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Phenotypic plasticity in light-induced flavonoids varies among tissues in *Silene littorea* (Caryophyllaceae)

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Abstract

Plants respond to environmental stimuli in a diversity of ways including the production of secondary metabolites. Biosynthesis of plant phenolics, including flavonoids, is frequently activated in response to a variety of abiotic and biotic stressors (e.g. extreme temperatures, high radiation, pathogens, etc.). This induced reaction is typically assumed to be a plastic response, but the components attributable to plasticity *vs* genetic variance in these components are poorly understood. Here, we investigate the variation in flavonoid production (anthocyanins and flavones) in petals, calyces, leaves and stems of *Silene littorea*. We performed a common garden experiment with maternal families from three populations in which plants were exposed to different light treatments (sun exposure and shade). The synthesis of both anthocyanins and flavones is mostly plastic, showing significant environmental effects in all photosynthetic tissues, with 23 to 42% of total phenotypic variance explained by light environment. However, non-photosynthetic petals showed considerably less plasticity in anthocyanin production in contrast with the accumulation of these compounds in photosynthetic tissues. The concentrations of anthocyanins in photosynthetic tissues increased when plants were exposed to sun, yet flavones are produced constitutively in both sun and shade treatments. Flavones exhibited approximately half the degree of phenotypic plasticity compared to anthocyanins in photosynthetic tissues. Overall, these results provide new insights into the degree of tissue-specific plasticity and flavonoid-specific response. Variable plasticity between flavonoids types in petals and photosynthetic tissues may allow this annual plant to differentially respond to changing light environments, while maintaining constitutive petal color in response to pollinators.

Keywords: flavonoids, light environment, plasticity, reaction norm, shade, within-individual variation.

Highlights

- Light exposure increased the production of anthocyanins and flavones.
- Production of anthocyanins and flavones is mostly plastic in photosynthetic tissues.
- Petals showed much lower flavonoid plasticity than photosynthetic tissues.
- Anthocyanin biosynthesis is more plastic than flavone production.

1. Introduction

Plant species are exposed to a range of environments over both time and space. Across an environmentally variable geographic range, selection will often lead to local adaptation to regional environmental conditions (Linhart and Grant, 1996). Alternatively, environmental heterogeneity within a population over time can drive phenotypic plasticity, a means of expressing alternative phenotypes in response to environmental changes (Schlichting and Pigliucci, 1998; Matesanz and Valladares, 2014). In plants, phenotypic plasticity is induced by variation in a great deal of environmental factors, such as water availability (Sultan and Bazzaz, 1993; Nilson and Assmann, 2010), light heterogeneity (Valladares and Niinemets, 2008; Matos et al., 2009), temperature variations (Vogler et al., 1999; Atkin et al., 2006), among many other factors. Thus, plants modify biochemical, anatomical, morphological and physiological phenotypes within an individual's lifetime in response to environmental factors (reviewed in Sultan, 2000; Atkin et al., 2006; Nicotra et al., 2010). Most of the literature has focused in those traits directly involved in functions related to photosynthesis, respiration and development (i.e. primary metabolites). However, limited information exists on the degree of plasticity in the synthesis of secondary metabolites, which exert a key role in plant response to environmental changes (Metlen et al., 2009; Di Ferdinando et al., 2014; Castagna et al., 2017).

Phenolic compounds, particularly flavonoids, are an important group of plant secondary metabolites (Crozier et al., 2006). The accumulation of different types of flavonoids, such as flavonols, flavones, or anthocyanins help plants to cope with a wide variety of biotic and abiotic stressors (e.g. wounding, extreme temperatures, exposure to visible or UV radiation, pathogens, etc.; reviewed in Mouradov and Spangenberg, 2014; Landi et al., 2015; Jiang et al., 2016). Anthocyanins are one of the end products of the

69 flavonoid pathway, and are found in many flowers and fruits but also in vegetative organs
70 (Steyn et al., 2002; Tanaka et al., 2008; Landi et al., 2015). Variation in anthocyanin content
71 is commonly constitutive in petals and fruits, probably due to its obligate mutualism with
72 pollinators and seed dispersers (Schaefer and Ruxton, 2011); but in the rest of the plant, the
73 accumulation of both anthocyanins and non-anthocyanin flavonoids can be highly variable
74 and often respond transiently to environmental signals (Jaakola and Hohtola, 2010; Di
75 Ferdinando et al., 2014; Del Valle et al., 2015).
76 The ability to generate protective flavonoids against a wide range of biotic or abiotic
77 stressors may confer a selective advantage in changing environments (Landi et al., 2015). The
78 synthesis of these compounds after an environmental stress is commonly assumed as a plastic
79 response (e.g. Smith, 1990; Steyn et al., 2002). However, little is known about the genetic and
80 heritable (i.e. the interaction of genotypic and environmental effects) components of this
81 plasticity (but see Lacey and Herr, 2005; Jaakola and Hohtola, 2010; Lacey et al., 2010;
82 Koski and Ashman, 2013). The knowledge of these three components of phenotypic variance
83 (genetic, environment and their interaction) is critical to understand the influence of natural
84 selection in the production of stress-induced flavonoids (Vogler et al., 1999; Murren et al.,
85 2015). Given that regulation of the flavonoid biosynthesis is tissue-specific (Albert et al.,
86 2014) and the phenotypic plasticity takes places at the intra-individual level (de Kroon et al.,
87 2005; Herrera, 2009), studies on the components of phenotypic variance in the flavonoid
88 production must be assessed in different plant tissues.
89 High radiation, including both VIS and UV light, is one of the most important factors
90 affecting flavonoid production. Due to their absorption spectrum and antioxidant properties,
91 flavonoids prevent damages to both photosynthetic apparatus and DNA caused by excessive
92 solar irradiance (Treutter, 2006; Pollastri and Tattini, 2011; Landi et al., 2015). In the last

93 years, the genetic mechanisms underlying the light-induced flavonoid accumulation have
94 been elucidated (reviewed in Albert et al., 2014; Xu et al., 2015). These studies point to a
95 fundamental role played by MBW complexes, a family of transcriptional regulatory genes
96 that act in the flavonoid biosynthetic pathway. Yet, how do tissues in which anthocyanins
97 play a role other than stress-response (e.g., petals for pollinator attraction), respond (or not) to
98 changing light conditions?
99 In this study, we experimentally investigated the degree of phenotypic plasticity in
100 flavonoid production throughout the aboveground tissues of *Silene littorea* Brot. when
101 exposed to different light environments. We selected this species because it accumulates
102 anthocyanins and other flavonoids in petals and photosynthetic tissues (calyces, leaves and
103 stems) (Del Valle et al., 2015; Fig. 1). In addition, *S. littorea* shows a latitudinal pattern in
104 flavonoids accumulation in most plant tissues: increasing flavonoids in southern populations
105 of the range area positively correlated with solar exposure and temperature and negatively
106 correlated with rainfall (Del Valle et al., 2015). However, it is still unknown if the differential
107 accumulation of flavonoids is explained by phenotypic plasticity or if it is caused by locally
108 adapted genotypes. To disentangle these two possibilities, we performed a common garden
109 experiment using extreme light conditions (sun and shade treatments). In this experiment, we
110 used seeds from three geographically distinct populations that are exposed to different degrees
111 of solar exposure in their native habitat (number of sunny days, level of UV radiation, solar
112 exposure intensity, etc.). In light of the cost and limits of phenotypic plasticity and local
113 adaptation in plants (Pigliucci, 2005; Valladares et al., 2007; Murren et al., 2015), we
114 postulate the following non-mutually exclusive predictions for the accumulation of flavonoids
115 in different tissues of *S. littorea*: 1) if flavonoid production is influenced by phenotypic
116 plasticity, then we predict high levels of variance attributable to the environment (light

117 treatment), with plants showing the same flavonoid quantity in each light treatment
118 independently of their home environments, and 2) if local adaptation or phylogenetic inertia
119 constrains flavonoid accumulation, then we expect differences among genotypes from
120 different populations, with most of the variance attributable to the genotype. Given that a
121 prevalent role of phenotypic plasticity has been found, we also analyzed whether the plastic
122 response varied among different plant tissues (petals, calyces, leaves and stems) and between
123 the anthocyanins and non-anthocyanins flavonoids.
124
125 **2. Materials and Methods**
126 *2.1. Study system and experimental design*
127 *Silene littorea* is an annual plant that inhabits foredune habitats from the northwestern
128 to the southeastern Iberian Peninsula (Casimiro-Soriguer et al., 2016). Populations of this
129 species share similar soil properties and vegetation composition, but are exposed to
130 heterogeneous climatic factors (temperature, precipitation, solar radiation) along the
131 latitudinal gradient where it grows (Del Valle et al., 2015). Previous HPLC-DAD-MS^n
132 analyses using the conditions described in Alcalde-Eon et al. (2013 and 2016) showed that
133 flavonoid profiles of *S. littorea* are composed by anthocyanins (cyanidin derivatives) in both
134 reproductive and vegetative tissues and flavones (isovitexin derivatives in petals and
135 isoorientin derivatives in calyces, leaves and stems; Del Valle J.C. and Alcalde-Eon C., unp.
136 results).
137 Seeds were obtained from previous crosses performed at the University Pablo de
138 Olavide greenhouse using nine maternal families of each population [Furnas (42º 38' 15" N,
139 9º 2' 21" W) and Barra (42º 15' 35'" N, 8º 50' 25" W; located 40 km south of Furnas)] (Buide
140 et al., 2018). In addition, seeds from six maternal families from the Sines population (37º 55'

17" N, 8º 48' 17" W; located 480 km southern Barra) were also used. These seeds were germinated and grown in autumn of 2013 following the procedure described in Buide et al. (2018). The surviving seedlings were planted in pots filled with approximately 2.5 L of an equal mixture of standard substrate (80-90% organic material, pH = 6.5) and beach sand. Before blooming (February 2014), pots were transferred into the experimental garden outside the greenhouse and assigned to shade (a bench covered with a nylon shade black cloth that reduced about 95% solar radiation) and natural sunlight (hereafter sun treatment; an uncovered bench with plants exposed to natural solar radiation) treatments. Shading experiment produces a reduction of xx% and XX% of UVA/B radiation and total transmitted sunlight, respectively. To control the influence of maternal family, half-siblings were equally assigned to each light environment. In total, data were obtained for 84 plants in sun and 51 in shade treatments from a total of 24 maternal families (Table S1). The mean photosynthetic photon flux throughout the experiment ranged from 15.86 to 36.10 µmol m^{-2} s^{-1} for the shade treatment and 205 to 1011.5 µmol m^{-2} s^{-1} for the sun exposure treatment. Light measurements were collected three times per day (0900, 1200 and 1500) at the start, middle, and the end of the experiment.

2.2. Flavonoids quantification

At the end of the flowering period (end of May 2014), flavonoids present in petals, calyces, leaves and stems were quantified using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). For each plant, samples of petal, calyx (four petals and the calyx of the same flower), leaf (collected from the middle region of the stem) and stem (1 cm length section) were selected. All samples were collected the same day (from ?:00 to ?:00h) and were preserved in 1 ml of CH_3OH:H_2O (v 70:30)

containing 1% HCl and stored at -20º C in the dark. We followed the procedure described in Del Valle et al. (2015) for the flavonoid extractions. Anthocyanin and flavone concentrations were estimated as A_{520} and A_{350}, respectively; in photosynthetic organs, anthocyanin concentration was corrected as $A_{520} - (0.24 \times A_{653})$ to compensate for the small overlap absorption by chlorophyll (Del Valle et al., 2015). Anthocyanins and flavones were quantified using five-point calibration curves of cyanidin-3-glucoside chloride and isovitexin and isoorientin standards (Sigma-Aldrich, Steinheim, Germany) and expressed as cyanidin-3-glucoside, isovitexin and isoorientin equivalents in fresh weight, respectively.

Three replicates of 200 µL were measured for each plant tissue sampled from the 135 plants used in the common garden experiment. The accuracy of flavonoid concentrations estimated spectrometrically was verified through HPLC-DAD-MS^n detection of anthocyanins and flavones in petals of 21 individuals ($r > 0.99$, $P < 0.0001$ and $r > 0.84$, $P < 0.001$, respectively; Del Valle J.C. and Alcalde-Eon C., unp. results).

2.3. Statistical analysis

Generalized linear mixed models (GLMMs) with Gaussian link functions were used to test the effect of light treatment (sun and shade) on the accumulation of anthocyanins and flavones in each plant tissue, considering treatment and population as fixed factors and maternal family as a random factor. Pairwise comparisons between light treatments were carried out using the "*multcomp*" R-package with Bonferroni adjustment (Hothorn et al., 2008). Flavonoid concentrations were log-transformed prior to conduct the GLMMs analysis. The relationship between the concentrations of anthocyanins and flavones in each plant organ was assessed with Pearson correlations.

188 A univariate analysis of variance (ANOVA) was carried out with maternal family as a
189 random factor (G), environment (E) (in this study, sun and shade treatments) as a fixed factor,
190 and the interaction of maternal and environment factors (GxE). For both anthocyanins and
191 flavones concentrations, we estimated the components of phenotypic variance (V_p)
192 corresponding to genotype (V_G), environment (V_E) and interaction (V_{GxE}). According to
193 Vogler et al. (1999), the genetic determination (V_G) was estimated as the variance of maternal
194 family divided by total corrected variance. Similarly, the environmental plasticity (V_E) was
195 calculated as the ratio of phenotypic variance explained by changes in the environment over
196 the total corrected variance. The heritable component of plasticity (V_{GxE}) was obtained by
197 dividing the variance of the interaction of genotype and environment factors by the total
198 corrected variance. Anthocyanin and flavone concentrations were log transformed to improve
199 normality prior to conducting the ANOVA analyses. We also used least square means of
200 phenotypic responses of a given genotype along the light environments for graphically
201 representing the reaction norms (Schlichting and Pigliucci, 1998).
202 To compare the phenotypic plasticity among flavonoid types and tissues, the
203 simplified relative distance plasticity index ($RDPI_s$) was used (Valladares et al. 2006). This
204 index can be obtained as:

$$RDPI_s = \sum (d_{ij} \rightarrow i'j') / (x_{i'j'} + x_{ij})) \; / \; n$$

205 where $(d_{ij} \rightarrow i'j') / (x_{i'j'} + x_{ij}))$ is the relative distances for the maternal families
206 exposed to shade and sun environments, and n is the total number of distances. The mean
207 phenotypic values for sibs of each maternal family in each light treatment, i.e. replicates, were
208 considered (Valladares et al., 2006). $RDPI_s$ values span from 0 (no plasticity) to 1 (maximal
209 plasticity), which allows for statistical comparisons of phenotypic plasticity among flavonoid

types (anthocyanins and flavones), plant tissues and populations. These differences were tested using ANOVA analyses with Student's t tests for post hoc RDPI$_s$'s comparisons.

Estimates of components of variance, ANOVAs and Student's t tests were carried out in SPSS v. 22.0 (Armonk, NY, IBM Corp.). Least square means and GLMMs analyses were performed in R v3.4.0 (R Core Team, 2017) using the R libraries "*lsmeans*" and "*lme4*", respectively (Bates et al., 2015; Lenth, 2016).

3. Results

3.1. Effects of light environments in the flavonoid production

In general, petal anthocyanin concentration was at least five times higher than in the photosynthetic plant tissues, particularly in the leaves where the quantities were very low (Fig. 2). In petals, plants from both light treatments showed no statistical differences in the accumulation of anthocyanins (Table 1). However, in the rest of tissues, plants in the sun had between 5 and 35 times more anthocyanins than those in the shade; these differences were especially noticeable in calyces and stems (Fig. 2). Plant tissues showed no significant differences in the accumulation of anthocyanins among populations, except in the stems which showed higher overall levels in Furnas (Table 1).

The flavone concentrations were at least five times higher than those of anthocyanins across all tissue types (Fig. 2). Similar to the anthocyanins, the concentration of flavones in the petals was approximately four times higher than flavones in the photosynthetic plant tissues. Plants in the sun displayed significant higher concentrations of flavones in all plant tissues compared to plants of the shade treatment (Table 1). Plants tissues showed significant differences in the accumulation of flavones among populations in the petals and calyces.

233 Within each population, the production of anthocyanins and flavones in each plant was
234 positively correlated in calyces ($r > 0.50$, $P = 0.011$), leaves ($r > 0.50$, $P = 0.003$) and stems (r
235 > 0.78, $P < 0.001$; Table S2). In petals, the correlation was not significant, except for plants
236 from Sines population ($r > 0.67$, $P < 0.001$; Table S2).
237
238 *3.2. Components of phenotypic variance and reaction norms*
239 Neither genotype (G), nor light environment (E) showed a significant effect on petal
240 anthocyanin accumulation (Table 2). The norms of reaction between light treatments show no
241 consistent pattern (Fig. 3A). In contrast, there was a significant environmental effect in all
242 photosynthetic tissues; reaction norms generally exhibited lines with decreasing slopes from
243 the sun to the shade environments (Fig. 3C, E, G). There is a moderate-high proportion of
244 phenotypic variance explained by the light treatments (V_E ranges from 0.23 in leaves to 0.42
245 in calyces). In calyces, norms of reaction converge at one point in the shade. Anthocyanins in
246 stems also varied significantly among genotypes, but had a low-moderate contribution to the
247 total phenotypic variance ($V_G = 0.19$). In leaves, anthocyanins showed significant GxE
248 interaction, having a low-moderate contribution to the total phenotypic variance ($V_{GxE} =$
249 0.16); this is explained by the lines with little or even positive slopes of connected genotypes
250 from Barra (Fig. 3E).
251 Flavones present in all tissues were significantly influenced by the light environment,
252 having a moderate-high contribution to the total phenotypic variance (V_E ranged from 0.27 in
253 stems to 0.37 in leaves; Table 2). In general, reaction norms of flavone production showed
254 lines with decreasing slopes from sun to shade environments (Fig. 3B, D, F, H). In petals and
255 calyces, the presence of flavones also varied significantly among genotypes, with a moderate

256 V_G in petals and calyces (V_G = 0.23 and 0.18, respectively); thus, parallel slopes were found
257 in most genotypes. Finally, there was also a significant GxE effect in stems (V_{GxE} = 0.20).
258
259 *3.3. Levels of plasticity among tissues, populations and flavonoid types*
260 $RDPI_s$ values varied significantly among tissues and flavonoid types, but no
261 significant differences were detected among populations (Tables 3 and S3). The production of
262 anthocyanins was much more plastic than those of flavones in calyces (among-population
263 mean; 0.92 *vs* 0.40), leaves (0.63 *vs* 0.42) and stems (0.70 *vs* 0.37); however, both types of
264 flavonoids showed similarly low $RDPI_s$ values in petals (0.14 *vs* 0.19; Fig. 4). These
265 heterogeneous differences in anthocyanins *vs* flavones plasticity among all tissues is
266 demonstrated by the significant tissue x flavonoid types interaction (Table 3).
267
268 **4. Discussion**
269 Our results add new insights into the degree of phenotypic plasticity across tissues for
270 the flavonoid production in *Silene littorea* under different light conditions, being petals less
271 plastic than photosynthetic tissues. In addition, anthocyanins and flavones showed different
272 degrees of plasticity: flavones displayed approximately half the degree of phenotypic
273 plasticity compared to anthocyanins in photosynthetic tissues, but not for petals. These results
274 agree with the current understanding of tissue-specific flavonoid gene regulation that allows
275 distinct function of flavonoids in different plant parts.
276 We have found that the synthesis of anthocyanins and flavones in calyxes, leaves and
277 stems of *S. littorea* is highly plastic. In general, the environmental effects of light exposure
278 explained most of the phenotypic plasticity found in this species. Little genetic variation for
279 flavonoid production was discovered (V_G). Although we sometimes found genotypes whose

reaction norms intersected, the genotype-by-environment interaction (V_{GxE}) was usually not significant. That is, there were mostly only small differences among genotypes in their response to the differing light environments. Our findings contrast with the results of anthocyanin accumulation in inflorescences of *Plantago lanceolata* at different temperature conditions, in which anthocyanin plasticity is at least partially genetically controlled (Lacey and Herr, 2005; 2007; Lacey et al., 2010). In the same way, the anthocyanin production in purple basil (*Ocimum basilicum*) is mostly genetic and favors the specialization for high light environments (Tattini et al., 2014). In nature, plants of *S. littorea* that are exposed to highly variable light conditions, both within- and among-populations. Specifically, plants can be found in habitats ranging from completely exposed sand dunes to the margins of nearly closed canopy coastal pine forest and understory shrubs. Furthermore, there is an increase of 31% in solar irradiance among populations at either end of the latitudinal gradient stretching the length of the Iberian Peninsula (Del Valle et al., 2015). In this scenario, high plasticity in the synthesis of light-induced flavonoids would be advantageous (Lande, 2009; Nicotra et al., 2010), allowing plants respond rapidly to changes in light availability.

Anthocyanin concentration in petals was not significantly affected by light environment. Thus, the plasticity level of anthocyanins in this tissue was low. However, the low anthocyanin plasticity in petals was not paralleled in photosynthetic tissues, being $RDPI_s$ from petals about one-fifth that of photosynthetic tissues. This may reflect some evolutionary constrains given that changes in anthocyanin contents might directly or indirectly influence the pollinator activity and affect plant fitness (Gómez, 2000; Schiestl and Johnson, 2013; Sletvold et al., 2016). Less plasticity in floral with respect to vegetative traits has been demonstrated in *Dalechampia scandens* in response to environmental variation, and assumed to support the Berg hypothesis, which proposes a decoupling and canalization of specialized

304 floral structures (Pélabon et al., 2011). In *Petunia hybrida*, plants can determine the level of
305 anthocyanin pigmentation through activation complexes, the *R2R3-MYB* transcription factors,
306 that differentially regulate anthocyanin production in petals and photosynthetic tissues (Albert
307 et al., 2011; 2014). In a previous study with *S. littorea*, Casimiro-Soriguer et al. (2016)
308 suggested that members of the same transcription factor family are responsible of the loss of
309 pigmentation in petals of white-flowered plants, which lack anthocyanins in petals but not in
310 the rest of the plant. In addition, *MYB* transcription factors were also involved in regulation of
311 anthocyanin synthesis in response to UV radiation (Del Valle, 2018). A tissue-specific
312 regulation of the anthocyanin production must grant *S. littorea* the ability to maintain their
313 floral traits without hindering the plastic response of anthocyanin production in calyces,
314 leaves and stems. Recently, it is suggested that anthocyanins may play similar photoprotective
315 functions than the previously posted to flavonols or flavones (Silva et al., 2016). In addition,
316 transient anthocyanin production may provide diverse protective roles in different
317 photosynthetic tissues (Kovinich et al., 2014).
318 We have found positive relationships between concentrations of flavones and
319 anthocyanins in calyces, leaves and stems. These results are in concert with our previous
320 findings, in which we suggested that the synthesis of both flavonoids were correlated, at least
321 in calyces (Del Valle et al., 2015). Because of the molecular structure, flavones absorb
322 maximally in the UV band, whereas anthocyanins absorb in the UV and green band of the
323 visible light (Giusti and Wrolstad, 2001; Lin and Harnly 2007). In addition, both types of
324 molecules possess important antioxidant properties, protecting leaves from oxidative stress
325 (Landi et al., 2015; Jiang et al., 2016; Silva et al., 2016). Thus, the coordinated plastic
326 response of anthocyanins and flavones may provide protection to excess solar radiation in the
327 UV and green regions of the visible spectrum. On the other hand, flavones showed around

half the plasticity levels than anthocyanins. These results provide more evidences of the metabolic plasticity within the flavonoid biosynthetic pathway, where anthocyanins are the last step and flavones are produced in mid-pathway secondary branches (Mouradov and Spangenberg, 2014; Tattini et al., 2014). In this regard, studies on *Arabidopsis* have demonstrated that flavonol and anthocyanin biosynthesis are independently regulated through specific *MYB* factors (Mehrtens et al., 2005; Stracke et al., 2007). Thus, the fact that different transcriptional factors are involved in the light-induced regulation of the synthesis of anthocyanins and flavones (Xu et al., 2015), may allow the coordinate response with different plasticity levels of both flavonoid groups found in *S. littorea*.

Flavone concentrations were always at least five times higher than that of anthocyanins, even in the shade treatment. In other words, the flavones are produced constitutively in both treatments in photosynthetic and non-photosynthetic tissues, which may suggest that flavones are also playing other non-photoprotective functions in *S. littorea*. Species of *Silene*, including *S. littorea*, frequently interact with seed predators, fungal pathogens, larval parasitoids and different herbivores, such as snails and grasshoppers (Prieto-Benítez et al., 2017; Buide, unp. data); thus, the presence of flavones might confer protection against these biotic agents (Jiang et al., 2016). Furthermore, levels of flavones in petals of *S. littorea* are higher than in calyces, leaves and stems. Flavones could act as copigments in petals, increasing color intensity and stability of anthocyanins, as is found in petals of *Iris ensata* (Yabuya et al., 1997). In addition, flavones may help the maintenance of epidermal cells; the absence of glicosilated isovitexin in mutant lines of *Silene latifolia* produce collapse of epidermal cells which originated plants with curled petals (van Brederode et al., 1982).

5. Conclusions

Although the synthesis of stress-induced flavonoids has been a research interest for many years, this study is the first to assess the plasticity in the synthesis of both anthocyanins and flavones in different plant tissues. Thus, we have found that production of anthocyanins and flavones is mainly a plastic response in photosynthetically active tissues of *S. littorea*, although this plasticity differs among plant tissues. Furthermore, we have found a higher plasticity in anthocyanins than in flavones. The increase of concentrations of both flavonoids when exposed to sun agrees with the photoprotective functions associated to these compounds, although the higher levels in both sun and shade in the case of flavones indicate that they could be playing other protective functions (Di Ferdinando et al., 2014; Landi et al., 2015). Because of the distribution range and habitat, *Silene littorea* and other species of Mediterranean Basin are exposed to a high spatio-temporal heterogeneity in environmental conditions (Del Valle et al., 2015; Narbona et al., 2018). Variable plasticity between flavonoid types in petals and photosynthetic tissues may allow *S. littorea* to differentially respond to selective pressures of pollinators and other biotic agents and changing light environments.

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Authors' contributions

EN and MB conceived the ideas and designed the experiment; JCD collected and analyzed the data; JCD, EN, JBW and MB wrote the article. All authors contributed critically to the drafts and gave final approval for publication.

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561

Table 1. Results from GLMMs testing the effect of light treatment, population and their interaction on the production of anthocyanins and flavones in each plant tissue. The interaction term is shown only when significant after model reduction applied to GLMMs.

Anthocyanins					
Source of variation	**SS**	**Numerator d.f.**	**Denominator d.f.**	***F***	***P***
Petals					
Treatment	0.34	1	111.28	2.13	0.15
Population	0.12	2	25.71	0.36	0.70
Calyces					
Treatment	16.92	1	113.00	132.08	< 0.001
Population	0.32	2	113.00	1.24	0.29
Treatment x Population	0.83	2	113.00	3.22	0.04
Leaves					
Treatment	1.31	1	109.51	75.17	< 0.001
Population	0.01	2	19.45	0.25	0.78
Treatment x Population	0.12	2	107.11	3.52	0.03
Stems					
Treatment	19.08	1	110.58	151.13	< 0.001
Population	3.41	2	17.02	13.52	< 0.001

Flavones

Source of variation	S.S.	Numerator d.f.	Denominator d.f.	*F*	*P*
Petals					
Treatment	3.84	1	107.27	147.05	< 0.001
Population	0.19	2	22.09	3.72	0.04
Calyces					
Treatment	15.42	1	102.70	98.30	< 0.001
Population	1.57	2	18.46	5.00	0.02
Leaves					
Treatment	20.51	1	111.48	189.27	< 0.001
Population	0.18	2	17.88	0.85	0.44
Stems					
Treatment	13.15	1	106.14	88.26	< 0.001
Population	0.95	2	9.88	3.20	0.09

562

563

Table 2. Variance partitioning of flavonoid concentrations in petals, calyces, leaves and stems of *S. littorea* plants. The analysis is an ANOVA with maternal genotype (G) as a random factor, environment (E) (sun or shade) as a fixed factor, and interaction (GxE). In bold is highlighted the significant phenotypic variation (V_P) components ($P < 0.05$).

		Anthocyanins					Flavones				
Tissue	Factor	S.S.	d.f.	M.S.	*P*	V_P	S.S.	d.f.	M.S.	*P*	V_P
Petals	G	8.54	23	0.37	0.110	0.27	2.26	23	0.10	0.004	**0.23**
	E	0.26	1	0.26	0.285	0.01	3.16	1	3.16	< 0.001	**0.32**
	GxE	4.43	20	0.22	0.358	0.14	0.64	21	0.03	0.286	0.07
Calyces	G	2.08	23	0.09	0.664	0.05	8.64	23	0.38	0.002	**0.18**
	E	16.11	1	16.11	< 0.001	**0.42**	14.45	1	14.45	< 0.001	**0.29**
	GxE	2.16	19	0.11	0.808	0.06	2.19	21	0.10	0.956	0.04
Leaves	G	0.47	23	0.02	0.874	0.12	3.93	23	0.17	0.456	0.09
	E	0.94	1	0.94	< 0.001	**0.23**	15.73	1	15.73	< 0.001	**0.37**
	GxE	0.67	20	0.03	0.017	**0.16**	3.28	20	0.16	0.157	0.08
Stems	G	42.88	23	1.87	0.004	**0.19**	12.71	23	0.55	0.413	0.24
	E	73.13	1	73.13	< 0.001	**0.32**	14.46	1	14.46	< 0.001	**0.27**
	GxE	10.79	20	0.54	0.941	0.05	10.62	21	0.51	0.003	**0.20**

S.S., sum of squares; d.f., degree of freedom; M.S., mean square.

564

Table 3. ANOVA for simplified relative distance plasticity index ($RDPI_s$) values of anthocyanins and flavones production in *S. littorea* plants among flavonoid types, tissues and populations.

Source of variation	**S.S.**	**d.f.**	***F***	***P***
Intercept	30.048	1	1124.864	< 0.001
Flavonoid	2.369	1	88.676	< 0.000
Tissue	5.273	3	65.796	< 0.001
Population	0.047	2	0.876	0.419
Flavonoid x tissue	1.547	3	19.306	< 0.001
Flavonoid x population	0.012	2	0.230	0.794
Tissue x population	0.311	6	1.940	0.079
Flavonoid x tissue x population	0.325	6	2.029	0.066
Error	3.633	136		

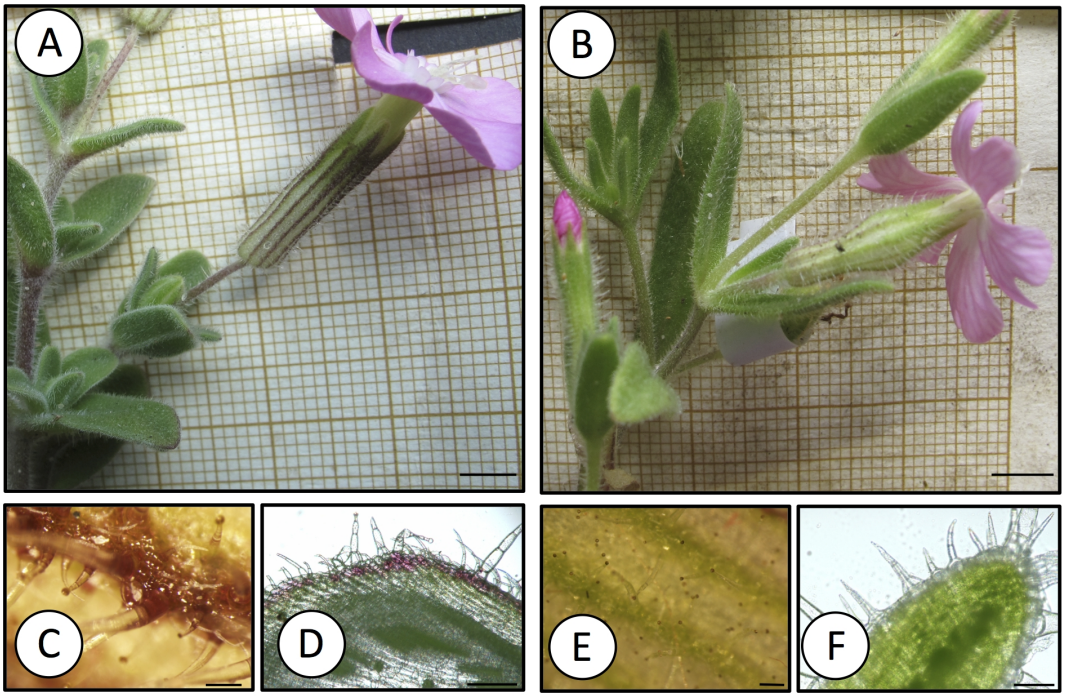
Figures captions

Fig. 1. Details of plants exposed to sun (A, C, D) and shade (B, E, F) treatments. C and E showed photographs of surface of the calyx ribs using stereo microscope. D and F showed photographs of cross section of leaf margin using microscope. Bar, 5 mm (A, B), 0.1 mm (E), 0.5 nm (C) and 0.2 nm (D, F).

Fig. 2. Comparisons of the concentrations of anthocyanins and flavones between the sun (white bars) and shade (grey bars) treatments in four plant tissues. Means and standard errors are presented. For plants from each population, results of pairwise comparisons of flavonoid concentrations between light treatments are shown when significant differences between treatments were detected using GLMMs (see Table 1). FW, fresh weight; **, $P < 0.01$; ***, $P < 0.001$.

Fig. 3. Reaction norms diagrams for anthocyanin (left column) and flavones (right column) present in four plant tissues in the sun and shade environments. Four functional subunits are depicted: petals (A, B), calyces (C, D), leaves (E, F) and stems (G, H). Triangles and green lines represent data from Sines location, circles and blue lines represent data from Barra location, and squares and red lines represent data from Furnas location. Data are least squares means; error bars represent ± SE.

Fig. 4. Differences in the simplified relative distance plasticity index ($RDPI_s$) values between anthocyanins (white bars) and flavones (grey bars) in four plant tissues. Among-population means and standard errors are presented. Results of Student t test comparing $RDPI_s$ values of both flavonoids types in each tissue are shown; ns, not significant; **, $P < 0.01$; ***, $P < 0.001$.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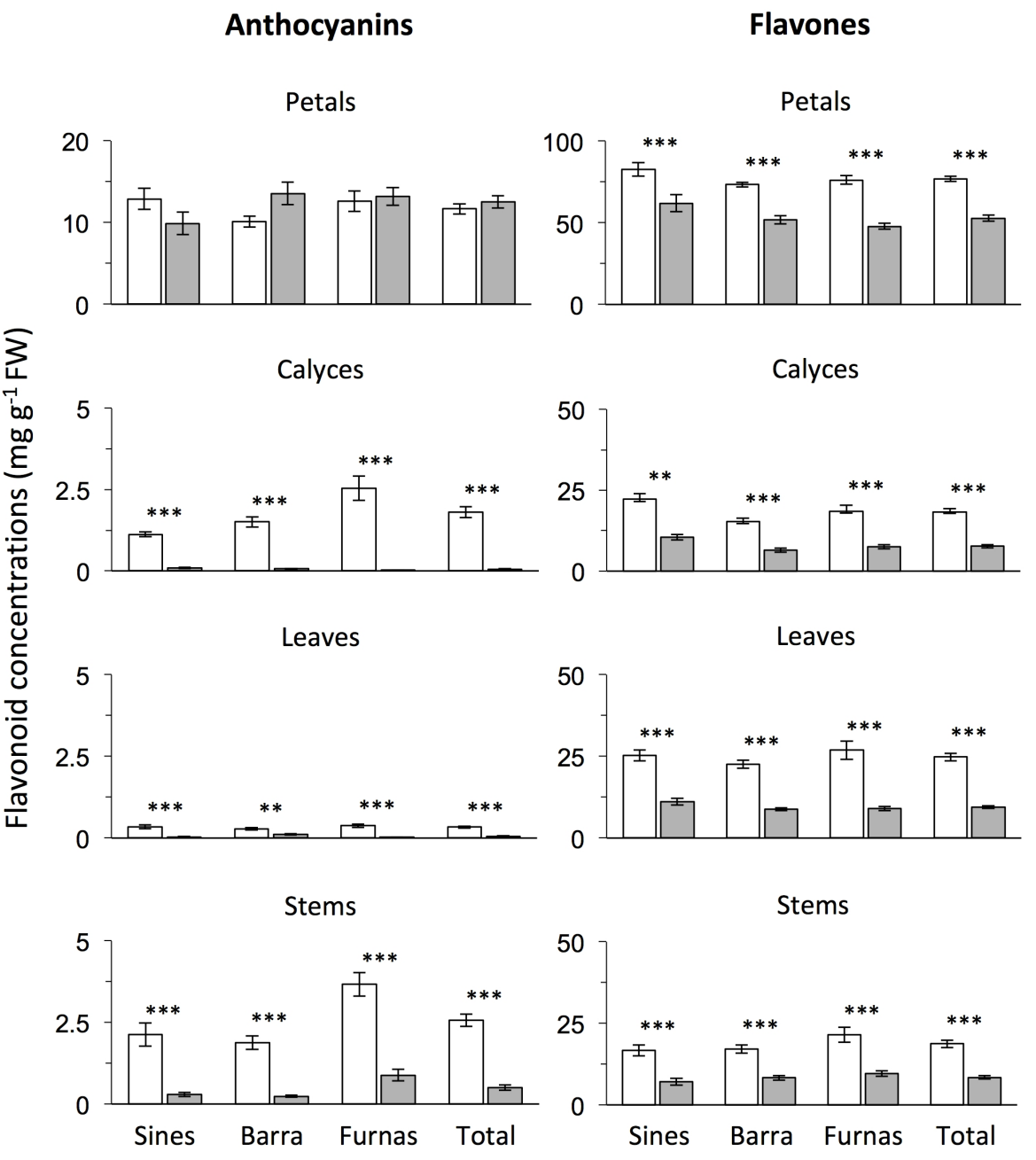
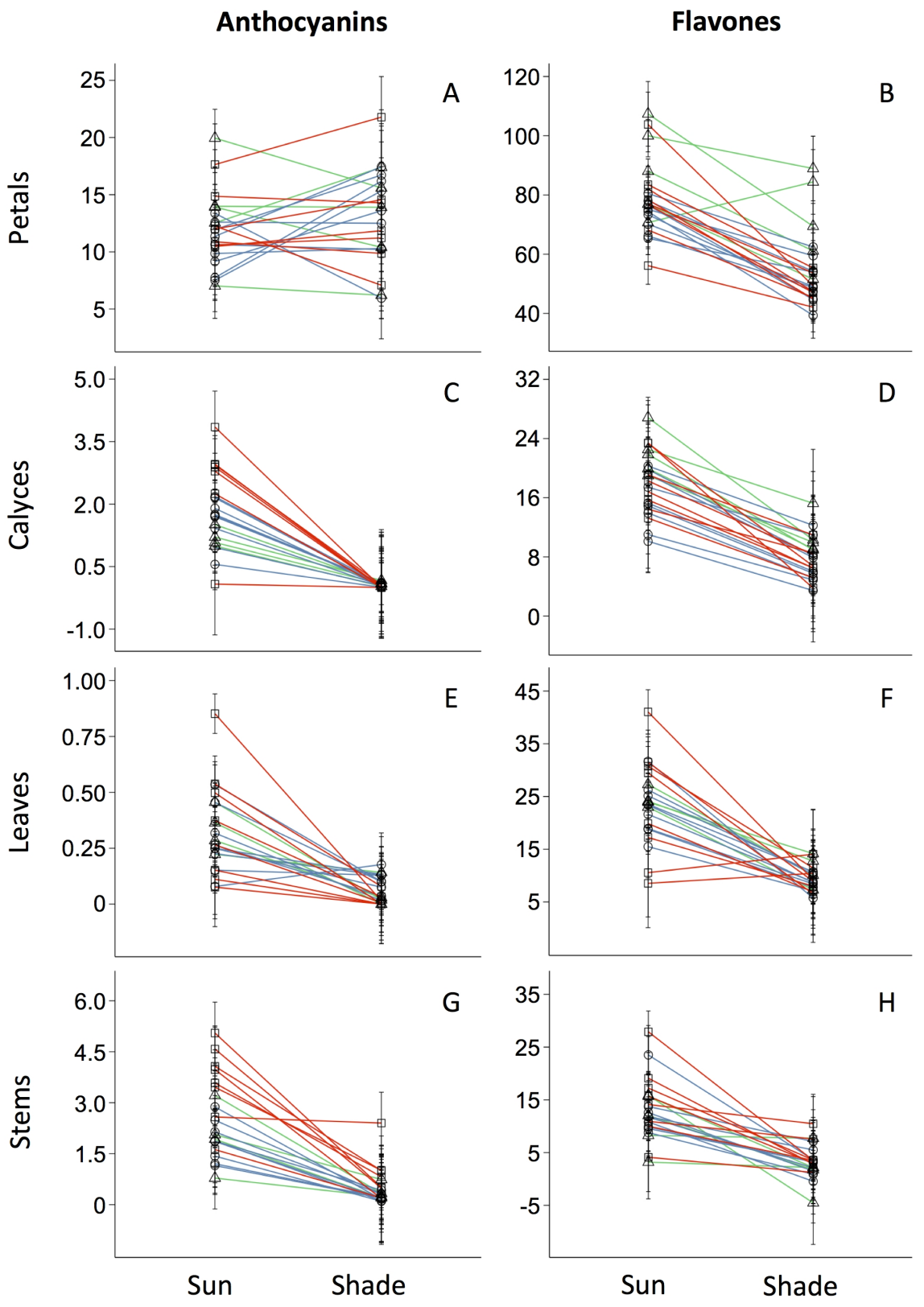

Anthocyanins
Flavones
Petals
Calyces
Leaves
Stems
Flavonoid concentrations (mg g-1 FW)
Sines
Barra
Furnas
Total


Anthocyanins
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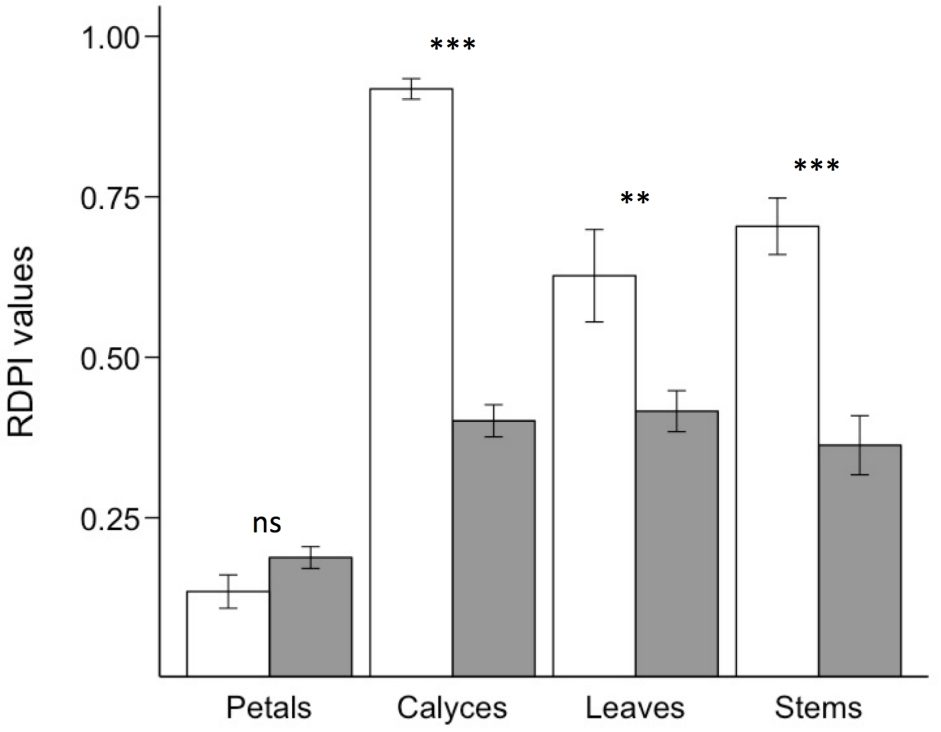
1.00
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RDPI values
***
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ns
Petals
Calyces
Leaves
Stems

Table S1. Number of plants for each maternal family, population and light environment (sun and shade).

Population	**Maternal family**	**Environment**	
		Sun	**Shade**
Sines	1	6	3
	2	1	2
	3	5	6
	4	3	0
	5	3	1
	6	4	1
Barra	7	3	1
	8	2	1
	9	4	2
	10	2	2
	11	2	2
	12	4	2
	13	4	3
	14	8	4
	15	4	2
Furnas	16	2	2
	17	2	2
	18	6	3
	19	3	2
	20	1	1
	21	1	1
	22	8	4
	23	4	4
	24	2	0
Total		84	51

Table S2. Pearson correlation coefficients of the comparison between concentrations of anthocyanin and flavones in each plant tissue of *S. littorea* plants from the three populations.

Population	**Petals**	**Calyces**	**Leaves**	**Stems**
Sines	0.67 ***	0.50 *	0.50 **	0.78 ***
Barra	-0.20	0.76 ***	0.58 ***	0.81 ***
Furnas	-0.14	0.87 ***	0.55 ***	0.82 ***
Total	0.30	0.71 ***	0.54 ***	0.81 ***

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

Table S3. Simplified relative distance plasticity index ($RDPI_s$) values obtained from flavonoid concentrations in petals, calyces, leaves and stems of *S. littorea* plants from each population. $RDPI_s$ values span from 0 (no plasticity) to 1 (maximal plasticity).

	Anthocyanins				**Flavones**			
Population	**Petals**	**Calyces**	**Leaves**	**Stems**	**Petals**	**Calyces**	**Leaves**	**Stems**
Sines	0.11 ± 0.03	0.86 ± 0.04	0.66 ± 0.22	0.66 ± 0.09	0.15 ± 0.03	0.37 ± 0.05	0.40 ± 0.06	0.40 ± 0.17
Barra	0.19 ± 0.05	0.93 ± 0.01	0.51 ± 0.09	0.78 ± 0.03	0.17 ± 0.03	0.39 ± 0.03	0.43 ± 0.04	0.37 ± 0.04
Furnas	0.09 ± 0.03	0.98 ± 0.01	0.87 ± 0.05	0.64 ± 0.10	0.23 ± 0.02	0.43 ± 0.05	0.42 ± 0.07	0.33 ± 0.07

Values are expressed as mean ± standard error.