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Do pollinators simultaneously select for inflorescence size and amount of floral scents? An experimental assessment on *Escallonia myrtoidea*

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Abstract Correlation among phenotypic traits may be explained by correlational selection, the simultaneous selection of more than one trait, or by genetic and/or developmental factors. In *Escallonia myrtoidea*, a tree with scented flowers from central Chile, inflorescence size and the amount of floral scents were positively correlated. Independent manipulation of scent and inflorescence size in a factorial design was used to assess the occurrence of pollinator-mediated correlational selection. Dependency on pollinators for seed set was also assessed. If pollinator-mediated correlational selection occurs, nonadditive effects of both traits are expected, albeit only when the effect of manipulating the state of such traits is disadvantageous with respect to the naturally occurring inflorescences, and provided that plants are not limited by pollinators for seed set and pollen export. *Escallonia myrtoidea* was very strongly pollinator-limited for seed set and pollen export. Pollinator-mediated additive effects were not observed in the frequency of visits by pollinators, pollen export, and seed set of *E. myrtoidea* after experiencing scent and inflorescence size manipulations. Consequently, there was no support for pollinator-mediated correlational selection between those traits, suggesting the prevalence of genetic and/or developmental factors.

Key words: floral scent, inflorescence size, pollination.

INTRODUCTION

The extent to which floral traits have been selected by pollinators is a central question in evolutionary ecology. Even though interest in this issue can be traced back to Darwin (1862), only recently has it been quantitatively assessed by determining the fitness of plants in relation to floral traits. An important feature determining the reproductive success of plants is inflorescence size. Factors described as favouring increased floral displays are: (i) inter and intraspecific competition for pollinators; (ii) potential for higher seed production; (iii) attraction of seed dispersers; and (iv) saturation of seed-eating predators (Geber 1985; Snow *et al*. 1996; and references therein). Nevertheless, a massive flower production may restrict the movement of pollinators among different individuals (Snow *et al*. 1996), and self-pollination may replace cross-pollination, and lead to higher inbreeding (Geber 1985; Snow *et al*. 1996). In self-incompatible plants, self-pollination may reduce female reproductive success by increasing the amount of self-incompatible pollen arriving at the stigmas, thus precluding fertilization of ovules with exogenous pollen (Snow *et al*. 1996). Self-pollination may also limit male reproductive success by wasting pollen in the same individual, which would otherwise be available for ovule siring in other plants in the neighbourhood (Snow *et al*. 1996).

Plants may increase their attractivity towards pollinators by increasing floral display or by increasing the production of floral scents. In fact, numerous ethological laboratory investigations indicate the importance of scents on pollinator behaviour and their likely effect on plant performance (Kunze & Gumbert 2001; Plepys 2002). Therefore, it is not surprising that intraspecific variation in floral scents influence the frequency of visits by pollinators in natural populations (Galen 1985; Galen *et al*. 1987; Odell *et al*. 1999; Miyake & Yafuso 2003).

The assessment of how scents interact with other traits, such as inflorescence size, still remains to be evaluated more accurately in natural populations. This omission is unfortunate because the interaction between chemical features and other plant traits may be particularly important for enticing pollinators because these usually respond to plant phenotype by associating numerous features displayed simultaneously, hence exerting correlational selection on

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those features (Meléndez-Ackerman *et al.* 1997; Gómez 2000; Kingsolver *et al.* 2001).

If inflorescence size correlates with the amount of scent, correlational selection, the simultaneous selection of more than one trait, exerted by pollinators on these traits might be suggested. However, a necessary condition for the occurrence of pollinator-mediated correlational selection is that plants are not be limited by pollinators for seed set and pollen export. If there is persistent limitation by pollinators through time due to ecological and/or evolutionary constraints, it would be more likely that genetic and/or developmental factors lead the expression of correlated traits. We describe herein studies on the interaction of floral display and scent production on the reproductive success of *Escallonia myrtoidea* (Escalloniaceae), an Andean tree from central Chile. A field experiment was performed based on the manipulation of floral scents and the number of flowers per inflorescence with a two-way factorial design. Then flowers were exposed to pollinators under natural field conditions, and reproductive success was assessed. If a close phenotypic correlation of these two floral traits occurs and correlational selection exerted by pollinators is indeed an explanation for the phenomenon, then we expected nonadditive effects of both traits on plant fitness, albeit only when the effect of manipulating the state of such traits is disadvantageous with respect to the naturally occurring flowers. In contrast, if there is no evidence supporting pollinator-mediated correlational selection, the most plausible explanation is that the correlation between floral scents and the number of flowers per inflorescence is mainly the consequence of genetic and/or developmental factors (Herrera 2001).

MATERIALS AND METHODS

Natural history and study site

Escallonia myrtoidea is a small tree that grows up to 6 m high that inhabits the Andes of central Chile from 30°S to 38°S and up to 2500 m a.s.l., mainly associated with humid habitats such as ravine bottoms, polar-facing slopes, and areas near river banks (Rodríguez *et al.* 1983). It bears hermaphroditic flowers which produce an average of 90 300 pollen grains, and 240 ovules per flower (Uslar 1982). Flowering and fruiting occur from December to March, during the austral summer, while seed dispersal starts in April (Rodríguez *et al.* 1983). The study was conducted at the Yerba Loca Natural Sanctuary (33°10′–33°22′S, 70°13′–70°24′W), where vegetation consists of sub-Andean mountain scrubland and high-Andean plant communities (Uslar 1982).

Assessment of pollinator limitations for seed set

In order to determine pollinator dependency and pollinator limitations for seed set on *E. myrtoidea*, four pollination trials were conducted plus a control for natural pollination (Kearns & Inouye 1993). Each treatment was performed on 50 flowers, made up of 7–8 flowers from each of seven inflorescences, with each inflorescence from a different tree. To test whether *E. myrtoidea* can set fruit in the absence of pollen (i.e. agamospermy), an apomixis test was performed in emasculated flower buds. These were bagged until seed dispersal to prevent any flower–pollinator interaction. Similarly, to test whether *E. myrtoidea* can set fruit after receiving pollen from the same flower without pollinators (i.e. autogamy), an automatic self-pollination test was performed by bagging nonemasculated flower buds until seed dispersal. To test whether *E. myrtoidea* can set fruit with pollen of the same individual carried by pollinators (i.e. geitonogamy), flower buds were previously emasculated, then hand pollinated with endogenous pollen at the time of stigmatic receptivity, and then bagged. To test whether *E. myrtoidea* can set fruit with pollen from other individuals (i.e. xenogamy), emasculated flower buds were hand cross-pollinated with exogenous pollen at the time of stigmatic receptivity. Results of these treatments were recorded by counting the number of seeds produced per crossed flower when the ovaries were ripe, prior to fruit dehiscence. The degree of self-incompatibility was determined through the index of self-incompatibility (ISI), the proportion of fruits produced per crossed flower in hand self-pollinated flowers and hand cross-pollinated flowers. ISI values range from 0 (self-incompatibility) to 1 (self-compatibility) (Ruiz-Zapata & Arroyo 1978). Pairwise comparisons between treatments were performed by applying a one-way ANOVA followed by Tukey HSD tests for balanced data. Analyses were performed with Statistica software package v. 6.0.

Analysis of floral scents

Floral volatiles were collected during three periods in the morning: 09.00–10.30 hours, 10.30–12.00 hours, 12.00–13.30 hours. Inflorescences were gathered and placed inside a glass jar provided with an inlet and an outlet. At the inlet, a compressed air cylinder containing synthetic air made from extra pure oxygen and nitrogen, with no detectable organic impurities, was attached through a regulator which controlled the air flow. At the outlet, a column was attached which contained Porapak Q (30 mg). Volatile entrainment (30 min with air flow of 250 mL min^{-1}) commenced immediately after the inflorescences had been severed from the plants. The volatiles adsorbed on the Porapak

Q were eluted with 1 mL of diethylether. These extracts were analysed in a GC-MS (gas chromatograph: Hewlett-Packard model HP5891; mass spectrometric detector with integrated data system: Hewlett-Packard model HP5972) with an Ultra 2 (25 m × 0.2 mm id) capillary column. Ionization by electron impact (70 eV) was carried out at 280°C. The GC oven was programmed to remain at 50°C for 10 min, to increase up to 280°C at a rate of 5°C min^{-1}, and then to remain at 280°C for 45 min. The identification of compounds in the chromatographic profiles was achieved by comparison of their mass spectra with a library database using a reverse search technique, which verified that main peaks in the reference spectrum were present in the unknown spectrum (Pesyna *et al.* 1976). Spectra were considered coincident if the similarity index was higher than 95% (Pesyna *et al.* 1976). Preliminary identifications were confirmed by coinjection of standards and comparison of retention times and mass spectra. GC peaks of coinjected compounds were considered coincident if retention times did not differ by more than ±0.03 min

Assessment of pollinator-mediated correlational selection

The mean number of flowers per inflorescence was determined to be 22.5 ± 12.5 (mean ± 0.5 SD, $n = 80$). We used 35 and 10 flowers as high and low flower treatments, corresponding to the mean number of flowers plus or minus 0.5 standard deviations. Fifteen individual trees of *E. myrtoidea* were randomly selected. In each tree, four inflorescences bearing only flower buds were randomly selected. These inflorescences were prepared for the four different treatments. For treatments which manipulated the number of flowers (I treatments), two inflorescences were randomly pruned leaving only a cohort of 10 fairly developed buds, for treatments which manipulated flower scents (S treatments), two inflorescences were randomly pruned leaving a cohort of 10 fairly developed buds, and another cohort with six very immature buds, which eventually served as a natural receptacle for injecting the experimental scent solution.

The manipulation of the number of flowers and of scents were factorially combined to conform the following four treatments:

1 Control (–S–I), bearing only 10 flower buds.
2 Inflorescence treatment (–S+I), in which inflorescences bore the cohort of 10 fairly developed buds and 25 added paper flowers.
3 Scent treatment (+S–I), in which inflorescences bore the cohort of 10 fairly developed buds, and the cohort of six immature buds.
4 Inflorescence and scent treatment (+S+I), in which inflorescences bore the cohort of 10 fairly developed buds, 25 added paper flowers, and the cohort of six immature buds.

Inflorescences in each experimental treatment were enclosed in a tulle-mesh bag until the cohorts of 10 fairly developed buds had become sexually mature flowers. At this point, the cohorts of six immature buds had not yet developed into flowers (and were not yet producing scents). The inflorescences were then uncovered, and scent extracts were injected into the immature buds of the two +S treatments. Injections (10 μL of volatile extract, whose concentration was adjusted to correspond to the floral scent produced by 25 flowers) were performed at the onset of the following three periods: 09.00–10.30 hours, 10.30–12.00 hours, and 12.00–13.30 hours, by injecting the corresponding volatile extracts collected during these periods (Table 1).

To check the role of the artificial flowers in attracting pollinators, a pilot experiment was performed to compare the frequency of foraging approaches in a radius of 5 cm surrounding inflorescences containing artificial flowers and natural inflorescences bearing the same number of open flowers. No difference was detected between the two types of inflorescences (Mann–Whitney Test: $U = 185.5$, $P = 0.69$, $n = 20$ inflorescences per treatment, observations performed during 20 periods of 10 min at the time of highest pollinator activity).

The frequency of visits by pollinators to the 60 experimental inflorescences was recorded during the two-day life span of the flower cohorts, between 8.00 and 14.00 hours. Each inflorescence was observed twice daily for 15 min each time. The average of the four records generated a single estimate of the frequency of visits by floral visitors per inflorescence. A visit was counted when floral visitors touched the reproductive structures of flowers. Flowers were bagged from 14.00 to 8.00 hours, so there was a narrow window for interaction with pollinators, only at the peak time of pollinator activity (Valdivia & Niemeyer, pers. obs., 2004).

At the end of the two-day period, five of the 10 senescent flowers were excised from the experimental inflorescences to evaluate the number of pollen grains exported from the anthers (i.e. proximal fitness evaluation of the male function), and five were left in the inflorescence and bagged in order to assess seed production (i.e. realized female function).

To assess the number of pollen grains exported from the flowers in the first group of five flowers, pollen grains remaining in the anthers were first counted. The anthers (five per flower) were mechanically destroyed in FAA solution (formaldehyde : acetic acid : ethanol, 90:5:5) where they were stored in order to release all pollen grains adhered to the walls of anthers. Then the solvent was evaporated at 60°C and the remaining anther tissues were suspended in 5 mL of lactophenol

Table 1. Volatile compounds produced by *Escallonia myrtoidea* during the periods of highest pollinator activity

Compounds	Kovats index	Relative amount (%) at the period 09.00–10.30 hours	Relative amount (%) at the period 10.30–12.00 hours	Relative amount (%) at the period 12.00–13.30 hours
Unknown	869	1.4	4	22.6
Unknown	876	nd	nd	2.8
iso-Amyl-acetate	882	14.6	15.5	35.7
Nonane	900	nd	nd	1.8
Amyl-acetate	918	nd	2.9	nd
Camphene	954	nd	3.4	0.6
Benzaldehyde	963	nd	3.3	nd
Sabinene	976	nd	3.1	nd
3-Hexen-1-yl acetate	1006	2.3	4.3	1.6
Hexyl acetate	1013	nd	4.5	1.2
Limonene	1032	5.9	0.9	1.3
(*E*)-Ocimene	1051	1.6	9.2	13.8
Lilac aldehyde B	1145	40.6	14.9	1.2
Lilac aldehyde C	1153	21.0	6.2	1.4
Lilac aldehyde D	1168	11.1	3.5	nd
Copaene	1385	nd	3.4	nd
Caryophyllene	1433	1.5	16.5	16.0
δ-Cadinene	1533	nd	4.4	nd

nd, not detected.

Table 2. Tests to determine pollinator dependency and pollinator limitations for seed set in *Escallonia myrtoidea* (see *Materials and Methods* for description of tests)

Pollination trial	Seed set (number of seeds/exposed flower)
Agamospermy test	19.7 ± 4.4
Autogamy test	23.5 ± 5.5
Geitonogamy test	8.8 ± 1.6
Natural pollination test	22.8 ± 6.2
Xenogamy test	176.7 ± 8.0

(lactic acid : glycerine : H_2O : phenol, 1:1:1:1) containing methylene blue to improve the pollen suspension homogeneity (Kearns & Inouye 1993). Pollen from each flower suspended in lactophenol was counted in six aliquots using a haemacitometer. The average of the six counts generated a single estimate of the number of remaining pollen grains per flower. In addition to the experimental flowers, five other flower buds were collected from each study tree at the end of the experimental period. These buds were used to determine the mean number of pollen grains produced per flower for each experimental tree, using the method described above. An estimation of the number of pollen grains removed by pollinators was obtained as the difference between the mean number of pollen grains produced by any flower and the number of pollen grains remaining in the experimental flowers, on each tree.

To assess seed production, when fruits of the second group of five flowers were ripe, they were excised and the number of seeds per flower exposed to each treatment was determined.

Analyses were performed by using ANCOVA with Type III SS error because this procedure is appropriate for testing hypotheses in the presence of interactions. The identity of each tree was considered as a covariable because treatments were all performed in each tree. All analyses were performed with Statistica software package v. 6.0.

RESULTS

Pollinator limitations for seed set

Seed set in *E. myrtoidea* differed significantly depending on the origin of pollen as well as on the presence of a pollen vector (ANOVA: $F_{4,245} = 157.3$, $P < 0.001$). Seed set in flowers with anthers excised and excluded from pollinators (i.e. agamospermy test) was not significantly different from seed set in flowers exposed to natural pollination (Tukey HSD test: $P = 0.996$) (Table 2). Similarly, seed set in flowers excluded from pollinators but having their respective anthers (i.e. autogamy test) was similar to seed set in hand-crossed flowers with pollen of the same individual (i.e. geitonogamy test), and in flowers exposed to natural pollination ($P = 0.999$ and 0.526, respectively, Table 2). However, seed set in flowers hand-crossed with exogenous pollen (i.e. xenogamy test) was 7.8 times higher

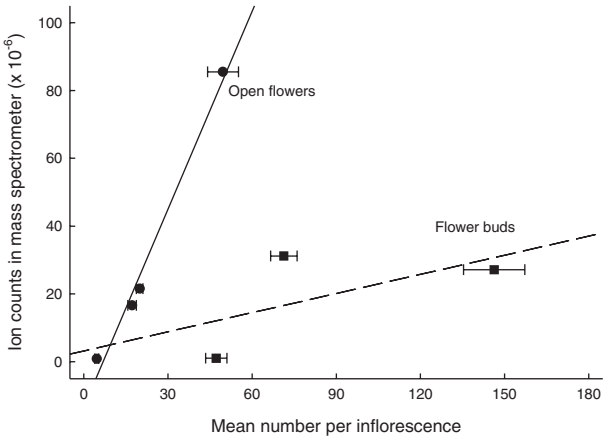

Fig. 1. Relation between floral scents and inflorescence size in *Escallonia myrtoidea*. Seven groups of 10 inflorescences each were harvested. Four groups contained different numbers of open flowers and flower buds, and three groups contained different numbers of flower buds only. Volatiles from each group of inflorescences were entrained during 30 min with air at a flow of 250 mL min^{-1}, between 10.00 and 12.00 hours, and the corresponding extracts were prepared and analysed as described in Materials and Methods. Bars represent standard errors. The line for open flowers was corrected for the presence of buds by using the correlation line for flower buds.

than seed set attained by natural pollination ($P < 0.001$, Table 2). The index of self-incompatibility (ISI) was 0.05 (Ruiz-Zapata & Arroyo 1978), thus showing that *E. myrtoidea* is a self-incompatible tree strongly limited by pollinators and therefore with few possibilities of facing any pollinator-mediated selection pressure.

Inflorescence size and amount of scent

The amount of floral scent was positively correlated with the number of flowers per inflorescence, although not with the number of flower buds (Fig. 1; regression analysis: $y = -13.23 + 1.94^{\star}x$, $F_{1,2} = 137.22$, $P = 0.007$ for floral scent and mean number of open flowers; $y = 3.18 + 1.19^{\star}x$, $F_{1,1} = 0.54$, $P = 0.596$ for floral scent and mean number of buds; where x is the ion counts in the mass detector and y is the mean number of flower structures). In addition, the mean number of flowers per inflorescence differed significantly among individuals (one-way ANOVA: $F_{1,11} = 57.08$, $P < 0.01$; $n = 10$ inflorescences per individual, 12 individuals). Therefore, there was interindividual variability in inflorescence size and in the amount of floral scents of *E. myrtoidea*.

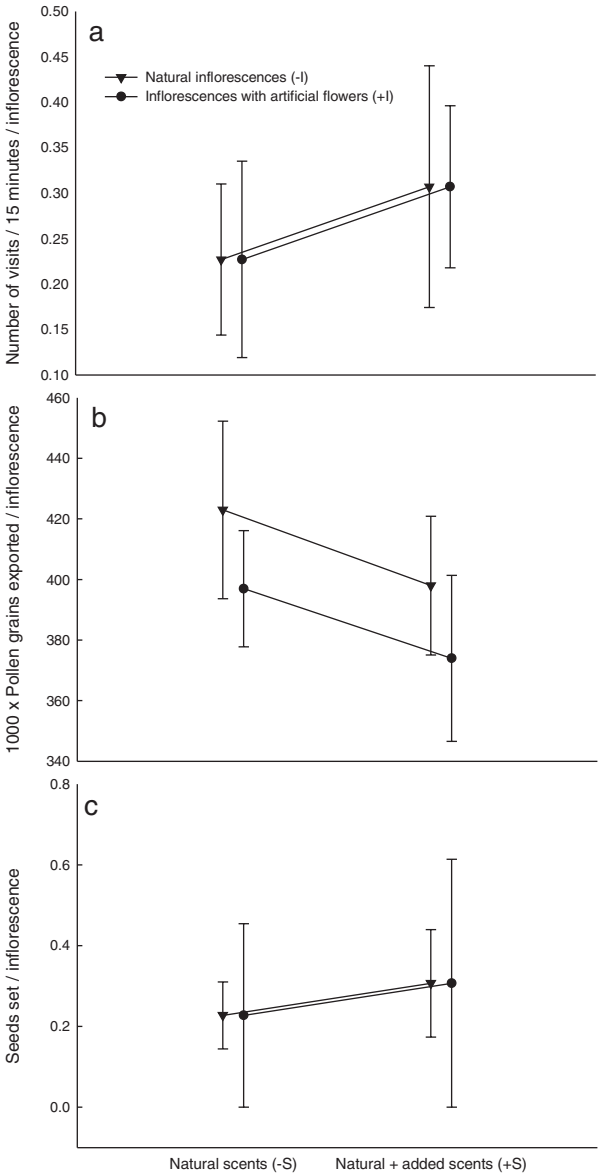

Fig. 2. Effects of inflorescence and scent sizes of *Escallonia myrtoidea* on: (a) frequency of visits by pollinators; (b) pollen export; and (c) seed production. For easier reading, the *X*-axis for the scent treatments have been slightly displaced.

Frequency of visits by floral visitors

Only diptera and hymenoptera were found visiting the flowers. Table 3a shows that there was no effect of either number of flowers or amount of floral scent on the number of visits (Fig. 2a).

Pollen export and seed set

There was no effect of either number of flowers or amount of floral scent on pollen export (Table 3b, Fig. 2a) or on seed set (Table 3c, Fig. 2c).

Table 3. Summary of ANCOVAs testing the effects of the amount of scents and the inflorescence size on (a) frequency of visits by floral visitors; (b) pollen exported from flowers (i.e. male function estimated); and (c) seed set per flower (i.e. female function realized). The individual tree bearing the experimental treatments was considered as covariable

	d.f.	MS	F	P
(a) Frequency of visits by floral visitors				
Tree	1	0.026	0.165	0.686
Inflorescence	1	0.192	1.221	0.274
Scent	1	0.016	0.106	0.746
Inflorescence x Scent	1	0.016	0.106	0.746
Error	55	0.158		
(b) Pollen export				
Tree	1	$5.48*10^{9}$	0.580	0.449
Inflorescence	1	$9.21*10^{9}$	0.975	0.328
Scent	1	$8.48*10^{9}$	0.897	0.348
Inflorescence x Scent	1	$9.81*10^{9}$	0.001	0.974
Error	55	$5.20*10^{11}$		
(c) Seed set				
Tree	1	11107	0.396	0.532
Inflorescence	1	60674	2.166	0.147
Scent	1	8	<0.001	0.987
Inflorescence x Scent	1	960	0.034	0.854
Error	55	28012		

DISCUSSION

Flower traits are often assumed to be under a strong selection exerted by pollinators which usually respond to plant phenotype by associating numerous features displayed simultaneously (Meléndez-Ackerman *et al.* 1997; Gómez 2000). In spite of the linear correlation found between inflorescence size and scent, significant pollinator-mediated nonadditive effects in the female reproductive success of *E. myrtoidea* after experiencing scent and inflorescence size manipulations, were not observed. These findings suggest that genetic and/or developmental factors rather than correlational selection determine the correlated expression of floral scents and inflorescence size.

Genetic control of floral scents is fairly well known (Dudareva & Pichersky 2000). Similarly, the genetic control of inflorescence size is not uncommon (Doust & Kellogg 2002; Kellogg 2004). However, numerous discrepancies have emerged with regard to the relative importance of female and male reproductive success in the evolution of inflorescence size (Fishbein & Venable 1996). While some authors claim that both the female and male functions are equally important for the evolution of inflorescence size (Shannon & Wyatt 1986; Broyles & Wyatt 1990), others claim that inflorescence size is under the strongest selective pressures through the male function (i.e. pollen export for ovule siring) rather than female function (i.e. seed production) (Fishbein & Venable 1996). Beyond the discrepancies about the relative importance of the female and male functions, both theoretical models (Schoen & Dubuc 1990) and empirical assessments (Fishbein & Venable 1996) have pointed that small- and medium-sized inflorescences may be favoured over large ones in a variety of ways. In *E. myrtoidea*, however, manipulation of inflorescence size had no effect on either female function or male function, and the range of inflorescence manipulation seemed not to be important for the frequency of visits by pollinators, and consequently for the reproductive success of plants.

It is worthwhile noting that during the assessment of pollinator dependency and pollinator limitations for seed set, it was found that flowers borne by a wide range of inflorescence sizes and exposed to natural pollination, did not differ in seed set with respect to flowers in the geitonogamy test, in contrast with seed set attained in the hand cross-pollination test (xenogamy test). These facts suggest that *E. myrtoidea* was strongly pollinator-limited for seed setting when the present study was conducted (*sensu* Bierzychudek 1981; but see Zimmerman & Pyke 1988), and that most likely no manipulation of inflorescence size and amount of scents would have achieved a significant change in seed set and pollen export. In this regard, selection of floral traits depends on variations in the abundance of pollinators at temporal and spatial scales, hence rendering it difficult to assess net selection on reproductive traits within a single season and site (Schemske & Horvitz 1989; Eckhart 1991; Herrera 2001).

In the present case, there is no reason to suspect a constant pollinator assemblage across years considering the great variability of rainfall, and hence in plant and pollinator abundance, among years in the

Mediterranean Andean mountains (Hajek & di Castri 1975). However, when this study was conducted, rainfall was not a limiting factor for growth. Hence, limitations by pollinators for seed set might seemingly be permanent across years, thereby leading us to conclude that genetic and/or developmental factors are the most likely explanation for the correlated expression of volatile compounds and the amount of floral scents. This is most likely due to ecological and/or evolutionary constraints, given the increasing body of work reporting that variations in flower or inflorescence traits are sometimes inconsequential for the reproductive success of plants (see Herrera 2001 for a comment). From the evolutionary point of view, it is differences in the reproductive success between individuals, rather than between inflorescences, that are important. Therefore, results of this study, based on between-inflorescence comparisons, need to be corroborated in a between-tree context before rigorous evolutionary interpretations can be offered.

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