success, through the fruit set and seed viability results. An initial set of 100 bud flowers that was subjected to losses due to herbivory at successive stages and natural reproductive success were used.

Statistical analyses

The data was analyzed using generalized linear models (GLM). Likelihood ratio tests were performed in each analyses enabling to compare the full model with a restricted model where the explanatory variables of interest are omitted and by calculating P values using the χ^2 distribution. When significant differences were found, differences between levels of each effect were analyzed using multiple comparisons of means with Tukey contrasts. All statistical analyses were carried in R version 3.1.1 (R Core Team 2014) with the packages `pscl` and `multcomp`.

Differences among populations on floral display were analyzed using GLM with the dependent variable (number of opened flowers) adjusted to a Poisson distribution with a log link function to model responses.

Data of floral rewards was assembled in groups of two consecutive verticillasters, sequentially from the base to the top. Differences in nectar variables (volume of nectar, sugar concentration and sugar mass) among flower position and flower age (young with stigmatic lobes closed versus old with stigmatic lobes open) were assessed using GLM

with a nested design, including flower position and flower age nested within flower position as factors. This approach enabled to assess differences in flower rewards along the inflorescence and differences in flower rewards according with the age, removing at the same time the effect of flower position. Nectar volume and sugar concentration (the later, arcsin transformed) were adjusted to a Gaussian distribution with an identity link function, whereas sugar mass was adjusted to a Gamma distribution with an inverse link function.

Differences in stigmatic receptivity and pollen germinability along the flower lifespan were investigated using GLM with the response variables (presence/absence of developed fruit for stigma receptivity and presence/absence of pollen tube for pollen germinability) being adjusted to a binomial distribution and a logit link function.

Differences among all pollination treatments made in Santa Clara population on fruit set, seed set, seed weight and seed viability were analyzed using GLM; fruit set (presence/absence of developed fruit) and seed viability (full/empty seed) were adjusted to a binomial distribution with a logit link function to model responses, seed set (number of nutlets per fruit) was adjusted to a Poisson distribution with a log function, and seed weight to a Gamma distribution with inverse link function. The effect of pollen supplementation on the same response variables (fruit set, seed set, seed viability and seed weight) was further analyzed in all populations using GLM with population and pollination treatment (open pollination and pollen supplement) as factors; because the interaction between pollination treatments and population was significant, differences between pollination treatments were analyzed separately for each population. Response variables were modelled as described above.

Differences in herbivory (presence/absence of damaged or eaten structures) were assessed among populations and among reproductive stages within populations using GLM with a nested design including population and reproductive stage nested within population as factors. If the later factor was significant, differences in herbivory between reproductive stages were analyzed separately for each population. Herbivory was adjusted to a binomial distribution with a logit link function to model responses.

Results

Floral lifespan and display

The mean flower lifespan of *S. sclareoides* was 3 days (2.94 ± 0.16 , 34; mean \pm SE, n). From the observation of the flowers it was possible to detect that the pollen was available in the anthers until the middle of the second day

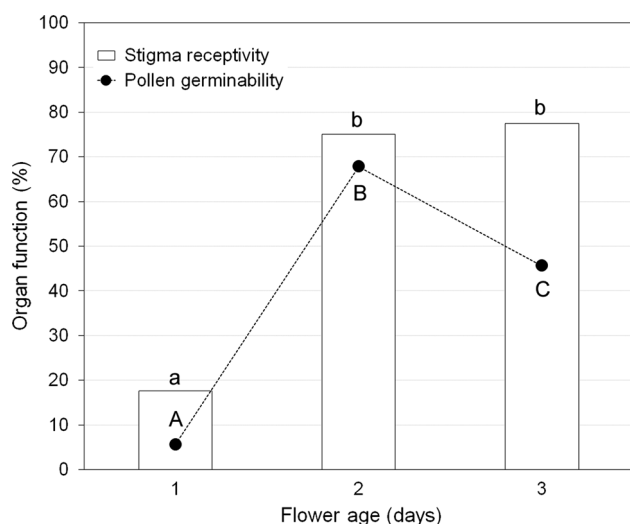


Fig. 2 Sexual functioning of *Salvia sclareoides* flowers. Stigma receptivity (white bars) is given as the percentage of flowers that set fruit, and pollen germination (black dots) is given as the percentage of germinated pollen grains over stigmas, both according to flower age. Different letters reveal statistically significant differences in female function (lower case) and male (upper case) according with flower age ($P < 0.05$)

and that the stigmatic lobes started to open in the second day, being fully expanded in the third day of the flower life (see details of male of female function in section “[Flower sexual functioning](#)”).

Concerning the floral display, *S. sclareoides* individuals had a mean number of open flowers per plant per day of 9.79 ± 0.56 ($n = 131$). No statistically significant differences were observed in floral display among populations ($\chi^2_{2,129} = 3.05$, $P = 0.218$; Santa Clara: 11.92 ± 1.93 , 38; Poço das Casas: 8.16 ± 0.59 , 31; Lôgo de Deus: 9.30 ± 0.68 , 62). Interestingly, Santa Clara population revealed a high variability in this trait having plants with three up to 48 simultaneously open flowers.

Floral rewards

From all the flowers used for nectar quantification, 11.9 % had no reward of this type. For the others, on average, the flowers produced $2.17 \pm 0.23 \mu\text{l}$ of nectar with 25.8 ± 1.6 % ($n = 52$) of sugar concentration. No significant differences were observed in nectar rewards along the inflorescence ($\chi^2_{4,56} = 17.71$, $P = 0.199$, $\chi^2_{4,50} = 0.08$, $P = 0.277$, and $\chi^2_{4,50} = 0.91$, $P = 0.361$ for nectar volume, sugar concentration and mass, respectively), neither between young and old flowers within verticillasters ($\chi^2_{4,56} = 1.85$, $P = 0.960$, $\chi^2_{4,50} = 0.01$, $P = 0.980$, and $\chi^2_{4,50} = 0.45$, $P = 0.709$ for nectar volume, sugar concentration and mass, respectively). The mean number of pollen grains produced per flower was $15,181 \pm 897$ ($n = 10$).

Flower sexual functioning

Stigmatic receptivity increased significantly from low values in the first day to high levels of receptivity in the second and third days ($\chi^2_{2,71} = 19.31$, $P < 0.001$; Fig. 2). This result is in accordance with stigmatic lobes opening, being the later a morphological indicator of stigmatic receptivity. Pollen was dehiscent in day one, however, pollen germinability was shown to significantly increase until day two, decreasing significantly afterwards ($\chi^2_{2,1379} = 328.01$, $P < 0.001$; Fig. 2). An overlapping period of both functions was observed in the day two of flower lifespan (Fig. 2).

Breeding system

With exception of seed set ($\chi^2_{5,125} = 3.59$, $P = 0.610$), pollination treatments significantly affected all the fitness variables of *S. sclareoides* (fruit set: $\chi^2_{5,636} = 56.91$, $P < 0.001$; seed weight: $\chi^2_{5,216} = 3.66$, $P = 0.028$; seed viability: $\chi^2_{5,358} = 67.18$, $P < 0.001$; Fig. 3). Spontaneous autogamy produced significantly less fruits than the other treatments ($P < 0.05$; Fig. 3a) and lead to a low self-fertilization index (SFI = 0.19), but no significant differences were found for the remaining variables (Fig. 3c, d). Obligated autogamy and geitonogamous pollinations resulted in similar values for all reproductive variables ($P < 0.05$), being in agreement with the overlap in male and female functions obtained for flower functioning. Also, these selfing treatments did not differ statistically from the xenogamous treatment for all measures of fitness (Fig. 3), resulting in a high self-compatibility index (SCI = 0.705) and selfing rates ($S = 1.20$) and indicating the absence of a self-incompatibility system. Despite not statistically significant, most fitness variables were lower in open pollination (natural conditions) than in other pollination treatments (Fig. 3; see also section “[Pollen limitation](#)” for further details).

Low levels of inbreeding depression (δ) were obtained for fruit set, seed set and seed weight ($\delta_{\text{fruit set}} = -0.04$, $\delta_{\text{seed set}} = 0.09$, $\delta_{\text{seed weight}} = 0.15$). The flower of *S. sclareoides* had a P:O ratio of $3,795.2 \pm 194.1$.

Pollen limitation

Significant differences were observed between pollen supplemented and open pollinated flower for seed weight and seed viability, and among populations for fruit set, seed set and seed viability (Table 2); significant interactions between pollination treatment and population were observed for fruit set and seed viability (Table 2). Overall, supplementary pollinations resulted in a higher fitness than

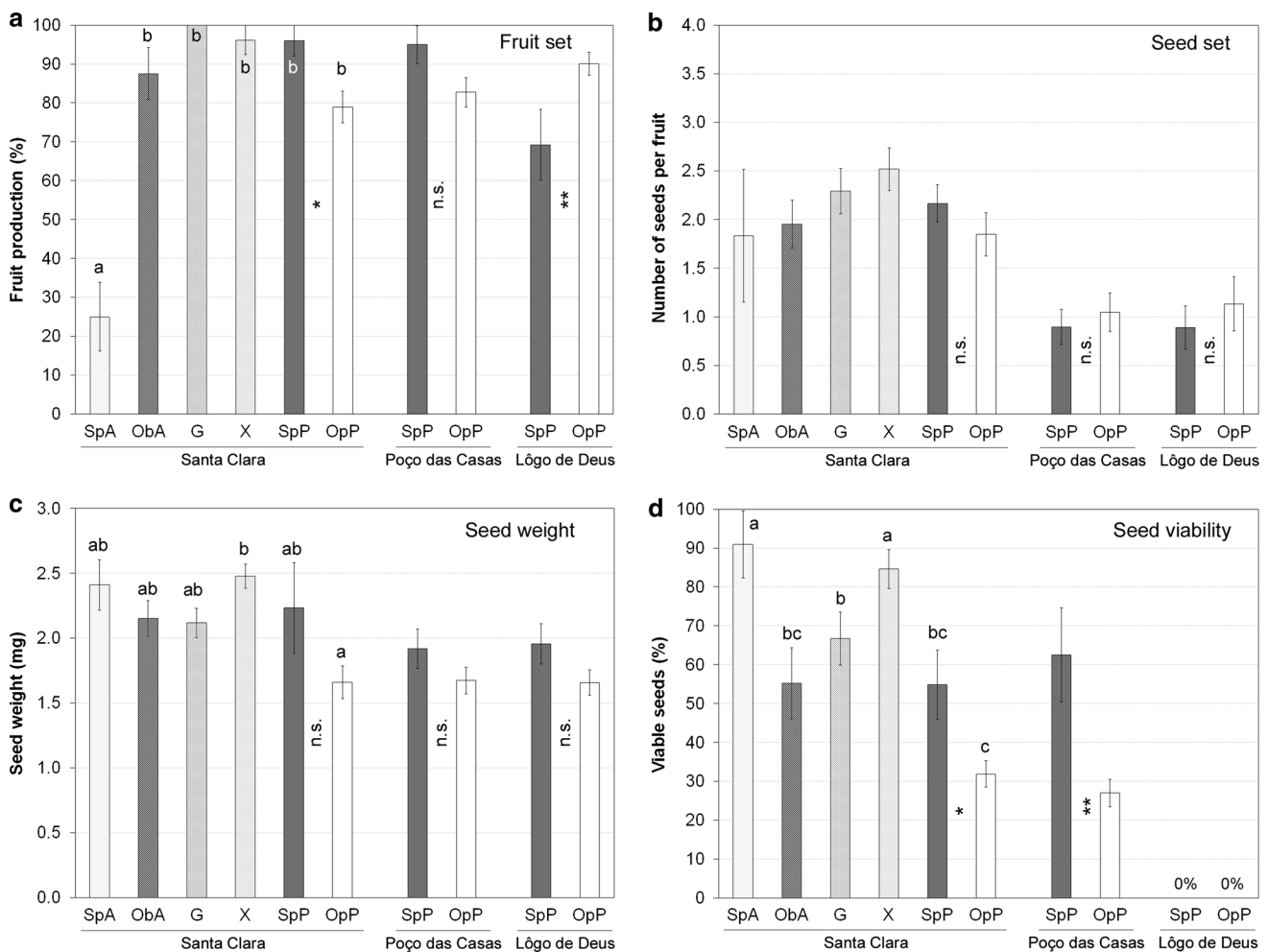


Fig. 3 Controlled hand pollination experiments performed in *Salvia sclareoides* to study the breeding system (Santa Clara population) and to assess pollen limitation (three populations studied). **a** fruit set, given as the percentage of flowers that set fruits after a given pollination treatment, **b** seed set, given as the mean number of seeds developed per fruit, **c** mean seed weight (mg), and **d** seed viability, given as the percentage of viable seeds. Pollination treatments: SpA—spontaneous autogamy, ObA—obligated autogamy, G—geitonogamy, X—xenogamy, SpP—supplementary pollination, OpP—open

pollination. Values are given as mean \pm SE. Statistical analyses for breeding system: different letters reveal statistically significant differences among treatments performed in Santa Clara population ($P < 0.05$), and lack of letters indicates non significant differences; statistical analyses for pollen limitation: the significance for the comparison between supplementary pollination and open pollination within each population is given as follows: ** $P < 0.01$, *** $P < 0.05$ and n.s. not significant

the open pollination treatment; however, significant differences were only observed for fruit set and seed viability (Fig. 3). Supplementary pollination increased fruit production in Poço das Casas and Santa Clara populations, but it was only significant higher in the latter population ($\chi^2_{1,263} = 2.75$, $P = 0.097$, $\chi^2_{1,138} = 5.84$, $P = 0.016$, respectively); contrarily to the expected, the opposite pattern was observed in Lôgo de Deus ($\chi^2_{1,139} = 8.43$, $P = 0.004$, Fig. 3a). The effect of pollen supplementation was also significant at the level of seed viability, with Poço das Casas and Santa Clara populations presenting higher seed viability after pollen supplementation than open pollinated flowers ($\chi^2_{1,178} = 7.90$, $P = 0.005$, $\chi^2_{1,218} = 5.88$,

$P = 0.015$, respectively). Again, in Lôgo de Deus unexpected results were observed with all the seeds studied being empty, regardless of the pollination treatment (Fig. 3d).

Herbivory of sexual structures

The percentage of herbivory varied significantly among the populations studied ($\chi^2_{2,3274} = 251.97$, $P < 0.001$) and among the different development stages of the reproductive unit within each population ($\chi^2_{6,3274} = 101.95$, $P < 0.001$) (Fig. 4). Poço das Casas was significantly more affected by herbivory than the other two populations ($P < 0.05$), while

Table 2 Statistical analysed of pollen limitation experiment made in the three populations studied

Response variable	Factors	χ^2	P
Fruit set	Treatment	0.02	0.903
	Population	42.26	<0.001
	Treatment population*	16.87	<0.001
Seed set	Treatment	0.00	0.934
	Population	21.31	<0.001
	Treatment population*	1.36	0.506
Seed weight	Treatment	12.72	<0.001
	Population	13.28	0.001
	Treatment population*	7.06	0.029
Seed viability	Treatment	2.12	0.015
	Population	0.21	0.751
	Treatment population*	0.17	0.794

The effect of pollen supplementation on fruit set, seed set, seed viability and seed weight was analyzed using GLM with population and pollination treatment (open pollination and pollen supplement) as factors. For details on statistical approach see “Statistical analyses” section

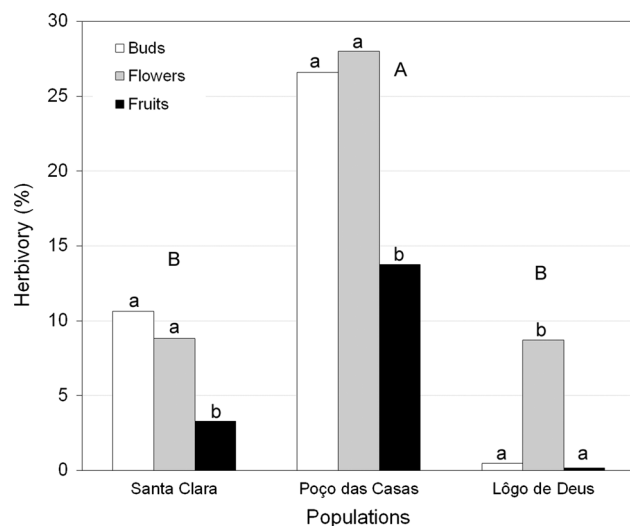


Fig. 4 Herbivory of sexual structures of *Salvia sclareoides* in the three populations studied. The levels of herbivory are given as percentage of buds (white bars), flowers (grey bars) and fruits (black bars) eaten or damaged tissues. Different upper case letters reveal statistically significant differences in the total level of herbivory between populations ($P < 0.05$); different lower case letters reveal statistically significant differences in the level of herbivory between developmental stages within each population ($P < 0.05$)

Lôgo de Deus was the less attacked population (Fig. 4). In Poço das Casas and Santa Clara populations the levels of herbivory were significantly higher in the flower buds and flowers, significantly decreasing in the fruit stage; in Lôgo de Deus, mostly flowers were attacked by herbivores ($P < 0.05$; Fig. 4).

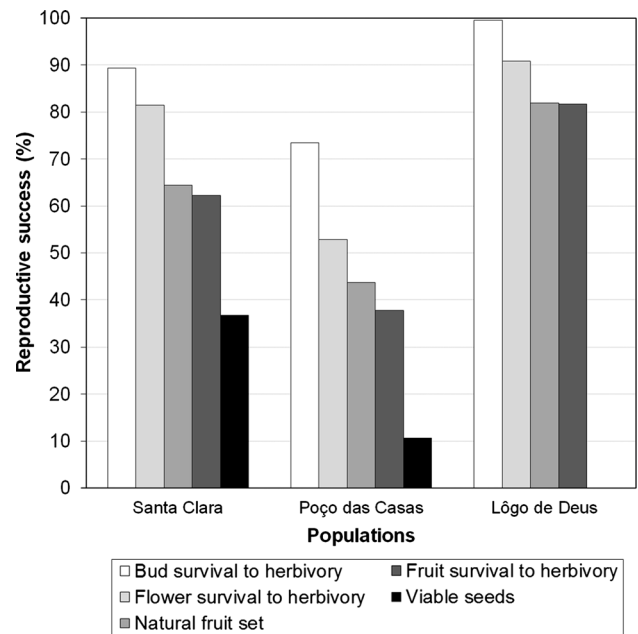


Fig. 5 Reproductive success of *Salvia sclareoides* in the three populations studied calculated from an initial set of 100 bud flowers that were subjected to losses due to herbivory at successive stages (herbivory of buds, flowers and fruits) and natural reproductive success (natural fruit production and number of viable seeds)

Reproductive success

The combined analysis of the variables affecting the reproductive success of *S. sclareoides* enabled to observe that in the populations of Poço das Casas and Santa Clara the decrease in reproduce success was gradual and similar in all the stages studied, while in Lôgo de Deus the rates of herbivory were low and the reduced reproductive success was mostly due to offspring quality, namely low seed viability (Fig. 5). Overall, Santa Clara was the population with a higher overall reproductive success (Fig. 5).

Discussion

Flower: rewards and floral display

The bilabiate flower of *S. sclareoides* shows a melittophilous syndrome with a one-way construction for nototribic pollen deposition (Westerkamp and Claßen-Bockhoff 2007), offering nectar at the base of corolla tube as a reward for pollinators (similarly to many *Salvia* species; Faegri and Pijl 1979; Haque and Ghoshal 1981; Wester and Claßen-Bockhoff 2006). This morphology was suggested to have evolved as a mechanism to improve the fit between the pollinator and the flower and to avoid undesirable pollen collectors (Claßen-Bockhoff 2007; Westerkamp and

Claßen-Bockhoff 2007). Nectar might thus be seen as an important reward to guarantee pollinator fidelity and species from Lamiaceae have been described as consistent nectar producers in Mediterranean ecosystems (Herrera 1985; Petanidou and Vokou 1993). Nectar production can also vary along the flower lifespan, favoring male or female functions (e.g., Aizen and Basilio 1998; Leshem et al. 2011) and/or along the inflorescence, most probably due to resource limitation (e.g., Stpiczynska 2003; but see Navarro 1998). In *S. sclareoides* none of these patterns were observed. Similar patterns of nectar production along the flower lifespan and inflorescence, together with other floral traits, such as, inflorescences with several simultaneously open flowers, could reflect an investment in producing attractive floral displays for pollinator attraction. Indeed, field observations confirm a positive correlation between floral display and visitation rates in this species (A. Jorge unpublished data), confirming the role of floral displays in advertisement. Because early flowering species, such as *S. sclareoides*, are usually subjected to low pollination services, an investment in attractiveness and reward production might be advantageous under competition for pollination services (Petanidou and Vokou 1993; Dafni 1991). However, a larger floral display may also lead to higher levels of geitonogamy lowering the opportunity for outcrossing (Harder and Barrett 1995), especially considering that this species is self-compatible.

Flower lifespan and functioning

Flower longevity is a key characteristic for plant reproduction and it is determined by a trade-off between the cost to maintain the flower open and the pollen dispersal and receipt rates (i.e., male and female accrual rates; Ashman and Schoen 1994). The flowers of *S. sclareoides* presented a short lifespan of 3 days, comparable with the flower lifespans described in other congeneric species (Navarro 1997; Petanidou and Vokou 1993; Aximoff and Freitas 2010; Leshem et al. 2011). Short lifespan is favored when flowers have high fitness accrual rates and high floral maintenance costs (Ashman and Schoen 1996).

Many flowering plants, including some *Salvia* species (Navarro 1997; Leshem et al. 2011), evolved to decrease sexual interference and promote outcrossing by separating male and female functions in time, i.e., through dichogamy (Lloyd and Webb 1986). Indeed, protandry is common in Lamiaceae family (East 1940; Owens and Uberta-Jiménez 1992; Navarro 1997; Hidalgo and Uberta 2001; Rodríguez-Riaño and Dafni 2007; Leshem et al. 2011; Castro et al. in press); despite that, the efficiency of protandry in avoiding interference and self-pollen deposition has been questioned (Navarro 1997; Rodríguez-Riaño and Dafni 2007). Our observations revealed that the pollen of *S. sclareoides* is

readily available in the dehiscent anthers since corolla opening, while the stigmatic lobes expand only in the second day, pointing to a protandrous species. This feature allows pollen dispersal to other flowers, decreasing the levels of within flower-pollination, especially at day one when the stigma is not receptive. Additionally, the dispersal schedules of pollen grains (starting in day one) do not coincide with their maturation schedules (day two-onwards as observed in other *Salvia* species; Navarro 1997; Leshem et al. 2011). This mismatch between pollen dispersal schedules and pollen germinability is interesting and at the same time difficult to explain. One might hypothesize that if the pollen does not germinate it can be secondarily transported by pollinators (Inouye et al. 1994) even if deposited in receptive stigmas (considering that the stigmas are dry). If this is true, this could be advantageous in species with large floral displays, such as *S. sclareoides*, where geitonogamous pollen transfer occurs because it might promote pollen dispersal to other plants during day one, before pollen becomes mature.

Despite all the above, from a functional point of view, the maximum pollen germinability coincided with the peak of stigmatic receptivity; consequently, there is an overlap in maturation schedules of both sexual functions. Thus, pollinators could mediate within flower and within inflorescence pollen transfer with successful pollen germination and fertilization. A similar result was found in *S. verbenaca*, allowing this species to self-fertilize if no outcrossing pollen arrives to the stigmas in the meantime (Navarro 1997). This strategy can be advantageous especially in early flowering plants when pollinators are scarce and competition for their services is high, and in the absence of mates for example, when dispersed to new places, because it constitutes a mechanism of reproductive assurance (e.g., Baker 1955; Schoen et al. 1996; Kalisz and Vogler 2003; Moeller and Geber 2007).

Breeding system

Under natural conditions, *S. sclareoides* presented a relatively high fruit set, similarly to what was observed in the sympatric *S. verbenaca* (Navarro 1997) and in other *Salvia* species (Haque and Ghoshal 1981; Aximoff and Freitas 2010; Zhang et al. 2011). However, the exclusion of pollinators significantly decreased fruit set revealing that this species needs pollination vectors to carry pollen and significantly increase reproductive success. Still, it is interesting to note that in their absence, a few plants were able to produce fruits; these genotypes that were able to spontaneously self-fertilize in the absence of pollination vectors could be in advantage in years of pollinator scarcity (Schoen et al. 1996; Kalisz and Vogler 2003) and, thus, be important individuals for the dynamics of *S. sclareoides* populations.

Fruit production in controlled hand pollination experiments involving autogamous and geitonogamous pollinations demonstrated that *S. sclareoides* is a self-compatible species, as already observed in several species of *Salvia* (Haque and Ghoshal 1981; Navarro 1997; Sales et al. 2010; Ohashi 2002; Leshem et al. 2011). Being a self-compatible plant with several flowers simultaneously open, there are several opportunities for selfing, including selfing mediated by pollinators, within the flower or among flowers of the same plant, and spontaneous selfing. If pollen is not dispersed in the first day, selfing will be competing with outcrossing, as both pollen germination and stigmatic receptivity within flower coincide; even when dispersed in the first day, selfing could occur by pollination of receptive flowers of the same plant (geitonogamy). Spontaneous selfing most probably results from maturation of the pistil that curls the stigmatic lobes backwards touching the anthers, similarly to the closely related *S. verbenaca* (Navarro 1997). Still, the mechanism of spontaneous selfing is still unknown in *S. sclareoides*. Previous works in the genus have shown a great range in spontaneous selfing rates, with *S. verbenaca* revealing high values (Navarro 1997), while in others the ability to self-pollinate is rare (Haque and Ghoshal 1981; Leshem et al. 2011).

Although not significant, xenogamous pollination seemed to be more advantageous for progeny in terms of seed quality, measured as seed weight and seed viability. The comparison between xenogamy, supplementary pollination and open pollination, revealed an interesting pattern in female fitness. Overall, xenogamy presented a better performance than open pollination, with the supplementary pollination presenting intermediate values, especially in seed quality. This may suggest that pollinator's behavior may clog the stigmas with pollen of less quality (e.g., pollen with lower viability due to aging) or with interspecific pollen loads, reducing the final reproductive success of *S. sclareoides*. In the future it will be interesting to study the composition of stigmatic pollen loads and the viability of the pollen transported by pollinators. It could also be interesting to assess if different pollen loads composed of selfing and outcrossing pollen and different timings of selfing versus outcrossing pollination have different fitness outcomes in subsequent developmental stages such as, seed germination, and seedling survival and growth. The results will help understand the role and importance of each strategy for the species survival.

Following the conservative indicator of Cruden (1977) for the breeding system, *S. sclareoides* is between facultative xenogamy and xenogamy. This is in accordance with the results from flower functioning (protandrous flower with partial overlap in male and female functions) and breeding system (self-compatible species that relies on pollination vectors to increase fruit production). *Salvia*

verbenaca was also suggested to be a facultative xenogamous plant (Navarro 1997). The most recent review on mixed mating systems estimated that 42 % of the flowering plants have this type of breeding system (Goodwillie et al. 2005). Despite this high estimate, the mechanisms that drive the mixed mating strategy are still poorly understood. Plants with this type of mating are considered to be in advantage over the plants with pure mating strategies, as they combine the possibility to cross their genes, while guaranteeing reproduction in the absence of pollinators or potential mates (Goodwillie et al. 2005). Still, the negative impacts of selfing, such as inbreeding depression, can detrimentally affect and drive the mating system (Barrett 1998). However, in our case this did not seem to have strong impacts, as the levels of inbreeding depression were low in the studied population, at least in the stages evaluated; nonetheless, it would be interesting to explore the occurrence of inbreeding depression in later stages and in populations varying in the frequency of selfing.

Pollen limitation and reproductive success in natural populations

Usually, flowering plants produce more flowers and ovules than fruits and seeds, and this is frequently associated with inadequate pollen receipt (Knight et al. 2005), due to quantity (pollen receipt is insufficient), or quality (pollen receipt has poor quality) deficiencies (Aizen and Harder 2007). When this occurs, a plant suffers from pollen limitation. Individuals of *S. sclareoides* with supplementary pollination had a higher reproductive success than plants with natural open pollination and, thus, *S. sclareoides* seems to suffer of some degree of quantitative pollen limitation (at least in some populations), and more importantly, limitation due to pollen quality. The later was revealed by a consistent pattern of lower seed weight after natural pollination and, more importantly, by significantly lower seed viability after natural pollination. At population level, the results of Poço das Casas and Santa Clara are consistent for all reproductive success measurements, while Lôgo de Deus population revealed a different scenario, as no viable seeds were obtained in both treatments. This result can be due to several factors: in this population, there is a high density of individuals in a very small area and thus, it can be expected that most individuals are siblings or genetically closely related, leading to crosses between closely related individuals, which may result in inbreeding depression in this population; also, this population can have limited resources for seed development due to plant density or soil properties (Navarro 1998). In the future, it will be very useful to study the effect of stigmatic clogging and pollen quality in seed viability, and to study the level of inbreeding depression and the genetic structure in these populations.

Besides pollen quantity and quality determined by pollen vectors, there is another interaction that may detrimentally affect the natural reproductive success of the plant, i.e., herbivores of sexual structures (McCall and Irwin 2006). Herbivory, and in particular the herbivory of sexual structures, is common in natural systems and can affect male and female plant fitness directly through organ consumption/damage (e.g., Oguro and Sakai 2009; Rodríguez-Rodríguez and Valido 2011) and/or indirectly through changes in species interactions (e.g., Krupnick et al. 1999; Cardel and Koptur 2010). In this study, we document damages to flowers and fruits of *S. sclareoides* that might have an impact in the reproductive success of this species. Poço das Casas was the most damaged population, and in an overall view, buds were the reproductive units that suffered the most; the sum of herbivory and seed viability results in presumably less than half of *S. sclareoides* individuals having the chance to leave progeny. Recent studies of the entomofauna of *S. sclareoides* habitats describe two phytophagous insects that destroy the seeds of this plant species [*Stegobium paniceum* L. and a new species of *Systole* (*Pseudosystole*); Lopes 2013]. Further studies are needed to understand the role of this antagonistic interaction for the dynamics of *S. sclareoides* populations.

Concluding remarks

Salvia sclareoides is an early flowering species with protandrous short-lived flowers. The inflorescences have several simultaneously open flowers and nectar evenly distributed along the inflorescence, a floral display associated with pollinator attraction. This species is a facultative xenogamous species, being able to produce offspring with self and outcross pollen, but relying on pollination vectors to mediate pollen transfer and significantly increase the reproductive success. Still, this species had some ability of spontaneous selfing, which might be selectively advantageous as a reproductive assurance mechanism. Xenogamous pollination seemed to have superior quality than self-pollen for progeny, although further studies should evaluate the impacts of this result in subsequent stages of the offspring development. This species has quantitative, but more importantly, qualitative limitation of pollination services. Also, reproductive structures were significantly damaged by herbivores and these losses might have a detrimental impact for reproductive fitness. These results provide useful information about the reproductive system of this species and open new research avenues, such as, to understand the factors involved in the quantitative and qualitative pollen limitation, including pollinator behavior and efficiency and

quality of the pollen delivered, and the contribution and consequences of selfing for the structure and dynamics of *S. sclareoides* populations.

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