

# Pollinator-mediated selection and experimental manipulation of the flower phenotype in *Chloraea blettioides*

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## Summary

1. While many studies of flower evolution have relied on the assessment of selection coefficients, these estimates represent correlative evidence of selection. Few studies have combined correlative with experimental evidence, and hence the mechanisms underlying selection processes are not well understood.

2. In this study we quantified the magnitude, direction and form of pollinator-mediated selection acting upon morphological traits in the Chilean endemic orchid *Chloraea blettioides*. Results revealed strong selection differentials for plant height and flower number, and absence of total selection for morphological flower characters. Multivariate analysis of selection revealed a strong and positive directional selection gradient for flower number.

3. The labellar lobe and flower tube were under negative correlational selection, indicating that selection promoted alternative combinations of traits. Plants with long floral tubes and small labellar lobes, and short floral tubes with large labellar lobes were selected for. Interestingly, selection on flower number increased at a higher rate through the female function than through the male function, which suggests that size-dependent resource limitation may be more important than pollinator-mediated selection for fruit set.

4. In addition to estimates of phenotypic selection, we evaluated through phenotype modification the impact of pollinators on the correlation and potential integration of flower traits coming from different whorls. We removed the tip of the lateral sepals and the labellar lobe in a  $2 \times 2$  factorial design.

5. Results revealed that phenotypic modification had no effect on male and female fitness. The absence of effect was consistent with the low and non-significant selection coefficients. Nevertheless, the observation of significant correlational selection for labellar lobe and flower tube suggests that experimental studies performed on these characters may be of interest to evaluate the functional and adaptive integration of flower design in orchids.

**Key-words:** Chile, correlational selection, fitness, floral display, flower evolution, male and female success, phenotypic selection, pollination, pollinia, sex function

## Introduction

Many studies have documented significant pollinator-mediated selection on quantitative floral traits, hence confirming that pollinators are an important selective force that moulds the evolution of the flower phenotype (see reviews in Herrera, Castellanos & Medrano 2006; Harder & Johnson 2009). Directional and stabilizing/disruptive selection are the most common selection functions for flower characters described in the literature (e.g. Galen 1989; Campbell *et al.* 1991;

Conner, Rush & Jenetten 1996; Maad 2000; Medel, Botton-Mahan & Kalin-Arroyo 2003). Despite the availability of analytical tools for almost 30 years (Lande & Arnold 1983; Mitchell-Olds & Shaw 1987; Phillips & Arnold 1989), the prevalence of correlational selection is still a source of debate (Kingsolver *et al.* 2001). Even though correlational selection has been suggested as the most common type of natural selection (Schluter & Nychka 1994), only few studies have directly examined this type of selection on floral traits (e.g. Caruso 2000; Gómez 2000; Herrera 2001), and their results did not provide clear evidence of its occurrence in morphometric floral traits. The absence of significant

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correlational selection probably results from the relatively narrow range of the natural phenotypic variance in floral traits that is often tested (Herrera 2001), or because sample size is insufficient for reliably testing the significance of the cross-product selection estimates in quadratic selection models involving many phenotypic traits.

One way to circumvent the methodological restrictions consists of performing manipulative experiments on the flower phenotype that change the floral variation observed within populations. This procedure provides a complementary assessment of flower adaptation to the phenotypic selection analysis (Campbell 2009), and represents an additional source of evidence for flower function. For example, Herrera (2001) performed a manipulative experiment of the flower phenotype by increasing the floral variability of *Lavandula latifolia* (Labiatae), concluding that artificial variation of the corolla did not significantly influence male or female pollination success. These results did not support the hypothesis that pollinator-mediated correlational selection contributes to maintain the current level of corolla integration in *L. latifolia* (Herrera 2001). However, several studies measuring the impact of experimentally-induced variation of floral traits upon fitness have suggested a reduction in reproductive success after flower trait modification, hence suggesting an adaptive value for the floral traits under experimentation (e.g. Inoue 1986; Nilsson 1988; Andersson 1994; Herrera 2001; Malo, Leirana-Alcocer & Parra-Tabla 2001; Castellanos, Wilson & Thomson 2004; Boberg & Ågren 2009).

The Orchidaceae are one of the most diverse plant families, with c. 19 500 species (Dressler 1993). Most orchid species rely on insects for pollination and like other angiosperms they provide rewards to their pollinators (e.g. nectar, pollen) (Nilsson 1992); nevertheless, Ackerman (1986) estimated that one-third of the Orchidaceae – that is, between 6500 and 10 000 species – are deceptive in some way. Generalized food deception and sexual deception are the most common fraud mechanisms, reported in 38 and 18 genera, respectively (Jersáková, Johnson & Kindlmann 2006). Orchids have evolved complex and elaborate floral architectures, both those which reward and those which defraud their pollinators (e.g. Darwin 1862; Dressler 1990; Nilsson 1992). Even though Darwin considered orchids as the best group on which to study floral evolution because of their ‘endless variation’ of floral contrivances for sexual reproduction, few examples of natural selection in orchids exist at present (Tremblay *et al.* 2005).

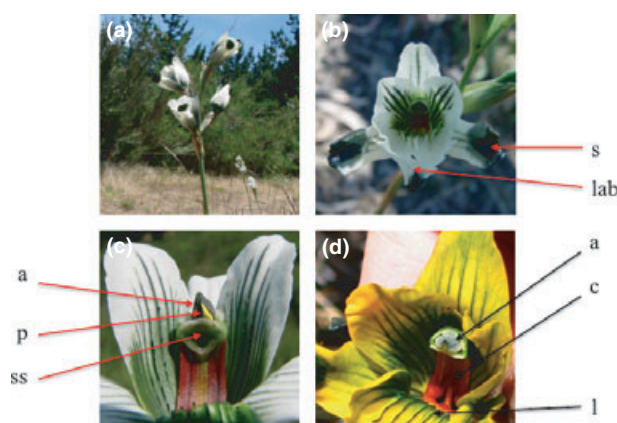
In this study, we examined the strength, sign, and shape of pollinator-mediated selection on floral and vegetative traits under natural conditions, and performed a manipulative experiment to assess the extent to which flower morphology influences the reproductive success of *Chloraea blettioides*. We contrasted the results from both approaches to evaluate the consistency of correlative and experimental evidence for pollinator-mediated selection. Our manipulation focused on floral traits that belong to different whorls: sepals that belong to the calyx and the labellum that is part of the corolla. As these floral structures probably work together in the process of attracting pollinators, we would expect them to be subject to

correlational selection. Therefore, the aims of this study are: (i) to evaluate pollinator-mediated selection on floral traits using male and female fitness components; (ii) and to explore the consistency of measurements of correlational selection using correlative and experimental approaches to the selection process.

## Materials and methods

### NATURAL HISTORY

*Chloraea blettioides* Lindl. is an endemic terrestrial orchid of Chile (Lehnebach 2003) (Fig. 1); it is found from c. 32° S to 38° S (Humaña, Cisternas & Valdivia 2008). The flowering season occurs from September to December; the pauciflorous inflorescence usually produces 1–5 large flowers (Fig. 1a), but sometimes individuals are observed with up to 10 flowers. Flowers are white with green apices and veins (Fig. 1b). The dorsal sepal is rounded and usually obtuse with a green spot at the tip. The lateral sepals narrow toward the dentate apex, which has a showy dark green spot that covers c. 30% of each sepal. The labellum (Fig. 1b) is shortly unguiculate, trilobed. The apex has a crest and a dark green spot. Green whole lamellae are present on the adaxial surface of the labellum probably, serving as guides for pollinators (Fig. 1b and d). The flowers of this plant have two pollinia (pollen in agglutinated masses). The column (androecium, style and stigmas fused) of *C. blettioides* is red and has two nectar channels at its base (Fig. 1c and d). Despite the showy spots, the conspicuous lamellae on the labellum, and the nectar channels of flowers, this orchid – as other species of its genus does not produce nectar. Thus, it is possible that this species is pollinated by deceit. A study of pollination performed on five taxa of the genus *Chloraea*, including *C. blettioides*, showed that these species are self-compatible, but none of them produced fruits through apomixis or autogamy (Humaña, Cisternas & Valdivia 2008). These results indicate that pollen and pollinators appear to be essential for reproduction in this genus. The insect visitors previously described in populations of *C. blettioides* are: the beetle *Arctodium* sp. (Coleoptera), the bee *Centris nigririma* (Hymenoptera), the bumblebee *Bombus terrestris* (Hymenoptera), ants of the genus *Camponotus* and *Solenopsis*



**Fig. 1.** *Chloraea blettioides* Lindl. in the study site: (a) Inflorescence of *C. blettioides*. (b) Flower parts: (s) lateral sepals, (lab) labellum. (c) Column detail: a pollinium (p) is observed within the anther (a), (ss) stigmatic surface. (d) *Chloraea blettioides* Lindl. var. *lutea*, (a) anther, (c) column, and (l) lamellar structures on the labellum.

(Hymenoptera), among others (Humaña, Cisternas & Valdivia 2008). Although all of these species were observed visiting flowers in our population, only *Centris nigerrima* was detected pollinating or with pollinia attached to its body.

*Chloraea bletioides*, a species that does not reproduce by autogamy and requires pollinators for its reproduction, is a suitable plant to perform studies of pollinator-mediated selection. Moreover, in this species (as in most orchids) both sexual components can be easily estimated: as pollen is transported as total packages by pollinators, male reproductive success can be evaluated through observations of pollinia removal. The correlation between pollinia removal and donation/paternity in orchids and other plants with pollinia (Broyles & Wyatt 1990; Nilsson, Rabakonandrianina & Pettersson 1992) suggests that this proxy is an appropriate estimator of siring success. In turn, female reproductive success can be evaluated as the number of fruits produced per plant (e.g. O'Connell & Johnston 1998; Maad 2000).

#### STUDY SITE AND FIELD PROCEDURES

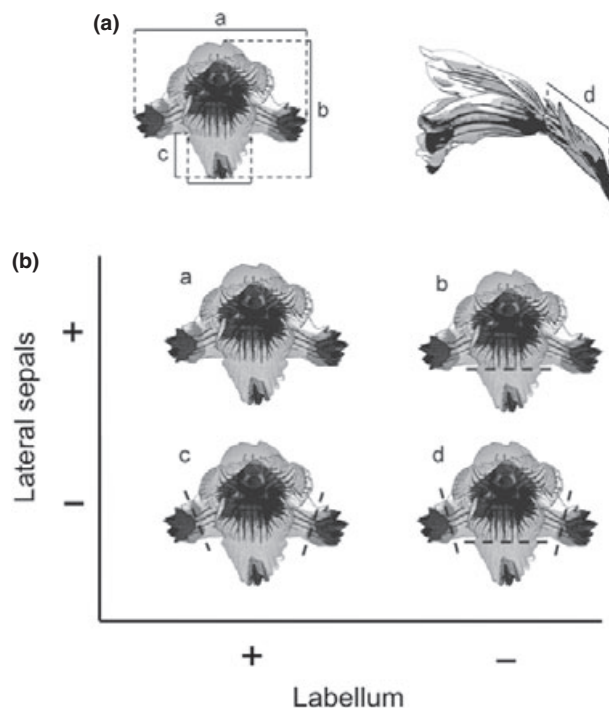
This study was conducted from December 2005 to March 2006, near Algarrobo, V Region (33°24'S, 71°38'W, elevation 101 m, in an area  $\approx 8000 \text{ m}^2$ ) in central Chile. Several endemic orchid species considered as scarce or rare grow in this site, including *Chloraea cristata*, *C. heteroglossa*, *C. Multiflora* and *Gavilea* sp. (personal observation). Plants of *C. bletioides* are usually found under pine trees (*Pinus radiata*), together with plants of *Chusquea cumingii* (Poaceae) and *Eryngium paniculatum* (Apiaceae).

We tagged 140 plants with closed flower buds of *C. bletioides*. Two buds were selected at random on each individual: one bud was used to take measurements of floral traits once it was completely open and the other bud was used in the manipulative experiment, modifying it according to the allocated treatment. To assess the importance of floral traits on reproductive success and hence the effect of selection on certain combinations of traits, several quantitative measures were made on 72 plants without altering the flower (one flower per plant). Furthermore, to evaluate the importance of structures belonging to different whorls on the reproductive success, a manipulative experiment was conducted on 124 flowers (one flower per plant) under a  $2 \times 2$  factorial design, modifying the lateral sepals and/or the labellar lobe (area of the central lobe of labellum) (see below). In *C. bletioides* flowers within plants exhibit small variation in height (mean coefficient of variation within plants (CV) = 8.8%), width (CV = 5.8%), labellar lobe length (CV = 7.0%), labellar lobe width (CV = 6.1%), and tube length (CV = 6.3%). Because of this, morphometric measurements were taken on one flower per plant assuming they represent the average flower for the whole individual.

We performed separate analyses for male and female sex function by considering the number of pollinia removed (0, 1 or 2) as an indicator of male reproductive success and fruit production (0 or 1) as an indicator of female reproductive success. For the selection analysis, the total number of pollinia removed and of fruits produced was counted in each plant ( $n = 72$ ). Unlike selection analysis, in the manipulative experiment pollinia removal and fruit production were recorded only in the experimental flowers.

#### ANALYSIS OF PHENOTYPIC SELECTION

To assess pollinator-mediated selection on floral traits, we recorded flower morphometric traits using a digital calliper (precision 0.01 mm). The following potentially explanatory morphological mea-



**Fig. 2.** (a) Floral traits measured in *Chloraea bletioides* ( $n = 72$  plants): a, flower width; b, flower height; c, area of the labellar lobe (length  $\times$  width of the central lobe of the labellum); d, floral tube length. In addition to these floral traits, we recorded for each plant the flower number and plant height. (b) Frontal view of a flower of *Chloraea bletioides*, showing the four floral experimental morphologies according to the factorial design with presence (+) and absence (-) of lateral sepals and labellar lobe. Plants ( $n = 124$ ) were assigned randomly to treatments. Final treatments consisted of: a, + area of the labellar lobe, + sepal tips (control) ( $n = 39$ ); b, - labellar lobe, + sepal tips ( $n = 26$ ); c, + labellar lobe, - sepal tips ( $n = 29$ ); d, - labellar lobe, -sepal tips ( $n = 30$ ).

surements were made ( $n = 72$  plants) (Fig. 2a): Flower width from a frontal view (measure of the length from tip to tip between the two lateral sepals), flower height from a frontal view (length between the tip of the labellum and the tip of the upper sepal), area of the labellar lobe (length and width of the central lobe of the labellum), and floral tube length. In addition to these floral traits, flower number was recorded and the total length (linear distance from the ground to the top of the highest flower) was measured for each plant. Initially, a larger number of traits were measured, but after preliminary scrutiny some characters were not considered for selection analysis because of lack of relationship with fitness or because of strong multicollinearity (see Table S1 in Supporting Information).

Regarding reproductive success, the number of fruits with seeds and the number of pollinia removed were recorded at the end of flowering season (March–April 2006). After the majority of fruits opened and the seeds were dispersed, wilted inflorescences of the measured plants were collected in paper bags and taken to the laboratory to confirm pollinia removal with the stereoscope. For each plant studied, male absolute fitness ( $W_m$ ) was estimated by the total number of pollinia removed and female absolute fitness ( $W_f$ ) by the total number of fruits produced. Male relative fitness ( $w_m$ ) was calculated as  $W_m/\bar{W}_m$ , where  $\bar{W}_m$  is the mean male absolute fitness. Female relative fitness ( $w_f$ ) was similarly calculated as  $W_f/\bar{W}_f$ , where  $\bar{W}_f$  is the mean female absolute fitness.

## MANIPULATIVE EXPERIMENT

To ensure that potential selection on flower traits occurred in relation to floral function, we modified traits beyond the range naturally observed in the population. In this way, we tested experimentally whether the floral traits under scrutiny represent relevant characters for pollinator attraction so that we can confidently ascribe adaptive function under putative selection. We focused on sepals and labellum, as these floral parts are what pollinators face when approaching flowers from a perpendicular perspective and because flower symmetry is not disrupted in any treatment. The sepals and labellum were experimentally manipulated in 124 plants according to a full two-way factorial design, with lateral sepals and labellum as the two factors involved. Two levels were used for each factor: control (without manipulation) and treatment (with manipulation). The manipulation of lateral sepals consisted of cutting their tips just above the dark green spots (c. 30% of the sepal from its distal extreme), while the manipulation of the labellum involved cutting off the labellar lobe at the height of the lamellae (c. 33% of the labellum) (see Fig. 2b). The cuts were made with fine dissection scissors when the flowers began to open, before they were fully open and pollinators had access to their interior. Although the design was completely balanced at the start of the experiment, some flowers had to be eliminated because they withered or were eaten by larvae. Once the experiment was assembled, the presence/absence of pollinia was periodically evaluated in each treatment and the fructification for each experimental flower was evaluated after the flowering season. At the end of the flowering season, all treated flowers were collected and taken to the laboratory to confirm pollinia removal.

As *C. blettioides* have showy floral spots at the tips of the lateral sepals and the labellar lobe, and these spots were cut in the experimental flowers, two multivariate logistic regressions were performed to detect possible relationships of spots with fruit production (0 = absence of fruit production, 1 = presence of fruit production) and pollinia removal (0 = pollinia not removed, 1 = pollinia removed). Results showed no significant relationship between sepal spot size with fruit production (overall model:  $\chi^2 = 1.81$ , d.f. = 5,  $P = 0.875$ ) or pollinia removal (overall model:  $\chi^2 = 2.68$ , d.f. = 5,  $P = 0.749$ ), suggesting that spots were of low importance for male or female fitness (Table S2).

## STATISTICAL ANALYSIS

Mean values and standard deviations were calculated for all characters and fitness measures (see Table S1). We estimated the differentials of total selection acting on traits. Similarly, gradients of directional, quadratic, and correlational selection were estimated using the multivariate model of Lande & Arnold (1983) (see review in Brodie, Moore & Janzen 1995). A sequential Bonferroni technique (Rice 1989) was used to assess the significance of differentials and selection gradients to a global value of  $\alpha = 0.05$ . This was done to minimize the Type I error in the tables of selection and as a complement to the original  $P$ -values that (together with the differentials and gradients) described selection patterns. The  $P$ -values of the selection differentials were combined for sex functions. Separate corrections of the original  $P$ -values were made for selection differentials (number of tests = 12) and selection gradients (number of tests = 54). To find general trends of selection through a sex function in the *C. blettioides* population, analysis of covariance (ANCOVA) was performed on the combined data set for traits showing statistical significance. To visual-

ize selection on two characters simultaneously (significant correlational gradients), we used SigmaPlot 9.01 for Windows (Systat Software, Inc 2004).

When using multiple regressions in selection analysis it is important to avoid characters that are intercorrelated (Lande & Arnold 1983; O'Connell & Johnston 1998; Maad 2000). The inclusion of highly intercorrelated variables often leads to dubious results (Zar 1999), as the standard errors of partial regression coefficients can be large, resulting in inaccurate point population estimations. In the study population all characters measured showed varying degrees of correlation ( $0.24 < r < 0.89$ ) (see Table S1). To perform the selection analysis the following morphological characters were used: flower height, flower width, floral tube length, area of the labellar lobe, total plant height, and flower number per plant. Flower height and width were chosen to observe whether individuals with flowers of different size showed differences in their reproductive success. Furthermore, these two traits were chosen to make comparisons between natural and experimental conditions, as flower height and width were modified when applying the manipulative treatments. The floral tube is an important trait in orchids as it is often related to fruit production and pollinia removal (Nilsson 1988). The labellum was included in the analysis because it serves as a landing platform for pollinators (Dressler 1990). Some studies have reported that plant characters such as height above-ground and flower number may influence pollinator visitation (O'Connell & Johnston 1998; Maad 2000; Aragón & Ackerman 2004; Tremblay *et al.* 2005). We included these variables to control for any plant factor influencing selection on flower characters.

Once the selection coefficients have been estimated it is necessary to assess the validity of model, ideally through an experimental approach to avoid the inherent weaknesses in the correlative approach. With an appropriate experimental design it is possible to vary the magnitude of character  $z_i$ , ensuring that the remaining characters are kept constant. In this way it is possible to observe the direct influence the change of the character on individual fitness (Mitchell-Olds & Shaw 1987). The aim of this approach is to eliminate multicollinearity (intercorrelation), the source of many problems with inference (Mitchell-Olds & Shaw 1987; Zar 1999). We used two-way ANOVA to test the significance of labellar lobe and sepal effects in four treatments: (i) control with flowers intact; (ii) labellar lobe removal; (iii) sepal tip removal; (iv) and labellar lobe and sepal tip removal (Fig. 2b). We did not apply more than one treatment per plant. As the values of male fitness (0, 1 and 2) present a Poisson distribution and the values of female fitness (0, 1) present a binomial distribution, we used GLM with logarithm link function and logit link function, respectively (McCullagh & Nelder 1989). All these analyses were performed in S-PLUS 7.0 for Windows (Insightful, Corp 2005).

## Results

## PHENOTYPIC SELECTION

Descriptive statistics of characters and fitness during the flowering season of 2005 are shown in Table S3. The 140 individuals that were measured produced 721 flowers, of which only 186 (25.8%) produced fruit. The 72 plants evaluated for pollinia removal produced a total of 359 flowers and exported 201 pollinia (28.0%).

Selection differentials for changes through the male and female functions are reported in Table 1. The results revealed total selection on flower number and plant height, but

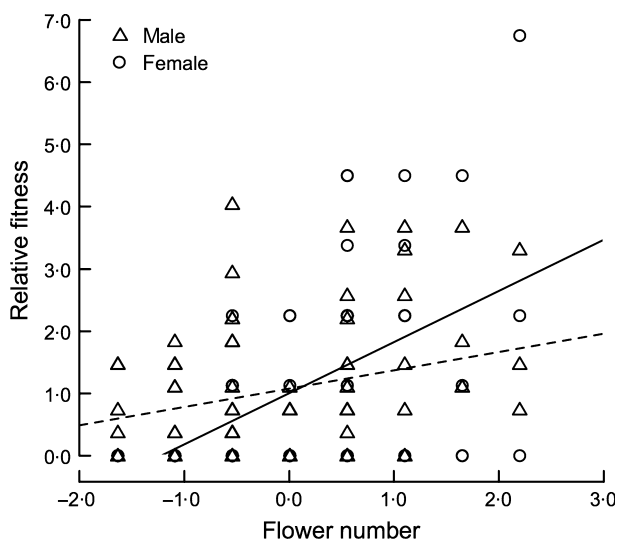
**Table 1.** Analysis of phenotypic selection for male and female functions on six characters in *Chloraea bletioides* during 2005. Differentials of selection ( $S_i$ ); and directional ( $\beta_i$ ), quadratic ( $\gamma_{ii}$ ), and correlational ( $\gamma_{ij}$ ) selection gradients  $\pm$  SE are shown. All parameters are standardized to unit variance. The statistical significance of differentials of selection was tested by the Pearson product-moment correlation of relative fitness (male and female, respectively) with characters. Boldface values are significant after Bonferroni adjustment

| Character <i>i</i>        | $S_i$            | Character <i>j</i>                  |                      |                                   |                                  |  |                                   |                                    |
|---------------------------|------------------|-------------------------------------|----------------------|-----------------------------------|----------------------------------|--|-----------------------------------|------------------------------------|
|                           |                  | $\beta_i \pm$ SE                    | $\gamma_{ii} \pm$ SE | Flower width $\gamma_{ij} \pm$ SE | Floral tube $\gamma_{ij} \pm$ SE | Area of the labellar lobe $\gamma_{ij} \pm$ SE | Plant height $\gamma_{ij} \pm$ SE | Flower number $\gamma_{ij} \pm$ SE |
| Male function             |                  |                                     |                      |                                   |                                  |  |                                   |                                    |
| Flower height             | 0.186            | 0.109 $\pm$ 0.133                   | -0.078 $\pm$ 0.276   | 0.349 $\pm$ 0.315                 | 0.003 $\pm$ 0.204                | -0.301 $\pm$ 0.222                             | -0.193 $\pm$ 0.226                | -0.044 $\pm$ 0.211                 |
| Flower width              | 0.131            | 0.012 $\pm$ 0.136                   | -0.138 $\pm$ 0.192   | -                                 | -0.229 $\pm$ 0.239               | 0.316 $\pm$ 0.219                              | 0.319 $\pm$ 0.234                 | -0.007 $\pm$ 0.226                 |
| Floral tube               | 0.114            | -0.012 $\pm$ 0.113                  | 0.341 $\pm$ 0.191    | -                                 | -                                | <b>-0.588</b> $\pm$ <b>0.163</b> ***           | 0.056 $\pm$ 0.246                 | 0.149 $\pm$ 0.196                  |
| Area of the labellar lobe | 0.224            | 0.146 $\pm$ 0.114                   | 0.322 $\pm$ 0.183    | -                                 | -                                | -  | -0.288 $\pm$ 0.185                | 0.285 $\pm$ 0.195                  |
| Plant height              | 0.280*           | 0.002 $\pm$ 0.120                   | 0.292 $\pm$ 0.243    | -                                 | -                                | -  | -                                 | -0.471 $\pm$ 0.255                 |
| Flower number             | <b>0.540</b> *** | <b>0.524</b> $\pm$ <b>0.115</b> *** | 0.020 $\pm$ 0.186    | -                                 | -                                | -  | -                                 | -                                  |
| Female function           |                  |                                     |                      |                                   |                                  |  |                                   |                                    |
| Flower height             | 0.099            | 0.013 $\pm$ 0.132                   | -0.060 $\pm$ 0.271   | 0.191 $\pm$ 0.310                 | 0.089 $\pm$ 0.201                | 0.033 $\pm$ 0.218                              | -0.296 $\pm$ 0.222                | -0.293 $\pm$ 0.207                 |
| Flower width              | 0.087            | 0.037 $\pm$ 0.136                   | -0.352 $\pm$ 0.189   | -                                 | 0.476 $\pm$ 0.235*               | -0.238 $\pm$ 0.215                             | 0.148 $\pm$ 0.230                 | 0.430 $\pm$ 0.222                  |
| Floral tube               | 0.013            | -0.122 $\pm$ 0.113                  | 0.111 $\pm$ 0.188    | -                                 | -                                | -0.482 $\pm$ 0.160**                           | -0.217 $\pm$ 0.241                | 0.294 $\pm$ 0.192                  |
| Area of the labellar lobe | 0.197            | 0.174 $\pm$ 0.113                   | 0.012 $\pm$ 0.180    | -                                 | -                                | -  | -0.190 $\pm$ 0.181                | 0.356 $\pm$ 0.192                  |
| Plant height              | <b>0.305</b> **  | 0.068 $\pm$ 0.120                   | 0.106 $\pm$ 0.239    | -                                 | -                                | -  | -                                 | -0.305 $\pm$ 0.250                 |
| Flower number             | <b>0.551</b> *** | <b>0.515</b> $\pm$ <b>0.114</b> *** | 0.345 $\pm$ 0.183    | -                                 | -                                | -  | -                                 | -                                  |

\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

absence of selection on corolla characters, which suggests that the rate of fitness gain occurs in relation to plant rather than flower characters. Likewise, flower number had a highly significant and positive directional selection gradient through male and female fitness (Table 1), indicating that plants with high flower number experienced an advantage through the two sexual functions. Interestingly, selection on flower number through the female function increased at a higher rate than through the male function (ANCOVA,  $F_{3,140} = 13.7$ ,  $P = 0.007$ , Fig. 3). The selection gradients remained significant after Bonferroni adjustment. The remaining characters did not show statistical significance. In addition, no character was under quadratic selection ( $\gamma_{ii}'$ ) in the two sexual functions, indicating that stabilizing and disruptive selection were not important forces in the population during the year of study (Table 1).

We detected two strong signals of correlational selection on pairs of traits. First, relative fruit number increased with a more positive correlation between flower width and floral tube length, although the association was no longer statistically significant after Bonferroni adjustment (Table 1). Second, selection acted against positive correlation between labellum area and floral tube length through both sexual functions. Flowers with large labellar lobes and short floral tubes, and flowers with small labellar lobes and long tubes were advantageous combinations. Only estimates through pollinia removal retained significance after Bonferroni correction. However, inspection of the level of rejection of the null hypothesis in the Bonferroni test for fruit number was  $P = 0.0033$  and the significance level observed was  $P = 0.0041$ , which suggests that the negative covariance observed was also probably under pollinator mediated selection.

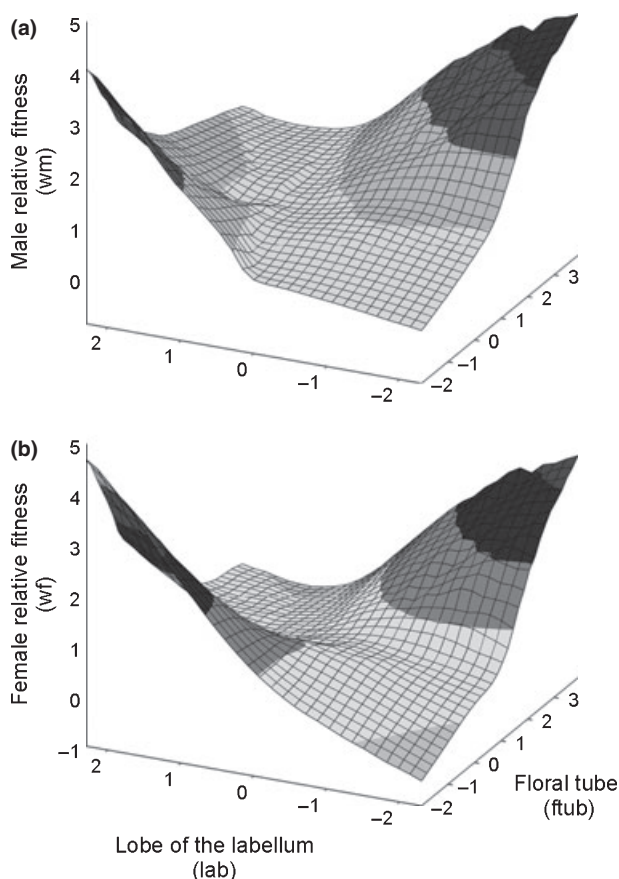


**Fig. 3.** Functional relationship between flower number and male (dashed line) and female (solid line) relative fitness. Relative fitness for each sexual function was calculated independently but expressed on the same scale for comparative purposes. The overall ANCOVA model was significant ( $F_{3,140} = 13.7$ ,  $P < 0.001$ ), and slopes differed between the sexual components (Sex:  $P = 0.707$ , Flower number:  $P < 0.001$ , Sex  $\times$  Flower number:  $P = 0.007$ ).

tion. Overall, these results indicate that plants with flowers showing a combination of large labellar lobes and short floral tubes as well as small labellar lobes and long tubes are consistently favoured through pollinia removal and fruit production (Fig. 4a and b).

#### MANIPULATIVE EXPERIMENT

The number of exported pollinia did not depend on the treatment involved (Fig. 2b) (a = 39%; b = 27%; c = 35%; d = 37%;  $\chi^2 = 6.3$ , d.f. = 3,  $P = 0.099$ ). Likewise, the number of fruits produced was unrelated to the treatment involved (a = 18%; b = 12%; c = 17%; d = 13%;  $\chi^2 = 0.67$ , d.f. = 3,  $P = 0.880$ ). These results indicate that the excision of the lateral sepals and the labellar lobe had no influence on pollinia removal or fruit production (Table 2). Similarly, the interaction between factors did not show statistical significance. Overall, these results indicate that pollina-



**Fig. 4.** Representation of the bivariate selection surface relating the area of the labellar lobe (all) with the floral tube (ftub) through (a) male relative fitness ( $w_m$ ) and (b) female relative fitness ( $w_f$ ). Traits are shown on a standardized scale (mean zero and unit variance). The surface is drawn according to all the standardized selection gradients [(a)  $w_m = 1.03 + (0.146 \text{ all}) - (0.012 \text{ ftub}) + 0.5((0.322 \text{ all}^2) + (0.341 \text{ ftub}^2)) - (0.588 \text{ all} \times \text{ftub})$ ; (b)  $w_f = 1.45 + (0.174 \text{ all}) - (0.122 \text{ ftub}) + 0.5((0.012 \text{ all}^2) + (0.111 \text{ ftub}^2)) - (0.482 \text{ all} \times \text{ftub})$ ]. The parameters for linear gradients were estimated separately from the quadratic model.

**Table 2.** Effects of the experimental manipulation of lateral sepals and the area of the labellar lobe on male and female fitness components in *Chloraea bletioides*. Degrees of freedom (d.f.), Student *t*-coefficients (*t*), and *P*-values are listed. Fruit production and pollinia removal were not affected by excision of the labellar lobe or sepals. Similarly, the interactive effect of the labellar lobe × lateral sepals on the two sexual components was not significant. For the different parameters, generalized linear models were adjusted using different error distributions and link functions (McCullagh & Nelder 1989)

| Response variable | Effects in model    | Test of significance |          |          |
|-------------------|---------------------|----------------------|----------|----------|
|                   |                     | d.f.                 | <i>t</i> | <i>P</i> |
| Fruit production  | Labellar lobe (LL)  | 1                    | −0.606   | 0.653    |
|                   | Lateral sepals (LS) | 1                    | 0.287    | 0.822    |
|                   | LL × LS             | 1                    | 0.028    | 0.983    |
| Pollinia removal  | Labellar lobe (LL)  | 1                    | −0.422   | 0.746    |
|                   | Lateral sepals (LS) | 1                    | 0.678    | 0.621    |
|                   | LL × LS             | 1                    | 0.695    | 0.613    |

tors were largely insensitive to the experimental modification of the flower phenotype, which is consistent with the absence of significant selection on individual floral characters (flower height, flower width and area of the labellar lobe), as well as on combinations of traits: height – flower width and flower width – labellar lobe (Table 1).

## Discussion

The percentage of fruits produced by the population of *Chloraea bletioides* (25.8%) is within the ranges reported for nectarless orchids of temperate zones world-wide (19.5–41.4%). This percentage is more consistent with records for Europe (27.7%) than with those in temperate zones of the southern hemisphere (41.4%) (Neiland & Wilcock 1998). The low fruit production in natural populations may be due to resource constraints (such as water or nutrients) (Stephenson 1981), or to the limitation imposed by pollinators (Bierzychudek 1981). Several authors have reported that fruit production in non-autogamous orchids is mainly pollinator limited (see Tremblay *et al.* 2005 for a review). In *C. bletioides* fruit production through hand pollination significantly exceeds natural fruit production (Humaña, Cisternas & Valdivia 2008). While 100% of flowers produce fruit after hand cross-pollination, this percentage decreases to 32.1% (Humaña, Cisternas & Valdivia 2008) and 25.8% (this study) in natural conditions, suggesting a role for pollen limitation and pollen vector abundance.

## PHENOTYPIC SELECTION

The results of this study show that flower number experienced positive direct selection. These results are consistent with previous evidence indicating that flower production in orchids is often a character under positive directional selection (e.g. Zimmerman & Aide 1989; Calvo 1990; Maad 2000). Our results are similar to those reported for orchids in temperate zones. For example, Firmage & Cole (1988) suggested that inflorescence size of *Platanthera blephariglottis* influenced male and female reproductive success in a parallel manner in small inflorescences (1–4 flowers). Likewise, in *Brassavola*

*nodosa* (Schemske 1980), a tropical orchid, male and female reproductive success increased disproportionately with respect to inflorescence size and the trends for both sexual components were parallel. The results obtained in *Chloraea bletioides* confirm the positive relationship between inflorescence size (flower number per inflorescence) and male and female reproductive success in orchids (Tremblay *et al.* 2005).

Although it is frequently suggested that selection on floral traits should act primarily through the male function (e.g. Queller 1983; Stanton, Snow & Handel 1986; but see Wilson *et al.* 1994), we found selection through the two sexual functions in this population. The magnitude and sign of the significant differentials and gradients of selection were relatively similar between the sexual components (Table 1), which is consistent with that observed in other orchid species in which no different selection patterns between sexual components have been detected (O'Connell & Johnston 1998; Maad 2000). All these results suggest that in Orchidaceae the selection upon floral characters acts indistinctly through both sexual functions (Nilsson 1992; Tremblay *et al.* 2005). However, unlike previous studies, our results provide evidence that the rate of fitness gain is not parallel between the two sexual functions (Fig. 3). Two potential explanations can be advanced to account for this pattern. First, in taxa with hermaphrodite flowers limited by pollen, the expectation is that floral display affects male and female reproductive success equally (Stanton & Preston 1988; Tremblay *et al.* 2005). This scenario is probably altered in orchids, in which each flower produces many ovules and pollen grains are bundled into pollinia that are delivered whole to the stigmas. The bundled pollen delivery is likely to reduce the potential variance in male mating success, relative to plants with pollen grains that are dispersed individually. In addition, resource limitation may also influence fruit set such that large and robust plants with more flowers may be better able to support the development of pollinated flowers to fruit than their smaller neighbours. This phenomenon would decelerate the male function with increasing flower number and accelerate the rate of fitness gain through the female function. A second potential explanation is based on the observation that the beetle *Arctodidium* sp. (Coleoptera)

was often found feeding on pollinia, which disintegrate the pollinia structure. If beetles are more attracted to plants with large flower number, it is likely that male mating success would tend to decrease with increasing flower number in *Chloraea bletioides*.

There was a consistent negative correlational selection between the labellar lobe and the floral tube for both male and female mating success. This result indicates that individuals with combinations of large labellar lobes and short floral tubes, as well as with small labellar lobes and long tubes were promoted through both sexual functions in this population. Since directional or stabilizing selection did not act upon either of the two traits separately, the bivariate surface of selection for this pair of traits can be drawn as a saddle with opposite combinations of labellar lobe size and floral tube showing the highest fitness values (Brodie 1992) (Fig. 4). Although correlational selection on reproductive characters in plants is probably of more common occurrence than previously described (Maad 2000), this study is one of the few assessments of multivariate selection in orchids (see Maad 2000; Tremblay *et al.* 2005; Benitez-Vieyra *et al.* 2006) and the only one that combines correlational and experimental approaches to phenotypic selection in this family. Few studies have attempted to measure correlational selection in orchids (see O'Connell & Johnston 1998; Maad 2000; Benitez-Vieyra *et al.* 2006), and most of them have focused on phenological, vegetative and floral characteristics. For example, O'Connell & Johnston (1998) found negative correlational selection for the interaction between plant height with opening date in one of two populations of *Cypripedium acaule*; Maad (2000) did not find evidence for this type of selection in *Platanthera bifolia*; and Benitez-Vieyra *et al.* (2006) detected a positive correlational selection coefficient on the cross-product nectary depth – flower number through the male function in *Cyclopogon elatus*.

#### EXPERIMENTAL REMOVAL OF FLOWER PARTS

Flower parts belonging to different whorls are believed to be of great importance for reproductive success in orchid species (Tremblay *et al.* 2005). Even though experimental manipulations of the flower phenotype have been previously carried out in the family (e.g. Inoue 1986; Nilsson 1988) to our knowledge only Malo, Leirana-Alcocer & Parra-Tabla (2001) modified parts belonging to different floral whorls to test the effect of florivory on pollination success. Our clipping experiment was designed to evaluate the functional role of the lateral sepals and the labellar lobe on the pollination process. While sepals would influence flower shape, the labellar lobe would serve mainly as a landing platform for pollinator insects, probably affecting their foraging efficiency. Orchid flowers often show a high shape complexity and specialization with their pollinators, which are mainly insects (Dressler 1990; Nilsson 1992). The labellum, which is a highly modified petal, is a floral part that plays a critical role in attracting pollinators (Dressler 1990; Schiestl *et al.* 2003). In *C. bletioides* it is probably the laminar structure on the adaxial surface of the

labellum that plays a role as a visual attractor ('nectar guides'), while the labellar lobe may serve as a support point for insects at landing (Fig. 1). It is likely that the lateral sepals contribute to the attraction of pollinators, enhancing the shape of the bottom half of the flower, thus increasing the attractiveness of the labellum. The experimental results of this study, however, did not reveal a significant functional role of those traits, suggesting that pollinator-mediated selection is not responsible for the high phenotypic correlation between the size of the lateral sepals and the area of the labellar lobe ( $r = 0.52$ ,  $P < 0.0001$ ) (see Table S1). This result was consistent with the absence of direct selection acting on characters (flower width and size of labellar lobe) and with the absence of correlational selection between flower height and width and/or flower width and area of the labellar lobe. Nevertheless, the consistent correlational selection on the labellar lobe and floral tube suggests that flower integration may occur through characters not directly involved in the same functional role. Whether phenotypic associations of flower traits in orchids result from correlational selection or developmental constraints need to be assessed in future studies.

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## Supporting information

Additional Supporting Information may be found in the online version of this article.

**Table S1.** Pearson product-moment correlation coefficients ( $r$ ) for the floral traits measured.

**Table S2.** Results from two logistic regressions for morphological traits on fruit production and pollinia removal.

**Table S3.** Number of individuals ( $n$ ), mean ( $\bar{X}$ ) and standard deviation ( $s$ ) of morphological characters and fitness measures in the population of *Chloraea bletioides* during the flowering season of 2005.

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