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Phenotypic and reproductive responses of an Andean violet to environmental variation across an elevational gradient

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Abstract

Environmental gradients in alpine systems may lead to differences in both abiotic conditions and species interactions in very short distances. This may lead to reproductive and phenotypic changes in plants to enhance fitness in each environment. In this study, we explored how the Central Andean *Viola maculata* responds to the elevation gradient, where it is distributed, with an expected increase in water availability and a decrease in pollinator availability with elevation. We hypothesized that: (1) plants would be more water-stressed at low elevations; (2) investment in and success of cleistogamous flowers (closed, self-pollinated) would increase with elevation; and (3) correlation patterns between floral and vegetative traits would vary along the gradient according to changes in biotic/abiotic selection pressures across sites. We partially confirmed the inverse gradient of water stress with elevation, with *V. maculata* populations in the lowest site experiencing lower soil moisture and showing thicker leaves and lower stomatal conductance. Cleistogamy was more prevalent and successful at the highest site, thus confirming the hypothesis of maintenance of a mixed-mating system as reproductive assurance. Correlation patterns between flower and leaf size differed across sites, with stronger vegetative–floral correlation at the lower sites and a weak correlation at the highest site. This finding disagrees with the notion of pollinators as drivers of correlation between floral and vegetative traits. Our study shows how a narrow gradient in an alpine system may affect not only reproductive and physiological responses in plants, but also floral and vegetative covariances.

Keywords Cleistogamy · Correlation pleiades · Drought · Floral–vegetative correlation · Phenotypic integration · *Viola maculata*

Introduction

Alpine ecosystems are highly heterogeneous, with environmental factors changing over very short distances (Körner 2003) and, therefore, are useful systems to study the patterns

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and process underlying adaptive phenotypic plasticity (von Wettberg et al. 2005; Gianoli and Valladares 2012; Hamann et al. 2017) and local adaptation in plants (Leimu and Fischer 2008), which are both important mechanisms enabling individual lineages and plant populations to maximize fitness in response to environmental heterogeneity. Small-scale differences in abiotic (e.g., nutrients, light, and water) and biotic (e.g., pollinators and seed dispersers) resources for plants may select for different ecological strategies. Such strategies would be reflected in distinct patterns of variation in physiological and morphological traits and biomass allocation along the elevational gradients. High heterogeneity at small spatial scales makes mountains large natural laboratories in which plant responses to different abiotic and biotic factors can be tested.

In the case of elevational gradients, as elevation increases, temperature and atmospheric pressure decrease steadily, while solar radiation increases (Körner 2003). Mountain ranges around the world, however, exhibit different trends

in moisture, growing season length, and cloudiness with elevation (Körner 2007). For example, in alpine habitats of Mediterranean-climate areas, drought may limit plant photosynthetic performance at low elevation (Loik and Redar 2003; Hernández-Fuentes et al. 2015), and low temperature together with high solar radiation early in the morning may cause photoinhibition, consequently reducing photosynthetic efficiency (Germino and Smith 2000). Low water environments select for sclerophyllous leaves with low SLA (specific leaf area, $cm^2 mg^{-1}$) and tend to reduce stomatal conductance to control for water loss (Lázaro-Nogal et al. 2015). Although stomatal density generally increases with aridity (e.g., Carlson et al. 2016), it may vary idiosyncratically along environmental gradients because of the simultaneous influence of several factors (e.g., soil moisture and wind exposure); hence, elevation-related patterns in stomatal density are difficult to predict (Wang et al. 2014).

Plants that cope with varying ecological factors associated with altitude may show reproductive mechanisms that enhance fitness in each environment. For example, high levels of autogamy (Arroyo et al. 2006) or a mixed-mating system strategy, such as the production of both cleistogamous (CL) and chasmogamous (CH) flowers, may provide reproductive assurance when pollinators are scarce (Culley 2002; Albert et al. 2011). The production of CL flowers (closed, self-pollinated) might compensate for decreased fruit set due to unsuccessful pollination of many CH flowers (showy, supposedly outcross-pollinated), thus conferring plants' reproductive assurance (Schoen and Lloyd 1984; Redbo-Torstensson and Berg 1995). Schoen and Lloyd (1984) proposed a model, where the CH/CL system would be favoured in a heterogeneous parental environment (mainly in terms of pollinator or flower–seed predator abundance) if individuals are exposed to a selection regime in which each flower type is favoured in a particular environment.

Plant traits are often correlated and selection should favour an integrated phenotype (Pigliucci 2003). Phenotypic integration, the pattern and magnitude of covariation among traits in a given organism (Gianoli and Palacio-López 2009), often increases with environmental stress and should be particularly observed among certain groups of characters that have been subjected to joint selection or are functionally or developmentally related (Schilichting 1989; Waitt and Levin 1993; Gianoli 2004). Thus, plants with specialized pollination should exhibit floral characters that strongly covary with one another but not with vegetative traits, a pattern known as correlation pleiades (Berg 1960). However, studies analysing phenotypic variation and covariation between floral and vegetative traits in species with specialized pollination found inconsistent evidence for Berg's hypothesis (Waitt and Levin 1993; Conner and Sterling 1996). Pélabon et al. (2013) found that the decoupling of phenotypic covariation between vegetative and floral traits in an outcrossing species

can be environment dependent. Brock and Weinig (2007) further suggested that vegetative plasticity may affect the expression of floral traits in some environments and that there may be environment-specific constraints on the evolution of floral and vegetative traits.

The Central Chilean Andes are under the influence of a mediterranean-type climate (Di Castri and Hajek 1976). Consequently, during the dry summer, plants at lower elevations are exposed to high temperatures and water stress (Cavieres et al. 2006), leading to a low photosynthetic performance (Hernández-Fuentes et al. 2015). By contrast, at higher elevations, water is not a limiting factor and the increase in temperature may have a positive effect on photosynthetic performance (Hernández-Fuentes et al. 2015). Furthermore, ecological studies in this region have reported that pollinator abundance (Arroyo et al. 1985; Arroyo and Squeo 1990), community-level flower visitation rate (Arroyo et al. 1985), and pollination-networks' organization (Ramos-Jiliberto et al. 2010) decline with elevation. Under this scenario, responses of floral traits to selection from the abiotic environment may conflict with pollinator-mediated selection. At low elevation, reduced water availability should lead to smaller flowers and leaves (Carroll et al. 2001; Caruso 2006), but selection pressure exerted by a richer and more abundant pollinator fauna might favour larger flowers (Suárez et al. 2009; Lázaro and Totland 2014), decoupling vegetative and floral traits.

In the present study, we aimed at understanding the phenotypic and reproductive responses of an Andean violet (*Viola maculata* Cav.) to environmental variation across an elevational gradient, in which both water stress and pollinator abundance should decrease with elevation. Specifically, the goals of this study were: (1) to evaluate how does this violet species physiologically varied to the environmental conditions along the gradient; (2) to assess whether this species present different strategies in each environment by varying the proportion of flower types produced (CH/CL) and/or by delaying selfing in CH flowers to assure reproduction; (3) to evaluate whether the relation between floral and leaf traits change along the elevational gradient, according to changes in abiotic and biotic conditions; and (4) to evaluate how phenotypic integration changes with environmental stress. Our hypotheses were: (1) plants would be more water-stressed at low elevations and, therefore, would show increased water-saving physiological responses; (2) to enhance reproductive output at each environment, cleistogamy would occur more frequently at higher elevations and chasmogamy at lower elevations; (3) a decoupling between vegetative and floral traits at low elevation, where a higher pollinator selection pressure is expected (this assumes selection forces of opposite sign acting on vegetative and floral traits and pollinator-mediated selection on floral size); and (4) since phenotypic integration often increases with environmental stress, we

expected a higher phenotypic integration in the site, where plants showed decreased physiological performance.

Methods

Study species and study sites

Viola maculata Cav. (Violaceae) is an alpine perennial rhizomatous herb, distributed along the southern part of the Andes (33°S–55°S) at elevations from 1600 to 2500 m. This species grows in grasslands, steppes, and stony places, often under the protection of other herbs or rocks. Leaves are alternate, ovate-to-lanceolate petiolate. *Viola maculata* produces chasmogamous zygomorphic yellow flowers from December to February, on 6–12 cm long peduncles. At the end of the chasmogamous flowering period, it starts producing cleistogamous flowers on 2 cm peduncles (J. Seguí, personal observation; first time recorded in the species). This plant species has clonal (vegetative) growth, with the production of ramets through horizontal growth (339 ± 30 cm²; $n = 34$); for this reason, for all the data collected in this study, we selected ramets with a minimum separation between them of 1 m, so we could ensure their independence. According to Lovett Doust (1981), it presents "phalanx" growth, whereby ramets are packed close to each other and the plant stays rooted in the same place on a year-to-year basis.

We conducted this study on three sites located along an altitudinal gradient in Central Chilean Andes, in Altos de Lircay National Reserve (35°36′07.0″S 70°56′49.0″W), spanning the whole altitudinal range of the species in the Reserve, ranging from 1950 to 2350 m (Fig. S1). The low-alpine site was located at 1950 m, close to the timberline of *Nothofagus antartica* and *N. obliqua*, and accompanied by *Chusquea coleou* Desv. and *Festuca pallescens* (St.-Yves) Parodi. The mid-alpine site, located at 2200 m, was accompanied by *Mulinum echinus* DC and *Berberis empetrifolia* Lam. Finally, the highest site, at 2350 m, was a sub-nival site close to the summit of "El Cerro Peine", and accompanied by *B. empetrifolia* Lam. and *F. pallescens* (St.-Yves). *Viola maculata* phenology at the lowest site was advanced about 2 weeks with respect to the two highest sites (Fig. S2). The vegetation cover decreased with elevation (Fig. S1).

Soil moisture and physiological traits

We measured soil moisture at the study sites by means of a moisture sensor (Irrometer® Company, Riverside, California), which measures soil water tension in centibars (cb), i.e., the effort required by root systems to extract water from the soil. We placed the tensiometer in a hole 20 cm deep, in the root zone of a *V. maculata* ramet, and waited between 30 and 60 min to take the measure. A high reading indicated

the dry end of the scale, whereas a low reading indicated the wet one. We took two measures for each elevation site, one at the end of December 2014 and another one at the end of January 2015. Measures across elevational sites were taken on the same day within the shortest time possible (≤ 4 h).

During the first week of February 2015, in 20 ramets per population, we measured the maximum photochemical efficiency of PSII (F_v/F_m), an estimate of photosynthetic performance (Maxwell and Johnson 2000), by means of a modulated fluorometer PAM-2000 and leaf clip holder 2030-B. In the same ramets, we also measured stomatal conductance (mmol m⁻² s⁻¹), a parameter that decreases with water stress (e.g., Lázaro-Nogal et al. 2015) using a leaf porometer (model SC-1, Decagon Devices, Inc., Pullman, WA, USA). We measured stomatal conductance and F_v/F_m between 14:30 and 17:30 h. The minimum and maximum dark-adapted fluorescence F_v/F_m were obtained after dark adaptation of the leaves for at least 20–25 min. During measurements, photosynthetically active radiation (PAR) was ~1900 (μmol/m² per second) at the lowest site, and ~2100 (μmol/m² per second) at the two upper sites. We also estimated the specific leaf area (SLA) from the ratio between leaf area and leaf dry weight (see flower and leaf traits section). SLA provides a possible means to adapt to local water availability, decreasing with water shortage. For example, Ramírez-Valiente et al. (2010) studied 13 cork oak populations in the Iberian Peninsula and reported that sclerophyllous leaves (low SLA) provide fitness benefits under water-limited environments. This because sclerophylly minimizes water loss while maintaining photosynthetic rates, thus allowing a conservative water use.

We also recorded the density of stomata and trichomes per mm² in the same 20 ramets per population. Stomata density reflects adaptive responses of plants to the environment at larger scales (Hetherington and Woodward 2003; Wang et al. 2014). We took a piece of epithelium of approximately 25 mm² from one leaf per stem and let it soak in 1.5% NaClO at 36 °C for 30 min, and then stained for 10 min in a solution of safranin 750 μg/ml. Finally, we photographed the sample using an optical PCE-MM200 Microscope, and digital images were used to count the number of stomata and trichomes in a 1 mm² grid, with the ImageJ software (ImageJ ver. 1.6).

Reproductive traits

To study the reproductive ecology of the species, we marked 39 ramets at the beginning of December 2014 (12 ramets at 1950 m; 14 ramets at 2200 m; and 13 ramets at 2350 m) and estimated floral display, reproductive success, selfing levels, and visitation rates. In these marked ramets, we estimated the floral display by recording the total number of cleistogamous (CL) and chasmogamous (CH) flowers produced

per ramet (flowers per ramet: 32.83 ± 4.74 at 1950 m, 15.53 ± 2.14 at 2200 m, and 35.84 ± 8.11 at 2350 m), as well as their reproductive output (from a total of 472 CL flowers and 586 CH flowers). The reproductive success in CH and CL flowers was compared by measuring fruit set (proportion of flowers setting fruits) and seeds per capsule across sites. To avoid seed loss in the field, we individually bagged fruits before ripening. Seed production was quantified in the lab by counting the number of seeds after fruits opened.

To evaluate selfing level (capacity to produce seeds by spontaneous self-pollination), in a total of 38 ramets, nearly, half of the CH flower buds available per ramet were bagged before anthesis with a mesh cloth that excluded floral visitors. Control flowers (open pollination) from the same ramet were also marked and left unbagged. To quantify the reproductive success of each treatment, we estimated the number of seeds produced per flower of the bagged and unbagged flowers.

We conducted a number of 30-min censuses in different ramets of *V. maculata* distributed along the gradient: total 450 min at 1950 m, total 300 min at 2200 m, and total 150 min at 2350 m. During each 30-min observation period, the researcher remained in front of a single ramet, recording all the insects that visited the flowers and the number of open CH flowers available per ramet. Only visitors touching the reproductive parts of the flowers were considered. We measured flower visitation rate as the number of visits per hour per flower. Censuses were carried out during December 2013 and January 2014, mostly from 12:00 to 17:00 h (low temperatures preclude insect activity before and after this time), avoiding windy days. Due to the high frequency of windy days and the time needed to arrive at the upper site, we censused visitors during fewer hours at this site.

Floral and leaf traits and their correlations

To characterize phenotypic variation in floral and leaf traits, we selected a total of 121 independent ramets in the altitudinal gradient (45, 40, and 36 ramets at 1950, 2200, and

2350 m, respectively) in January 2015. Within these ramets, we randomly chose a stem, counted the number of leaves and flowers, and selected a ripe leaf with a CH flower associated with its base (Fig. S3) to measure: (1) leaf area and dry mass; (2) flower peduncle length (cm; measured with a steel ruler); (3) area and dry mass of petals (measuring separately the upper, lateral, and anterior petals); and (4) area and dry mass of the five sepals, separately. We scanned each leaf (measured within a week from collection) and flower (preserved in alcohol) and calculated the area with the image analysis software ImageJ (ImageJ ver. 1.6). We weighed flowers and leaves after oven-drying (4 days at 60 °C). We obtained the floral and leaf total mass by multiplying the mass of the measured leaf and flower traits (mass of the five petals plus the five sepals) by the number of leaves and flowers in each stem, respectively. Finally, we obtained the coefficient of variation (standard deviation/mean) of each leaf and flower trait to estimate phenotypic variation within each site.

Statistical analyses

All the analyses were conducted in R (R 3.4.0, R Development Core Team, 2008), except otherwise indicated. We assessed differences among sites in mean values of physiological traits (stomatal conductance, F_v/F_m, stomata, and trichome density, SLA) and morphological traits (Table 1) using linear models, with log-transformed variables to meet the assumptions of normality. To compare reproductive success among sites and its interaction with treatment (selfing) and flower type (CH and CL), we used generalized linear mixed models (GLMM), including ramets as the random factor. The coefficient of variation (standard deviation/mean) of floral and leaf areas was obtained using the *cv* function of the raster package (Hijmans and Van Etten 2012) in R. For generalized and mixed models, we used error distribution and link functions that best fit the data: (1) binomial distribution for fruit set and CH/CL relative proportion of total flowers; (2) a Poisson

Table 1 Statistics and mean and standard errors of the floral and leaf traits measured at each elevation

Factors	ANOVA results	1950 m	2200 m	2350 m
Leaf area (cm²)	$F_{2,118} = 0.88, p = 0.41$	3.36 ± 0.19^a (37.95)	3.18 ± 0.24^a (47.91)	3.69 ± 0.27^a (45.66)
Petal area (cm²)	$F_{2,118} = 8.73, p < 0.001$	2.92 ± 0.08^a (19.22)	3.59 ± 0.13^b (24.20)	3.06 ± 0.13^a (25.87)
Sepal area (cm²)	$F_{2,118} = 1.02, p = 0.36$	0.54 ± 0.02^a (21.82)	0.52 ± 0.03^a (45.58)	0.53 ± 0.02^a (28.95)
Flower peduncle length (cm)	$F_{2,118} = 4.52, p < 0.05$	10.21 ± 0.33^a (22.19)	8.96 ± 0.31^b (21.99)	9.18 ± 0.35^b (22.54)
Number of leaves per stem	$F_{2,118} = 4.29, p < 0.05$	7.24 ± 0.48^a (45. 13)	6.4 ± 0.23^{ab} (23.08)	5.6 ± 0.18^b (19.65)
Number of flowers per stem	$F_{2,118} = 1.81, p = 0.16$	1.53 ± 0.14^a (59.96)	1.82 ± 0.15^a (53.92)	1.5 ± 0.12^a (49.12)
Leaf dry mass (g)	$F_{2,118} = 1.9, p = 0.15$	0.31 ± 0.02^a (50.37)	0.30 ± 0.06^a (64.72)	0.25 ± 0.02^a (53.78)
Flower dry mass (g)	$F_{2,118} = 3.7, p < 0.05$	0.011 ± 0.001^a (67.99)	0.016 ± 0.002^b (95.55)	0.010 ± 0.001^a (61.40)

The coefficient of variance is given in brackets. Different letters indicate significant differences within each elevation

distribution for seeds per capsule; and (3) a Gamma distribution for seeds per flower. After all the models, we performed a posteriori tests based on least-squares means comparisons among levels of significant factors, using the package lsmeans (Lenth 2016) in R.

To study how reproductive and vegetative traits were related at each elevation, we used three GLM models in which reproductive traits (petal area, flower peduncle length, and floral mass) were the response variables, whereas vegetative traits (leaf area and leaf mass) and their interaction with site were included as predictors. Floral and leaf traits showed variation inflation factor (VIF) values < 3, indicating no collinearity problems, and, therefore, could be included together in the analysis (Zuur et al. 2009).

We estimated phenotypic integration of floral and leaf characters using Wagner (1984) index of phenotypic integration (INT), which measures the variance among the eigenvalues of a phenotypic or genetic correlation matrix (Lázaro and Santamaría 2016). Each eigenvalue represents the amount of variation explained by a given principal component. A high variance among eigenvalues indicates high integration, because most of the phenotypic variation is accounted for by the first principal components, and hence, traits are strongly associated. We calculated phenotypic integration indices per population. Whenever we had several measurements for one character (i.e., the two lateral petals), we averaged them to obtain a single value per individual. The expected eigenvalue variance under the hypothesis of random covariation among traits is Exp (INT) = (number of traits − 1)/number of ramets. As the number of ramets measured varied among populations, Exp (INT) also did. Therefore, to compare populations, we calculated corrected integration indices (INTc) by subtracting from each value of INT its expected value Exp (INT) (Wagner 1984; Cheverud 1988). Bootstrapping was used to calculate the standard deviations and 95% confidence intervals of INTc in R. The INTc was considered to indicate significant phenotypic integration when its 95% confidence interval did not include zero (Herrera et al. 2002), and INTc was expressed as the percentage of the maximum possible value (the number of traits considered; Herrera et al. 2002).

Finally, we also studied the differences in the floral and leaf covariance matrix structure across sites following the 'jump-up' approach of Phillips and Arnold (1999). In this approach, based on Flury's (1988) hierarchical method, a model assuming heterogeneity of covariance matrices is sequentially compared, by means of likelihood ratio tests, with models that specify different relationships among the populations' covariance matrices (common principal components, partial principal components, proportionality, and equality). These tests were performed using 'The Common Principal Component Analysis' software by Patrick Phillips (http://pages.uoregon.edu/pphil/programs/cpc/cpc.htm).

Correlation matrices (table S1) were calculated in R with the package Hmisc (Harrell 2017).

Results

Soil moisture and physiological traits

Soil water tension, which is inversely proportional to soil moisture, was higher at the lowest site compared to the two other sites at the end of December (1950 m: 32 cb; 2200 m and 2350 m: 26 cb). However, at the end of January, soil water tension at the lowest site (64 cb) was only slightly higher than that at the upper site (62 cb), while it was lowest at the mid-elevation site (48 cb).

There were no differences in photosynthetic efficiency of PSII (F_v/F_m) among sites ($F_{2,59} = 1.85$, $p = 0.16$; Fig. 1a). Stomatal conductance increased with elevation ($F_{2,59} = 15.56$, $p < 0.001$; Fig. 1b) and specific leaf area (SLA, cm^2/g) was higher at the upper site ($F_{2,122} = 9.91$, $p < 0.0001$: Fig. 1c). Stomatal density was lower in plants at the upper site (Fig. 1d), while leaf trichome density did not differ among sites ($F_{2,58} = 0.17$, $p = 0.84$).

Reproductive traits

The GLMM showed that the proportion of CH and CL flowers varied across sites ($\chi^2 = 134.3$, $df = 2$, $p < 0.0001$; $R^2 = 0.32$). Ramets at the intermediate site (2200 m) produced the highest proportion of CH flowers (estimate: 1.87 ± 0.2, $p < 0.001$), while those at the highest site produced the lowest proportion of CH flowers (estimate: -0.30 ± 0.09). When we compared reproductive success between CH and CL flowers as estimated by fruit set, we found a significant interaction between site and flower type ($\chi^2 = 83.12$, $df = 2$, $p < 0.0001$; $R^2 = 0.32$). This was because fruit set of CL flowers was higher than fruit set of CH flowers at the upper site, whereas at the lowest sites, the fruit set of different flower types did not significantly differ (Fig. 2a). We also found a significant interaction between the type of fruit (CH or CL fruits) and site on the number of seeds per capsule ($\chi^2 = 6.66$, $df = 2$, $p = 0.03$; $R^2 = 0.41$), because there were no differences in seed number across sites in CL fruits, while CH fruits had more seeds at the lowest site compared to the upper ones (Fig. 2b). In addition, ramets with a higher proportion of chasmogamous flowers were less successful—in terms of fruit set—at the highest site ($r = -0.80$, $p < 0.001$), whereas at the other sites, CH:CL ratios in each ramet were not correlated with such measure of success (Fig. S4).

GLMM showed that bagged CH flowers produced fewer seeds per flower than unbagged ones ($\chi^2 = 38.7$, $df = 1$, $p < 0.001$, $R^2 = 0.54$), a pattern consistent across sites

Fig. 1 Bar plots of the physiological variables measured in *Viola maculata* at each site. Different letters indicate significant differences among sites within each model. **a** Photosynthetic efficiency (F_v/F_m), **b** stomatal conductance (mol H_2O/m^2 per seg), **c** specific leaf area (mm^2/ mg), **d** stomatal density (stomata per mm^2)
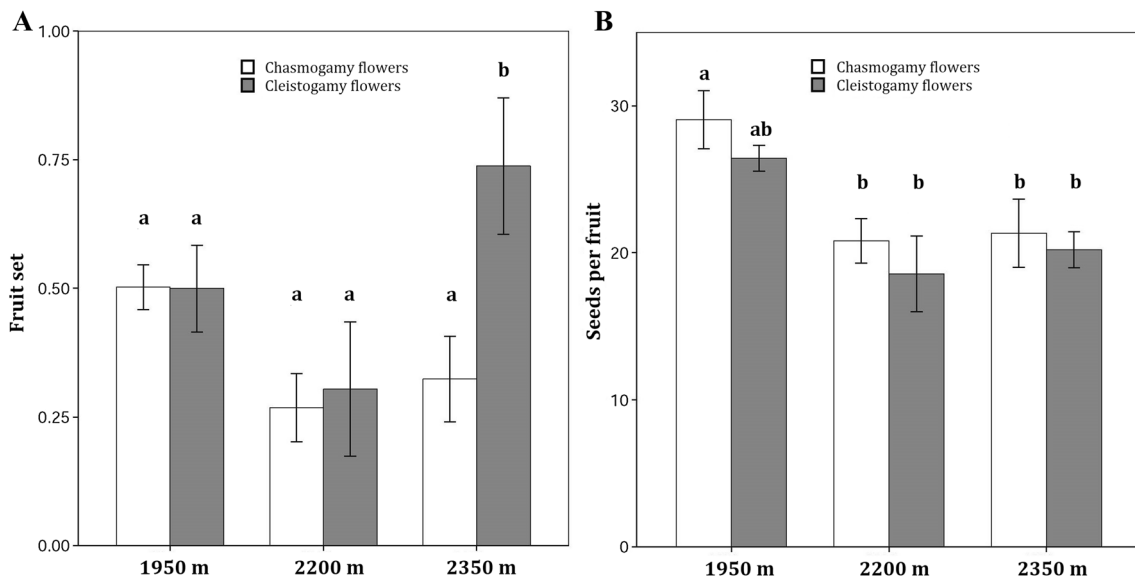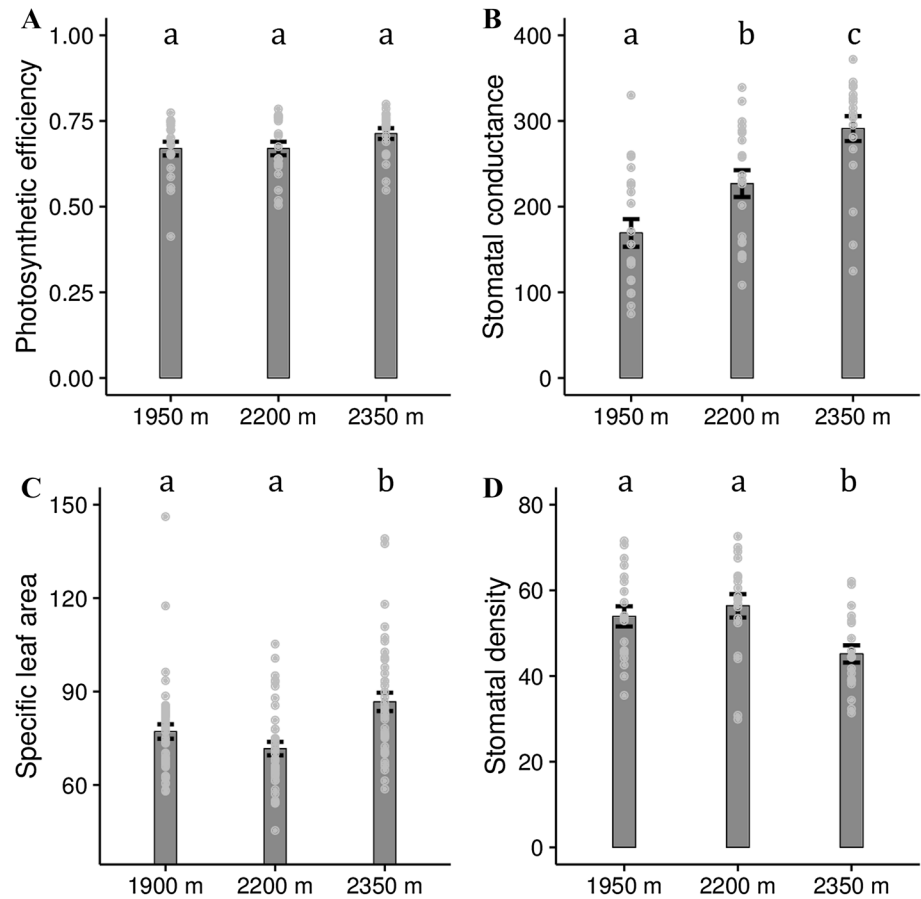

Fig. 2 **a** Mean ± SE fruit set (percentage of flowers setting fruits). Different letters indicate significant differences within sites. **b** Seeds per fruit in chasmogamous and cleistogamous flowers. Different capital letters indicate significant differences across sites for chasmogamy flowers, and different letters indicate significant differences across sites for cleistogamy flowers

(selfing × site; $\chi^2 = 3.05$, $df = 2$, $p = 0.22$). Autonomous selfing accounted for only 11.5% (0.81 ± 0.3 seeds per flower) of reproductive output of CH flowers (6.97 ± 1.5 seeds per flower). We observed very low visitation rates overall (0.4 visits per hour and flower), with most of the flower visits corresponding to ant species (8 interactions; 80% of the total visits). At 1950 m, we observed two interactions by a butterfly (*Vanessa* sp.), and two visits by ant species, at 2200 m, six visits by ant species, and at 2350 m, one visit by an ant. The overall very low visitation rates precluded performing the statistical analysis planned.

Floral and leaf traits and their correlations

Flowers were larger and heavier at the intermediate site (Table 1). By contrast, flower peduncles were longer at the lowest site (Table 1). Leaf area and dry mass per stem did not vary among study sites. The size of leaves and flowers was more constant at the lowest elevation (Table 1). Flower area increased with leaf area, but not consistently across sites (site × leaf area: $F_{2,120} = 3.38$, $p < 0.05$, $R^2 = 0.28$). Flower area increased with leaf area at the lowest site (estimate: 0.25 ± 0.08, $p < 0.01$), whereas there was no relationship between these two variables at the upper site (estimate: -0.22 ± 0.10, $p < 0.05$, Fig. 3a). Flower peduncle length was correlated with leaf area (estimate: 0.27 ± 0.07, $p < 0.001$), but this association tended to be stronger as elevation decreased (site × leaf area: $F_{2,120} = 2.69$, $p = 0.07$. $R^2 = 0.38$). By contrast, flower mass increased with leaf mass consistently in all sites ($F_{1,120} = 28.08$, $p < 0.0001$, $R^2 = 0.19$).

Significant phenotypic integration was found at the three elevations, as 95% confidence intervals of INTc did not include zero. However, such phenotypic integration did not differ significantly among populations (INTc showed overlapping confidence intervals), with the intermediate site showing slightly higher INTc (36.5%) than the low-elevation (30.66%) and high-elevation sites (25%). Although the magnitude of phenotypic integration (INTc) did not differ among their populations, its pattern did. Phillips and Arnold's (1999) analysis showed that the matrices for the study populations differed in principal components ($\chi^2 = 78.74$, $df = 30$, $p < 0.0001$), correlation structure ($\chi^2 = 137.95$, $df = 42$, $p < 0.0001$) and proportionality ($\chi^2 = 109.83$, $df = 40$, $p < 0.0001$). Significant correlations between floral and leaf traits were always positive in all three populations (Fig. 3b), indicating the absence of trade-offs between floral traits and leaf area. As expected, the size of upper, lateral, and anterior petals covaried in all sites. However, other trait correlations differed among sites. Leaf area was correlated with sepal area and petal area at the two lower sites, especially at the intermediate site, but not at the highest one. By contrast, flower peduncle length was highly correlated with petal area only at the highest elevation (Fig. 3b).

Discussion

The hypotheses of lower pollinator visitation rates and reduced water stress with elevation were partially supported by our results. Physiological data and soil moisture measurements partially show lower water stress at higher elevations. We did not find clear differences among sites in visitation rates (we would need more years of censuses). Nonetheless, reproductive traits indicate that cross pollination is more frequent at low elevation and intermediate elevation (CH flowers were larger, more abundant and successful than CL flowers), while self-pollination (cleistogamy) is more frequent and successful at high elevation.

Soil moisture and physiological traits

The Andes of central Chile present complex altitudinal gradients, where temperature and soil water availabilities vary inversely with elevation: temperature decreases and water availability increases with elevation (Cavieres et al. 2006). Our soil moisture measurements agree with this pattern, with drier soils at the lowest site, although it seems that at the end of the growing season, the upper site also became water-stressed, probably because of the end of the influence of snow melting.

We expected higher photoinhibition at the upper elevation due to lower temperatures and higher irradiance, but we found no differences across elevations in F_v/F_m. This may be related to the greater (drought-driven) stomatal closure at the lower sites, which also impairs photosynthetic performance by reduction of CO_2 uptake. Studies in elevation gradients show that SLA generally decreases with elevation (Körner 2003; Milla et al. 2009), mainly as a response to decreasing temperature and increasing light and nutrient stress. Our results found the opposite direction, with the lowest SLA at the lowest site, because of the greater water stress in this site. We found that stomatal conductance increased with elevation, which indicates that water stress in *V. maculata* decreased with elevation, a result that agrees with soil moisture data. The pattern of lower stomatal density at the upper site is also consistent with the general relationship between stomatal density and water availability (Carlson et al. 2016). It also suggests that this violet species is able to develop differential responses to the environment across relatively short distances, either by phenotypic plasticity or by genetically-based adaptations.
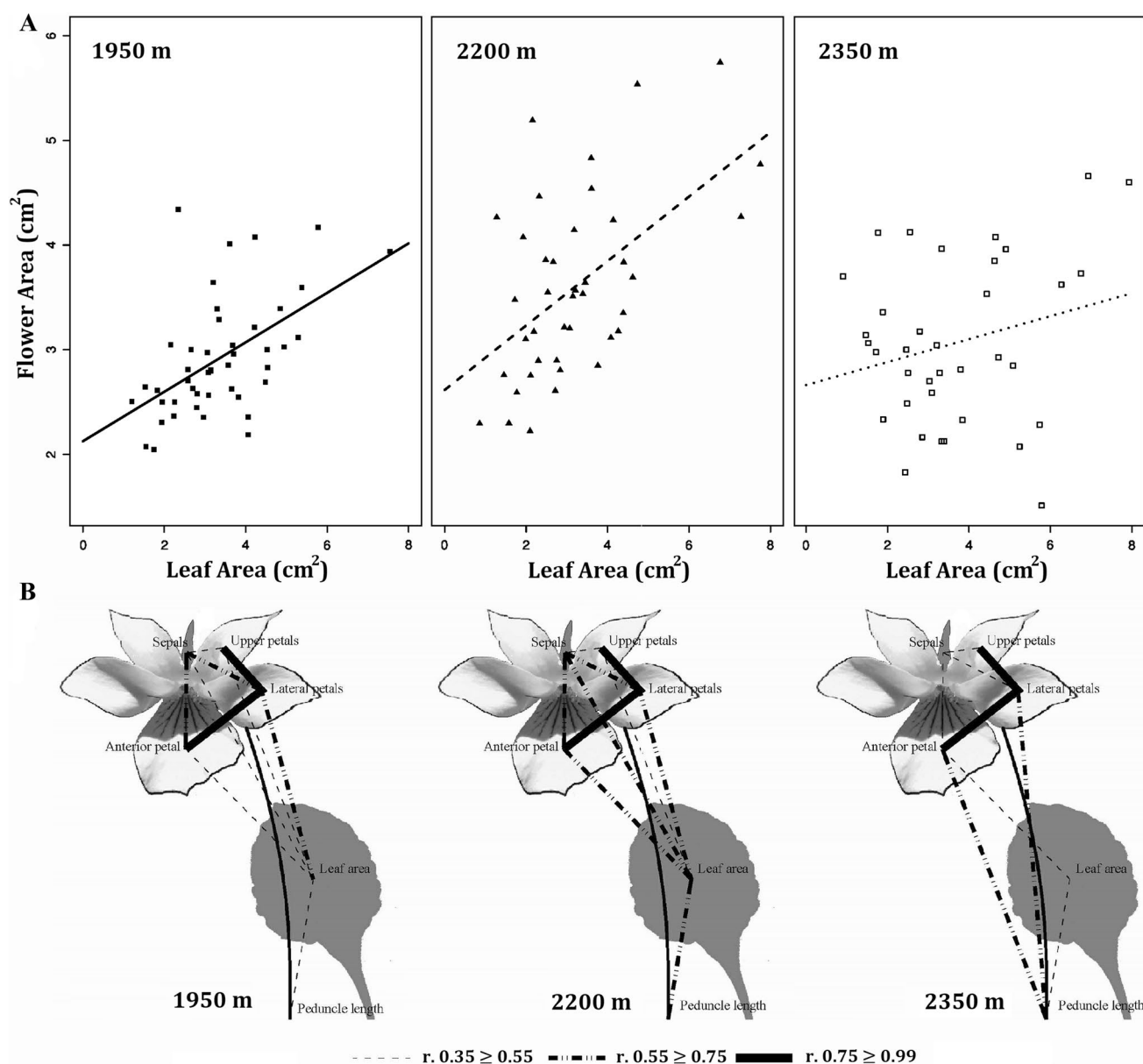
Fig. 3 a Partial residual plots showing the relationships between flower area and leaf area at each elevational site. The lines represent the best estimates for each study site, while the symbols represent the partial residuals of the model. **b** Patterns of correlations among plant traits in the three study populations of *Viola maculata*. Lines indicate significant positive correlations among floral and leaf traits

Reproductive traits

Our results supported the reproductive assurance hypothesis, as we found a high autonomous selfing capacity in the study species (although selfing level did not differ across sites), and different proportion and success of CH and CL flowers at each elevational site, but similar total reproductive output among sites. The pattern found of increased investment and fitness (fruit set) of CH and CL flowers at the lowest site and the upper sites, respectively, suggests

the occurrence of reproductive assurance adaptation (most likely genetically-based).

Several studies have reported that pollinator abundance and diversity tend to decrease above the treeline (Arroyo et al. 1985; Totland 1997) because of lower temperatures and increased cloudiness. In the Andes of central Chile, this decline in flower visitation rates has also been confirmed from the lower to the upper alpine zones (134 plant species included, Arroyo et al. 1985). We found a very low visitation rate and fruit set ($\leq 50\%$) in the CH flowers in all

sites, which could indicate the presence of pollen limitation in this alpine species. These results are consistent with the low pollinator/plant ratios and relatively high prevalence of specialized interactions described in Central Chile (Carvallo and Medel 2010; Carvallo et al. 2013) due to the depauperate species assemblages caused by geographical barriers in Mediterranean Andes. However, limited resource availability cannot be discarded as explanation (Totland and Eide 1999; Galen 2000; Muñoz et al. 2005), particularly in view of the especially dry summer of 2014–2015 in the Central Chilean Andes.

Floral and leaf traits and their correlations

Contrary to expected, we found no differences in leaf size across sites. We did find larger and heavier flowers at the intermediate site, where we also found the highest proportion of CH flowers per ramet and the higher floral integration (although INT was not significant across sites). Taken together, these results suggest that cross pollination might be favoured at this intermediate site, probably due to a higher pollinator abundance that we did not detect during the year studied. We would expect also larger flowers at the lowest elevation, but the co-occurrence of water stress and the growing season probably constrains flower size at this site, as has been shown in other systems (Galen 2000; Strauss and Whittall 2006).

Most studies addressing variation in floral traits across different environments have focused on how pollinators promote this variation (Herrera 2005; Lázaro and Totland 2014), but other factors may also contribute to floral size variation, such as environmental factors influencing resource availability (Strauss and Whittall 2006). Few studies have determined to what extent foliar responses to the environment are correlated with flower size (Armbruster et al. 1999; Pélabon et al. 2013), particularly in species with predominantly selfing or mixed-mating system (Brock and Weinig 2007). Both GLMs and matrix correlation analysis showed the same pattern, with flower traits (petal and sepal size, and flower peduncle length) and leaf size strongly correlated at the two lower sites, whereas they were weakly correlated at the highest site. These findings disagree with Berg's hypothesis (1960), since floral and vegetative traits are expected to be decoupled when they depend more on pollinators, enabling vegetative traits to respond plastically to environmental heterogeneity without disrupting the reproductive function of CH flowers. As the reproductive success of CH flowers was higher at the lowest elevation, and CH flower size and investment peaked at the intermediate elevation, we expected decoupling between vegetative and floral traits at these two sites, and not at the highest one.

Several studies have tested the correlation pleiades hypothesis between floral and leaf traits, but some have already reported results inconsistent with the hypothesis. Armbruster et al. (1999) found that four species with specialized pollination had floral traits decoupled from variation in vegetative traits; however, the same trend was also observed in three of four species with unspecialized pollination. Therefore, the correlation pattern cannot be predicted from the reproductive ecology of the plant. Lambrecht and Dawson (2007) found that the effect of moisture availability on leaf traits may mediate an indirect effect on floral size, with stronger correlations between leaf and floral size at wettest sites. Contrariwise, we found the stronger correlation at the driest site, which indicates that increased soil moisture does not always determine such correlation pattern. A study with the predominantly selfing *Arabidopsis thaliana* suggested that vegetative plasticity may affect the expression of floral traits under some environments, finding a decoupled correlation between floral and leaf traits under conditions resembling sunlit settings and a strong correlation under foliar shade treatment (Brock and Weinig 2007). Therefore, floral traits decoupling from vegetative traits observed at high elevation might be related to sunlight conditions along the elevational gradient, since solar radiation increases with altitude (Körner 2007), and vegetation neighbouring decreases (Fig. S1). Nonetheless, to discern which environmental factor is driving the correlation patterns observed, future studies under controlled conditions would be needed, considering factors such as temperature, humidity and light. Furthermore, despite strong vegetative–floral correlation could result in increased variation in floral morphology, which is likely to disrupt the reproductive function of flowers (Conner and Via 1993), reproduction would be assured by the production of CL flowers in the case of *V. maculata*. Finally, in terms of floral integration and correlations patterns among all traits, our results are consistent with our hypothesis of increasing phenotypic integration and correlation among traits with environmental stress, which result in a more "tight" phenotype at the lowest and intermediate sites (see Fig. 3b).

Conclusions

We confirmed partially the inverse gradient of water stress with elevation in Chilean Central Andes, with *V. maculata* populations in the lowest site experiencing lower soil moisture and showing thicker leaves, lower stomatal conductance, and higher stomatal density. Reproductive results support the hypothesis of mixed mating, by production of both CL and CH flowers, but that the proportion of CH flowers decreases with elevation, in line with the idea of increased reproductive assurance at higher elevation. Phenotypic integration did not differ across sites, but correlation patterns between flower and leaf size did with

stronger vegetative–floral correlation at the lower sites and a weak correlation at the highest site. The non-replicated nature of our study prevents drawing general conclusions about the study species or alpine violets; for instance, we cannot discern which environmental factor is influencing the different correlations found between floral and leaf traits along the gradient. Nevertheless, it seems that in this violet, species with mixed-mating system pollinators are not the main force decoupling floral–vegetative traits. Our study shows how a relatively narrow gradient in an alpine system may affect not only reproductive and physiological responses in plants, but also floral and vegetative covariance and phenotypic integration, with a tighter phenotype under more stressful conditions.

Author Contributions JS and EG conceived and designed the project. JS and CSL collected the data. JS and AL analyzed thedata. JS, EG, AT and AL wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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