

Deconstructing a floral phenotype: do pollinators select for corolla integration in *Lavandula latifolia*?

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Abstract

An experimental field study was undertaken to assess whether correlational selection generated by pollinators can account for observed phenotypic integration of the two corolla lips in the zygomorphic flowers of the insect-pollinated Mediterranean shrub, *Lavandula latifolia* (Labiatae). The experiment consisted of manipulating the upper and lower corolla lips according to a two-way factorial design with interaction, and involved the complete (lower lip) or extensive (distal two thirds of upper lip) excision of one or the two lips. Per-flower male (proportion of pollen grains removed) and female (number of pollen tubes down the style) pollination success, and maternal fecundity (seed set), of experimental flowers were evaluated after exposure to natural pollination in the field. Artificially induced corolla variation had no significant effects on either male or female pollination success, and two aberrant floral morphologies, characterized by lack of corolla integration, had a maternal fecundity advantage over normal two-lipped phenotypes. These results do not support a predominant role of correlational selection by pollinators in maintaining corolla integration in *L. latifolia*, but are instead consistent with the alternative view that integration is mainly the consequence of genetic and/or developmental factors.

Introduction

Natural selection has the capacity of modifying not only the mean (directional selection) and the variance (disruptive or stabilizing selection) of metric phenotypic traits, but also their covariances (Lande & Arnold, 1983; Phillips & Arnold, 1989; Brodie *et al.*, 1995). Although most discussions on the correlated evolution of traits have traditionally focused on its genetic (e.g. pleiotropy, linkage) and developmental causes (e.g. Grant, 1975; Lande, 1980; Falconer, 1989), covariance between traits can also be the adaptive product of correlational selection, arising when some particular combination of traits is favoured at the expense of other combinations (Endler, 1986, 1995). It has even been suggested that correlational selection on sets of characters may turn out to be

the most common method of operation of natural selection (Schluter & Nychka, 1994).

Correlational selection, and thus selection-mediated phenotypic integration, should be expected to involve most frequently traits whose functional effectiveness is closely tied to their ability to work together, and whose successful interaction is essential for the fitness of the individual bearing them. Floral characters of animal-pollinated plants clearly belong in this category because, as emphasized by Stebbins (1974, p. 50), for successful animal pollination '... the flower must become a highly integrated structure, with all of its parts precisely adjusted to one another'. As floral parts are functionally linked and fitness is determined by the interaction of two or more of them, pollinators may selectively modify the correlation between functionally linked floral traits by means of correlational selection (Armbruster, 1991; Armbruster & Schwaegerle, 1996; Armbruster *et al.*, 1999). This idea already lay at the core of Berg's (1959, 1960) 'correlation pleiades' hypothesis stating that, due

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to selection from pollinators, floral characters should be phenotypically less variable and exhibit greater integration (i.e. correlated variation) among themselves than vegetative ones. Berg's hypothesis has been examined, and partly validated, by a number of studies (e.g. Conner & Via, 1993; Waitt & Levin, 1993; Conner & Sterling, 1995, 1996; Armbruster *et al.*, 1999).

In recent years, many studies have measured selection by pollinators on quantitative floral traits by evaluating the fitness correlates of variation in floral traits, either occurring naturally or induced experimentally (e.g. Schemske & Horvitz, 1989; Campbell *et al.*, 1991; Herrera, 1993; Wilson, 1995a; Conner *et al.*, 1996; Conner & Rush, 1997). Nevertheless, although the theoretical basis and analytical tools necessary for the study of correlational selection have been around for some time (Lande & Arnold, 1983; Phillips & Arnold, 1989), only a few recent studies have directly examined correlational selection involving floral traits (O'Connell & Johnston, 1998; Caruso, 2000; Gómez, 2000; Maad, 2000). These investigations did not always obtain clear evidence of correlational selection on morphometric floral traits, possibly because they focused on the relatively narrow range of phenotypic variance of floral traits occurring naturally. Manipulative experiments artificially enhancing floral variability and designed specifically to test for the effects of trait interactions on pollination success may prove critical to assess whether adaptive processes (i.e. pollinator-mediated correlational selection on sets of traits), rather than purely genetic (linkage, pleiotropy) and developmental associations among characters, are ultimately responsible for the close integration of floral parts commonly observed in animal-pollinated plants.

This paper presents the results of an experimental field study aimed at elucidating whether selection from pollinators can account for integration of major corolla parts in the insect-pollinated shrub *Lavandula latifolia* Med. (Labiatae). Flowers of *L. latifolia* are zygomorphic, with the five corolla lobes arranged into two unequal lips. The upper lip is longer and stands in an upright position, while the lower lip is shorter and generally reflexed (Fig. 1). A field experiment was performed based on the simultaneous manipulation of the upper and lower lips according to a two-way factorial design with interaction. Manipulations involved the complete (lower lip) or extensive (upper lip) excision of one or the two lips. Flowers were exposed to pollinators under natural field conditions, and their reproductive success evaluated subsequently. Whether correlational selection exerted by pollinators is a plausible explanation for the close phenotypic correlation of these two corolla parts will be assessed by considering the interaction effect of manipulating the upper and lower lips on per-flower reproductive success. Correlational selection would be rejected as the main agent responsible for maintaining integration of corolla lips if either (1) the pollination consequences of manipulating one corolla lip were

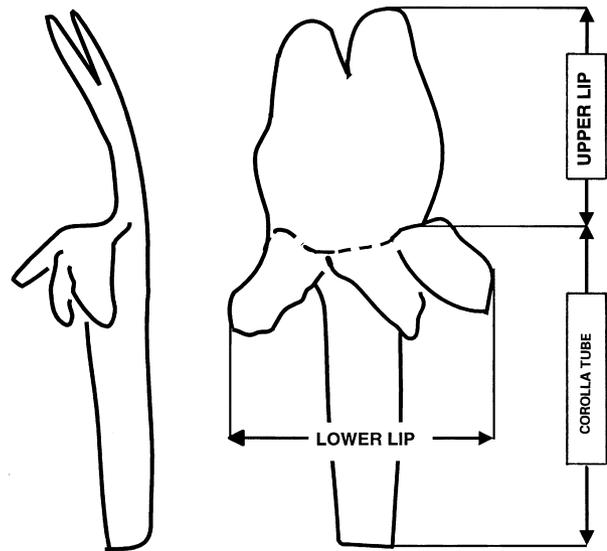


Fig. 1 Schematic diagram of a *Lavandula latifolia* corolla detached from the calyx, in side (left) and front (right) views, showing the three morphometric traits considered.

independent of the character state (manipulated or unmanipulated) of the other lip, i.e. no significant interaction effect between upper and lower lip manipulations; or (2) a significant interaction existed between the effects of upper and lower lip manipulations, yet the sign of the interaction indicated some disadvantage of naturally occurring phenotypes relative to artificial, comparatively non-integrated phenotypes. Alternatively, correlational selection would be supported as the main factor maintaining integration of corolla lips if a significant interaction effect existed whereby the naturally occurring corollas had a distinct advantage over artificially generated, non-integrated combinations of upper and lower lip lengths.

Materials and methods

Plant reproductive biology

Lavandula latifolia is a low evergreen shrub (up to 35 cm high) producing long-stalked (up to 1.25 m high) inflorescences in early summer. It is common in the understory of open mixed woodlands on limestone-derived soils at middle elevations in the eastern and south-eastern Iberian Peninsula (Suárez-Cervera & Seoane-Camba, 1986). The composition of the pollinator assemblage, the relation of the plant with pollinators, and other relevant aspects of its reproductive biology have been described in detail elsewhere (Herrera, 1987, 1988, 1989, 1991, 1995, 2000). In the Sierra de Cazorla study region (see below), *L. latifolia* flowers are pollinated by a diverse insect assemblage comprising nearly 80 bee, fly and butterfly

species. Flowering takes place in July–September. Flowers are hermaphroditic, protandrous, have pale-blue tubular corollas (tube length 7–8 mm), and are produced over a short (3–6 cm) terminal portion of the stalks in a dichasium-like arrangement (see Herrera, 2000, Plate 1, for photographs). Within individual inflorescences, flowers open at a slow rate and each flower lasts for 1.5–2.5 days. Most often there are only 2–4 flowers simultaneously open in each inflorescence. Flowers are self-compatible, but spontaneous autogamy occurs very infrequently due to protandry and, principally, to the spatial separation of anthers and stigma. In the absence of pollinators, <4% of flowers set fruit. Outcross pollination results in greater proportion of flowers setting fruit, and greater proportion of ovules yielding seeds per fruit, relative to self-pollination. Flowers have four ovules, each potentially producing an independent nutlet.

Study sites and experimental design

This study was carried out in the Parque Natural de Cazorla-Segura-Las Villas (Jaén province, south-eastern Spain). The experimental investigation was carried out during July–September 2000 at a *L. latifolia* population located around the Arroyo Aguaderillos, in the Reserva de Navahondona-Guahornillos, at 1160 m elevation. Quantitative data on corolla morphology were also obtained there, and from one additional population at Cruz de Quique (1275 m elevation, 8.8 km from Aguaderillos). About 20 female-stage flowers were collected from each of 20–25 plants at each population, and preserved in formaldehyde-acetic acid-ethyl alcohol (FAA) until measurement. Measurements were taken on corollas detached from the calyx under a dissecting microscope fitted with an ocular micrometer, and included length of the upper lip, width of the lower lip, and length of the corolla tube (Fig. 1). Preliminary comparisons revealed no noticeable differences in either absolute values or character correlations between corolla measurements taken on fresh and FAA-preserved flowers (C. M. Herrera, unpublished data).

Ten widely spaced plants were selected at the Aguaderillos population in late July 2000, and 10 inflorescences marked on each of them. For 2 weeks, all marked inflorescences were checked twice a day for new flowers, early in the morning and late in the afternoon. On each occasion, all new opening flowers were individually marked on the calyx using distinct combinations of small dots of indian ink, and randomly assigned to one of four experimental categories (see below). A total of 610 flowers were marked. Flower longevity was determined for all marked flowers to account for any possible effect of experimental treatments on length of exposure to pollinators.

Corolla manipulations of experimental flowers were conducted according to a complete two-way factorial design, with ‘Upper corolla lip’ and ‘Lower corolla lip’

being the two factors involved (‘UpperLip’ and ‘LowerLip’ hereafter). Two levels were used for each factor, namely Control (no manipulation) and Treatment (manipulation). In the case of UpperLip, the manipulation involved excising the distal two thirds, while for LowerLip the manipulation consisted of excising the whole lip. The four floral morphologies resulting from the combination of these two two-level factors are illustrated in Fig. 2. Corolla manipulations were done with fine scissors under magnifying glasses, and were always performed when the flower was found opening for the first time, most often at a stage when corolla lips were not completely unfolded and pollinators had not had access to the interior of the corolla. Due to the asynchronous flower opening and withering within individual inflorescences, it was impractical to keep perfectly balanced combinations of flower treatments in each inflorescence throughout the experimental period. To account for any possible effect of slight variations in the size of the floral display, a continuous record was kept of the total number of flowers open in each experimental inflorescence.

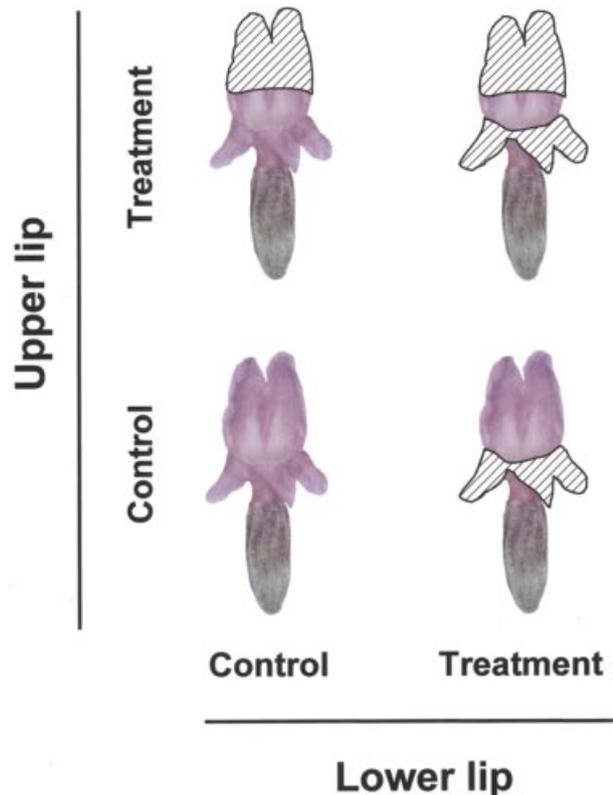


Fig. 2 Front view of a *Lavandula latifolia* flower illustrating the four corolla morphologies resulting from the factorial combination of the UpperLip and LowerLip two-level (control and treatment) experimental treatments. Hatched areas denote the part(s) of the corolla excised in each case.

Measuring reproductive success

The effect of corolla manipulation on per-flower reproductive success was assessed in terms of male (proportion of pollen grains removed) and female (number of pollen tubes in the style) pollination success, and in terms of realized female fecundity (seed set). Information on these three parameters could be obtained simultaneously for individual experimental flowers because of certain peculiarities of *L. latifolia* flowers. The end of the flower's functional life is marked by the contraction of the distal portion of the calyx, which clasps tightly around the middle of the corolla tube. Immediately afterwards, the corolla withers and quickly dries up, persisting firmly attached to the calyx until the maturation of the achenes, 4–6 weeks later. The two corolla lips generally rolled inwards as they dried up, and formed a plug-like structure that kept both the dry style and the anther remains (and thus presumably any remaining pollen grains) inside the tube of the persistent corolla until fruit maturation.

The corollas from two-thirds of experimental flowers were collected immediately after withering and placed singly in microcentrifuge tubes with 1 mL of 70% ethanol. The remaining third of withered corollas was collected in early September, when plants were checked again to determine seed set (see below). Splitting corolla collection into two separate batches was a safety strategy designed to reduce losses due to predation on developing seeds and to avoid some potential biases. Removal of the withered corolla immediately after anthesis drastically raises fruit infestation rate by the developing larvae of a Cecydomiid fly (from 3% up to 35% in 2000; C. M. Herrera, personal observation), which is a predator of developing seeds. Had all the withered corollas been collected immediately after flowering, artificially increased seed predation on experimental fruits would have considerably reduced the number of experimental flowers eventually maturing seeds. On the other hand, had the collection of all corollas been delayed until fruit maturation in September, pollen tube and pollen removal figures based on this late-collected material would have been biased by the early abscission of unpollinated or aborted flowers.

Martin's (1959) epifluorescence method was used to reveal pollen tubes in the styles from experimental flowers. Styles were kept at 65 °C for 20 min in 0.5 N NaOH for softening, rinsed in distilled water, and stained for 20 min at 65 °C in decolorized aniline blue. The number of pollen tubes reaching the lower third of the style was then counted under a fluorescence microscope. Anther remains were separated from the corolla and thoroughly crushed with a small plastic pestle in the 1-mL volume of ethanol in which the corolla had been stored since collection in the field. The internal surface of the corolla was also gently scraped into the same ethanol volume to liberate any adhered pollen. The

microcentrifuge tube was then vortexed for 30 s, its contents diluted in 75 mL of Isoton II[®] electrolyte solution, and rinsed with a further 0.5 mL of the same diluent. Four 1-mL aliquots were extracted and the number of pollen grains counted using a Beckman Coulter Z2 particle counter fitted with a 100- μ m-aperture tube. Particle size counting range was set to between 25 and 35 μ m, which encompassed the range of *L. latifolia* pollen grain diameter occurring in the study population. The four counts from each flower were averaged to obtain a single estimate of the number of pollen grains remaining. In addition to the withered corollas of experimental flowers, between 10 and 20 full-sized, closed flowers with undehisced anthers were also collected from each study plant at the end of the experimental period. These flowers were used to determine the mean number of pollen grains produced per flower for each experimental plant, using the same methods as with withered corollas from experimental flowers. From these figures, an average number of pollen grains produced per flower was obtained for each individual plant. An estimate of pollen removal for each experimental flower was obtained as the difference between its left-over grains and the estimated initial grains figure specific to the individual plant it belonged to.

Seed production by experimental flowers was determined in early September, when seeds had completed development but had not yet started to disperse. The number of enclosed ripe seeds was determined for all marked calices that persisted on the marked inflorescences. On the basis of prior knowledge on the species' reproductive biology, I assigned zero seeds to all missing calyces, as these were attributable to abscised fruits. Two experimental plants had all their developing fruits removed by harvester ants (*Messor capitatus*) before maturation. All seed production data were lost for these two plants, which were excluded from the corresponding analyses.

Statistical analyses

Three response variables were considered to assess the effects of corolla manipulations on reproductive performance of individual flowers: number of pollen tubes down the style, proportion of initial pollen grains removed, and seed set (=number of seeds eventually matured per flower). Significance of effects was tested by fitting generalized linear mixed models to the data (see, e.g. Herrera, 2000, for applications of this method in the context of plant reproduction studies). Computations were performed with SAS Macro program GLIMMIX (Version 25 September 1998 for SAS/STAT Version 8; available on the World Wide Web at www.sas.com/techsup/download/stat). This program iteratively calls SAS Procedure MIXED, which implements a generalization of the standard linear model which allows for proper incorporation of random effects (Littell *et al.*, 1996; SAS

Institute, 1996a, b). The distribution of pollen tubes down the style was modelled using a Poisson distribution of errors and logarithms as the link function. Proportion of pollen grains removed and seed set were both modelled as binomial responses, with logits used as the link function. Flowers of *L. latifolia* have a fixed complement of four ovules, which justifies modelling the number of seeds matured per flower as a binomial response variable (i.e. the probability of one ovule yielding a mature seed). The two experimental factors considered here (UpperLip and LowerLip) and their interaction were included as fixed effects in the models. To account statistically for possible individual differences among experimental plants in reproductive performance parameters and in their response to experimental treatments, plant identity was included as a random effect in the model, along with the two-way interactions Plant \times UpperLip and Plant \times LowerLip. As this study focuses on the consequences of corolla manipulations, covariance parameter estimates and significance tests associated with random effects in models will not be considered.

Results

Floral integration

The three morphometric corolla traits considered were positively and significantly correlated at the two populations, both when correlations were computed for flowers from all plants combined and when correlations were computed using plant means (Table 1). Irrespective of the method used to compute correlations, length of the upper lip and width of the lower lip were the two variables most closely correlated, and the correlation pattern between corolla traits was similar at the two populations. These results thus indicate that, of the three descriptive corolla traits considered, upper and lower

corolla lips are those consistently exhibiting the closest phenotypic integration.

Reproductive performance of individual flowers

Pollinators removed an average (± 1 SD) of $52.8 \pm 14.3\%$ of initial pollen grains from experimental flowers ($N = 484$ flowers; all treatments and plants combined), but there was considerable variability among flowers with regard to this magnitude, which ranged between 3.6 and 95.1% (mostly falling in the interval 40–70%; Fig. 3A).

The mean number of pollen tubes reaching the lower third of the style was 13.7 ± 6.6 ($N = 488$ flowers; all treatments and plants combined), and the range was 0–38 tubes. The vast majority of experimental flowers (97.8%) had at least one pollen tube in the basal portion of the style, with most flowers having between nine and 18 pollen tubes (Fig. 3B). Keeping in mind that *L. latifolia* flowers have four ovules, these figures indicate that the pollen tube/ovule ratio ranged between 0.25 and 9.5 in experimental flowers, most often falling in the range 2.0–4.5.

Flowers set an average of 0.95 ± 0.89 seeds ($N = 477$ flowers; all treatments and plants combined), and about two-thirds of experimental flowers eventually produced at least one seed (60.6%; Fig. 3C). Among these, variability in the number of seeds matured per fruit was small, with most fruits maturing either one or two seeds (Fig. 3C).

Treatment effects

There was no evidence that experimental manipulations of the corolla had any effect on either the male or female components of pollination success (Table 2). Neither the proportion of pollen grains removed nor the number of pollen tubes in the lower third of the style were significantly affected by the extensive reduction of the upper lip or the complete excision of the lower lip of the corolla. The UpperLip–LowerLip interaction effects on the proportion of pollen grains removed and the number of pollen tubes were likewise statistically non-significant (Table 2).

There was no significant main effect of either UpperLip or LowerLip on seed set (Table 2). Their interaction, however, was statistically significant, thus indicating that the effect of the manipulation of one corolla lip on seed set depended on the treatment level of the other lip (Fig. 4). To investigate this possibility further, I tested the statistical significance of simple main effects, or the effects of a given factor at different treatment levels of the other factor (Pedhazur, 1982, pp. 362–365). Among flowers with unmanipulated upper lips, the excision of the lower lip resulted in a marginally significant enhancement of mean seed set relative to control ones (from 0.83 to 1.01 seeds/flower, Fig. 4; $F_{1,449} = 2.78$, $P = 0.096$). A similar

Table 1 Pearson correlation coefficients (± 1 SE, obtained by bootstrapping) between three metric floral traits at two *Lavandula latifolia* populations. In each matrix, values above the diagonal correspond to correlations based on individual flowers, and values under the diagonal correspond to correlations based on individual plant means. All coefficients are statistically significant ($P < 0.01$ or better in all cases). Correlations between the two corolla lips are highlighted.

	Upper lip length	Lower lip width	Corolla tube length
A. Cruz de Quique ($N = 398$ flowers from 20 plants)			
Upper lip length	–	0.664 \pm 0.030	0.301 \pm 0.044
Lower lip width	0.802 \pm 0.092	–	0.484 \pm 0.040
Corolla tube length	0.380 \pm 0.165	0.538 \pm 0.148	–
B. Aguaderillos ($N = 465$ flowers from 25 plants)			
Upper lip length	–	0.532 \pm 0.033	0.208 \pm 0.052
Lower lip width	0.768 \pm 0.087	–	0.357 \pm 0.046
Corolla tube length	0.562 \pm 0.152	0.495 \pm 0.170	–

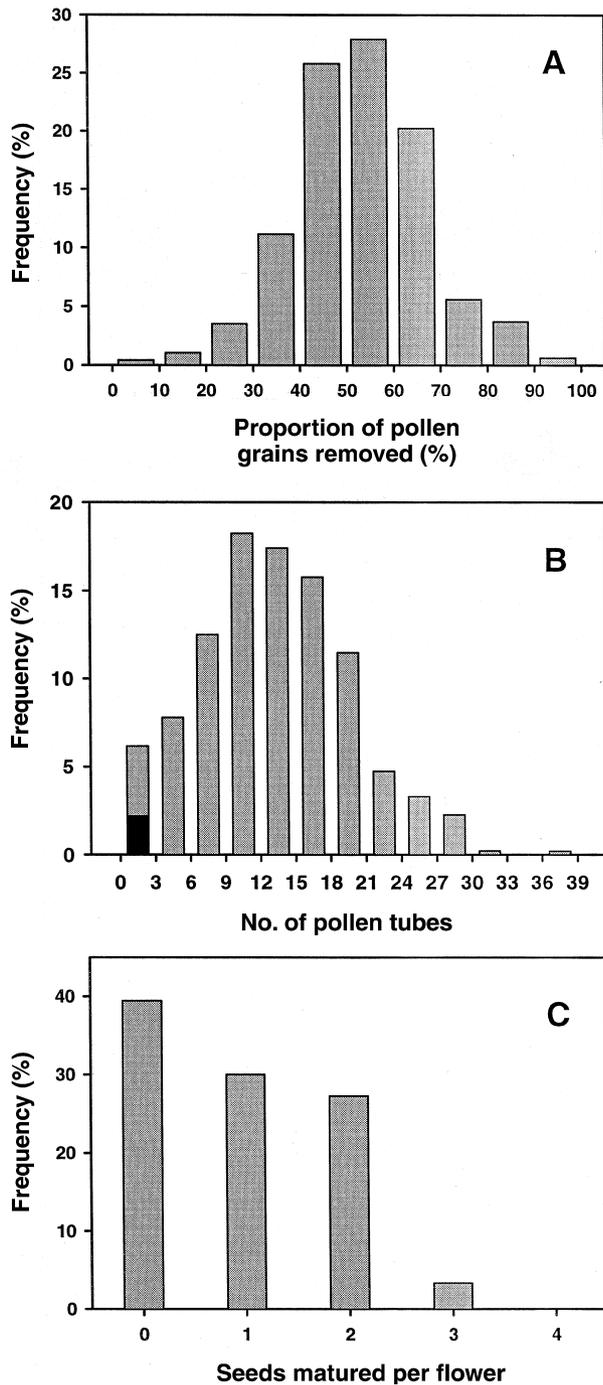


Fig. 3 Frequency distributions of (A) proportion of pollen grains removed ($N = 484$ flowers), (B) number of pollen tubes reaching the basal portion of the style ($N = 488$) and (C) number of mature seeds eventually produced per flower ($N = 477$), in experimental *Lavandula latifolia* flowers, all plants and treatments combined. In graph B, the filled portion in the leftmost bar represents the fraction of styles in the 0–3 bin with zero pollen tubes.

Table 2 Significance of the effect of factorial manipulation of the upper and lower corolla lips on the male and female components of reproductive performance of *Lavandula latifolia* flowers. Generalized linear mixed models were fitted using different error distributions and link functions for the different parameters (see Material and Methods: Statistical analyses).

Response variable	Effect in model	Significance test		
		d.f.	<i>F</i>	<i>P</i>
Proportion of pollen grains removed	Upper lip (UL)	1,9	1.51	0.25
	Lower lip (LL)	1,9	0.21	0.66
	UL \times LL	1,453	0.75	0.39
Number of pollen tubes	Upper lip	1,9	0.02	0.89
	Lower lip	1,9	0.64	0.44
	UL \times LL	1,457	1.20	0.27
Seed set*	Upper lip	1,8	0.31	0.59
	Lower lip	1,8	0.04	0.84
	UL \times LL	1,449	4.72	0.025

*Number of seeds eventually matured per flower.

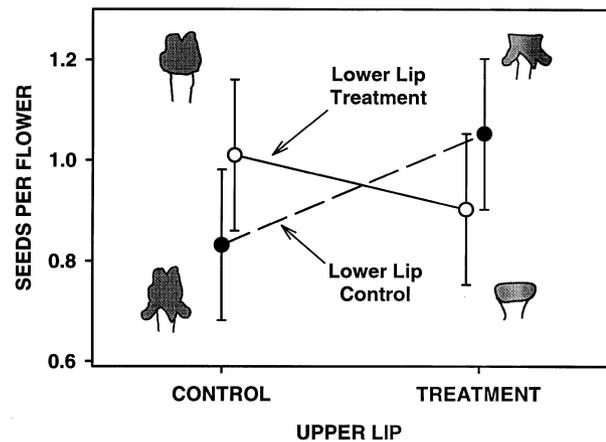


Fig. 4 Interaction graph depicting the statistically significant UpperLip–LowerLip interaction effect on the number of seeds per flower ('seed set' in Table 2). Dots represent model-adjusted class means, and vertical segments extend over ± 1 SE. The effect of the excision of a given corolla lip was dependent on the character state of the other lip. For flowers with unmanipulated upper lips, excision of the lower lip resulted in increased seed set. For flowers with excised upper lips, in contrast, excision of the lower lip resulted in decreased seed set.

situation occurred among flowers with unmanipulated lower lips, for which the shortening of the upper lip resulted also in a marginally significant enhancement of seed set (from 0.83 to 1.05 seeds/flower, Fig. 4; $F_{1,449} = 3.31$, $P = 0.069$).

Each of the statistical analyses summarized in Table 2 was repeated twice: (1) removing from the model all fixed–fixed and fixed–random interaction terms; and (2) adding to the model as a covariate the total number of

open flowers in the inflorescence of the focal flower at the time of marking. Results of these additional analyses were similar to those shown in Table 2, and are omitted.

Mean longevity of experimental flowers (± 1 SD) was 45 ± 17 h ($N = 610$ flowers). A linear mixed model was fitted to the flower longevity data having UpperLip, LowerLip, and their interaction as fixed effects, and plant identity and its interaction with UpperLip and LowerLip as random effects. Neither UpperLip ($F_{1,9} = 0.01$, $P = 0.95$), LowerLip ($F_{1,9} = 0.31$, $P = 0.59$), nor their interaction ($F_{1,576} = 1.53$, $P = 0.22$) had statistically significant effects on flower longevity.

Discussion

Upper lip length and lower lip width were the two corolla traits exhibiting the closest phenotypic integration at the two *L. latifolia* populations studied. Results of experimental corolla manipulations, however, are not consistent with the interpretation that the correlated variation of these two traits is the result of correlational selection exerted by pollinators. Manipulations modified drastically the relative proportions of the upper and lower corolla lips, yet these changes had no significant effects in terms of either male or female components of pollination success. Furthermore, the direction of the significant UpperLip–LowerLip interaction effect on realized maternal fecundity was opposite to that expected if correlational selection were responsible for observed integration. Experimentally de-integrated flowers with two aberrant morphologies tended to set more seeds per fruit than control flowers with normal two-lipped corollas. The potential significance of these results and possible interpretations are discussed in the following sections.

Pollination success

Proportional pollen removal and number of pollen tubes are valid descriptors of per-flower male and female pollination success in *L. latifolia*. Measuring realized male reproductive success in hermaphroditic plants requires tracking seed paternity using genetic markers (Snow, 1989; Wilson *et al.*, 1994; Ashman, 1998), yet pollen removal on a per flower basis is a useful comparative index to evaluate the male component of pollination success, as it provides an upper estimate of the number of pollen grains that become available for siring seeds (Young & Stanton, 1990; Wilson & Thomson, 1991; Conner *et al.*, 1995; Wilson, 1995a; J. Herrera, 1997). Using number of pollen tubes, rather than of pollen grains on the stigma, as a measurement of the female component of pollination success was motivated by the presumption that pollen grain counts on the dried stigmas of withered flowers could be unreliable, as an unknown fraction of pollen grains could have been dislodged from the stigma as it dried up. In the study site, the number of pollen tubes in the basal portion of the

style of female-stage *L. latifolia* flowers is closely correlated with the total number of pollen grains deposited on the stigma (C. M. Herrera, unpublished data; a similar relationship was reported by J. Herrera, 1997, for the congeneric *Lavandula stoechas*), hence observed variation among flowers in number of pollen tubes will closely reflect variation in pollen deposition.

Manipulations of corolla morphology performed in this study could have influenced the male and female pollination success of *L. latifolia* flowers through at least three different mechanisms. Firstly, experimental petal cuttings might have influenced pollinator foraging behaviour directly, regardless of the changes induced in corolla morphology. For example, petal cutting could have either enhanced or reduced the release of floral volatiles, which could in turn influence pollinator foraging. I do not have any data bearing directly on this possibility, but some indirect evidence tends to rule it out. If corolla cutting modified pollinator foraging through its effect on volatile emission, I would expect a significant decline in treatment effect from flowers with the two lips manipulated to those with only one lip cut, which was not observed (tested using *a posteriori* contrasts in generalized linear mixed model; results not shown).

The other two ways would be related to the induced changes in corolla morphology, and thus directly relevant to the objectives of this investigation. Manipulations of corolla morphology may modify the attractiveness of individual flowers to pollinators and, consequently, influencing the number of pollinator visits received over the entire life of the flower. All treatments reduced overall corolla size, which was expected to result in decreased pollinator attractiveness, as found in other insect-pollinated plants (e.g. Bell, 1985; Stanton & Preston, 1988; Schemske & Ågren, 1995; Conner & Rush, 1996; Ashman *et al.*, 2000; Philip & Hansen, 2000). Furthermore, manipulations disrupted heavily the shape of flowers in front view (i.e. as perceived by an approaching pollinator) in comparison to normal flowers, which could have also affected attractiveness to visitors. I did not measure pollinator visitation rates for this study, but the lack of any significant effect of corolla manipulation on *both* pollen removal and deposition suggests that the number of effective pollinator visits received by individual flowers over their lifespan was unaffected by experimental modifications of the corolla. Another mechanism would involve modifications in the amount of pollen removed and/or deposited per pollinator visit, which could have occurred even if corolla manipulation had no effect on overall pollinator attraction (Campbell *et al.*, 1991; Smith *et al.*, 1996; Temeles & Rankin, 2000). No data are available to evaluate directly this second possibility, but if corolla manipulation actually affected mean pollinating effectiveness per visit, the effect was too weak to translate into measurable differences in per-flower pollen removal and deposition.

It seems safe to conclude that, under the conditions of this study, variations in the morphology of the corolla were inconsequential for both male and female pollination success of individual *L. latifolia* flowers. One possible explanation for this finding could be that, during the study period, pollinators were so abundant in relation to the number of flowers available that they foraged indiscriminately over all floral phenotypes and included in their foraging bouts flowers with unpreferred corolla morphologies to obtain sufficient nectar or pollen returns. This would lead to pollinator visits becoming so frequent as to 'saturate' the pollen removal and deposition functions irrespective of floral phenotype (Wilson, 1995b). Some indirect evidence partly supports this view. For the vast majority of flowers, the number of pollen tubes was well in excess of the number of ovules, thus suggesting an effective saturation of the female function. For the male function, results are more difficult to interpret in relation to the saturation hypothesis. On one side, removal of pollen grains was far from complete, as only 53% of pollen grains were removed on average. This proportion is considerably lower than the 80–90% pollen removal reported for bee-pollinated flowers in general (Mitchell & Waser, 1992; Wilson, 1995a, b), and also lower than the 82% reported by J. Herrera (1987) for the congeneric, bee-pollinated *Lavandula stoechas*. These observations would contraindicate male function saturation of *L. latifolia* flowers in this study. But, on the other side, one would expect that mean number of undispersed pollen grains per flower will approach different asymptotic values depending on the specific composition of pollinators and their thoroughness at removing pollen. Casual observations on experimental plants during this study indicated that, in accordance with previous studies in the same locality (e.g. Herrera, 1987, 1988, 2000), flowers were visited by a diverse insect assemblage composed not only of pollen-gathering female bees, but also of nectar-feeding butterflies and male solitary bees that only passively remove pollen. The modest pollen removal figures found in this study might still reflect a situation of male saturation for the particular specific configuration of pollinators prevailing during the study period.

Maternal fecundity

Seed production per *L. latifolia* flower is generally insensitive to artificial increases in the quantity of pollen reaching the stigmas, but it does respond positively to experimental increases in resource availability and the quality (cross vs. self) of the pollen (Herrera, 1987, 1990, 1991, 2000). Results of the present study also suggest that failure of flowers to produce some seed was very rarely due to insufficient pollination. Flowers that eventually failed to set seed ($N = 147$) had a mean (± 1 SD) of 12.6 ± 7.3 pollen tubes in the style, and only 12% of them had pollen tubes/ovules ratios < 1 .

The only statistically significant effect of corolla manipulation found in this study was the interaction of UpperLip and LowerLip on seed set. Flowers that had either the upper lip alone or the lower lip alone manipulated tended to set more seeds than 'normal' unmanipulated flowers. This result cannot be related to differences between floral morphologies in pollen deposition, as there was no significant effect of UpperLip, LowerLip or their interaction on number of pollen tubes. Differences among floral morphologies in the quality of the pollen received may account for the observed results. In *L. latifolia*, even slight differences in pollinator composition may result in seed set differences, presumably because of variations in the proportion of selfed vs. outcrossed pollinations performed by different species (Herrera, 2000). The different pollinators of a given plant species may respond differentially to corolla variations (Johnson *et al.*, 1995; Thompson, 2001). Consequently, the seed set advantage of floral 'morphs' with either the upper or the lower lips manipulated found in this study may be due to these flowers being visited proportionally more often by species predominantly performing cross pollinations, like butterflies. Testing this hypothesis will require direct observations of pollinator foraging responses to floral manipulations. Preliminary data, however, suggest that this is a plausible possibility, as butterflies tend to select preferentially *L. latifolia* shrubs characterized by relatively longer upper lips and smaller lower lips (C. M. Herrera, unpublished data).

Floral integration

The experimental design used in this study was based on the premise that the two lips of the corolla played different roles in the pollination of *L. latifolia* flowers. While the conspicuous, vertical upper lip presumably acts enhancing insect attraction, the lower lip serves as a landing platform and foothold to pollinating insects (Herrera, 2000, Plate 1), thus probably affecting their foraging efficiency and flower handling times. The attractive role of the upper lip of Labiatae flowers was long ago demonstrated experimentally by Clements & Long (1923) for the insect-pollinated *Monarda fistulosa*. The influence of the lower lip of Labiatae flowers on pollinator handling time and pollen deposition and removal has been documented, for example, by Temeles & Rankin (2000) for the hummingbird-pollinated *Monarda didyma*. These observations thus lend biological sense to the experimental dissection of the effects of upper and lower lips attempted in this study, and provide biological and statistical justifications for questions about their possible interactions.

Results of this study are clearly contrary to the interpretation that correlated variation of upper and lower lips in *L. latifolia* corollas is the result of correlational selection exerted by pollinators. Artificial enhancement of morphological variation and the

phenotypic de-integration of the two lips was inconsequential in terms of pollination success. Even more importantly, two aberrant floral morphologies characterized by strong de-integration relative to the normal phenotype had a distinct realized maternal fecundity advantage over normal, two-lipped corollas. These results suggest that pleiotropic and/or developmental effects are possibly more important than pollinator selection in maintaining the phenotypic integration of the upper and lower corolla lips in *L. latifolia*. This interpretation is not at odds with what is known on the genetic basis of corolla morphology and relative size of corolla parts in other zygomorphic flowers. In *Antirrhinum majus* and *Linaria vulgaris*, single regulatory genes (*cycloidea* and *Lcyc*, respectively) are responsible for the zygomorphism characteristic of these species, which results from the arrangement of the five corolla lobes into two unequal, bilaterally symmetric parts (Coen & Nugent, 1994; Luo *et al.*, 1995; Cubas *et al.*, 1999). Mutations affecting these genes produce drastic transformations from zygomorphic corollas with two unequal lips to radially symmetrical ones with five similarly sized lobes. This suggests that, in these species, the relative dimensions of corolla parts (lips in the case of wild-type zygomorphs, lobes in the case of mutant actinomorpha) are most likely subject to strong pleiotropic effects.

Two aspects must be kept in mind when interpreting the results of this study. Firstly, the ability to detect selection on floral traits may be strongly dependent on variations in the abundance of pollinators, as emphasized by Wilson (1995b). I have censused *L. latifolia* pollinators at the study locality every year in the period 1982–1987 (Herrera, 1988), in 1991 (Herrera, 2000), and again during 1996–1998 (C. M. Herrera, unpublished data), thus spanning a 17-year period. These long-term data reveal that abundance and composition of the pollinators of *L. latifolia* fluctuate from year to year at the study locality, hence the likelihood of detecting selection may likewise vary. Some results shown here may therefore be contingent on pollinators being very abundant during the study year, as noted earlier. Were this the case, however, it must be emphasized that there are reasons to suspect that similar results would be obtained in other years as well, and are probably not unusual for the *L. latifolia* population studied. Although I did not conduct pollinator censuses during this study, my subjective impression in the field was that pollinator abundance in 2000 was not above the average levels for the period 1982–1998. A second aspect to be considered is that results of this investigation might be dependent on aspects of the experimental design used. From an evolutionary viewpoint, it is differences between individual plants in reproductive success, not between individual flowers, that matters. Results of this study, based on between-flower comparisons, need to be corroborated in a between-plant context before rigorous evolutionary inferences can be done. In short, it is not

possible to know if the irrelevance of corolla morphology for the pollination success of *L. latifolia* flowers found in this study would persist in years with fewer pollinators, or in populations where pollinators are consistently scarcer than in the Aguaderillos site (e.g. populations on arid slopes, located well away from permanent streams; Herrera, 1988), and/or if whole plants, rather than single flowers, were the experimental units chosen. Future studies will consider these two possibilities.

Closing remarks

Results reported in this paper join a growing list of manipulative experiments showing that variations in flower or inflorescence traits are sometimes inconsequential for male and/or female pollination success (e.g. Méndez & Obeso, 1992; Andersson, 1994; Wilson, 1994, 1995a; Johnson *et al.*, 1995; Wilson & Thomson, 1996; J. Herrera, 1997; Lamborn & Ollerton, 2000). No formal analysis has been so far conducted to determine whether the relative frequencies of 'positive' and 'negative' results in the literature on pollinator selection on floral traits is affected by publication biases and selective reporting (i.e. the statistical significance of an experimental outcome influencing its likelihood of being reported or published). Information from other fields of research, however, leads one to suspect that studies finding no significant effects of floral manipulations on pollination success are less likely to be reported than those finding significant effects (Palmer, 1999, and references therein). Detailed reporting of predominantly negative results (e.g. Wilson, 1995a; present study) will prove essential for the reliability of future meta-analyses on the frequency and significance of pollinator selection on floral phenotypic traits.

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