Decoupled phenotypic variation between floral and vegetative traits: distinguishing between developmental and environmental correlations

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INTRODUCTION

In flowering plants with specialized pollination, a tight fit is expected between floral and pollinator morphologies (Fenster et al., 2004; Armbruster et al., 2009), and variation in flower size is likely to be maladaptive because pollinators remain of more or less fixed size and behaviour (Stebbins, 1974; Armbruster et al., 2004, 2009). Berg (1959, 1960) hypothesized that the maintenance of the fit between the floral traits responsible for pollen transfer and the pollinators in the face of the phenotypic response of plants to environmental variation was allowed by the decoupling of the phenotypic variation between vegetative and floral traits. However, studies analysing phenotypic variation and covariation between these two types of traits yield inconsistent evidence for the Berg hypothesis. While several studies support the prediction that floral traits are less variable than vegetative traits (Fenster, 1991; Cresswell, 1998; Herrera, 2001; Hansen et al., 2007; Pérez-Barrales et al., 2007; Chalcoff et al., 2008; Van Kleunen et al., 2008; Rosas-Guerrero et al., 2010; Pélabon et al., 2011), others show that floral traits still respond to environmental variation, sometimes markedly (Vogler et al., 1999; Carroll et al., 2001; Dorken and Barrett, 2004; Brock et al., 2005; Mal and Lovett-Doust, 2005; Caruso, 2006; Brock and Weining, 2007; for a review, see Herrera 2009). Furthermore, several studies analysing the morphological integration of vegetative and floral traits confirm the decoupling of phenotypic variation between these two types of traits (Conner and Sterling, 1996; Magwene, 2001; Juenger et al., 2005; Chalcoff et al., 2008), while other studies observe positive correlations (Watt and Levin, 1993; Conner and Sterling, 1996). Following the influential book from Olson and Miller (1958) on morphological integration, the decoupling of the phenotypic variation between vegetative and floral traits has been generally inferred from patterns of phenotypic correlation within and among series of traits belonging to one or the other category (Armbruster et al., 2004; Pigliucci and Preston, 2004). This approach is not without problems, however. First, comparisons of non-homologous floral and vegetative traits are difficult, because variational properties strongly depend on the trait complexity and dimensionality (Hansen et al., 2007). Hallgrímsdóttir and Preston (2009) further argued that integration and modularity (the organization of complex organisms into quasi-independent parts) ‘cannot be reliably studied from phenotypic covariance patterns alone’, because of the superimposition of multiple determinants of phenotypic
covariance. Building on this argument, we suggest that part of the inconsistencies observed in the tests of the Berg hypothesis may have resulted from the effect of environmental variation on the relative magnitude of non-congruent sources of covariation that generate phenotypic correlations among traits.

Phenotypic correlations result from the combination of at least two, more or less independent, processes that generate covariation. On the one hand, homologous or structurally adjacent traits that share similar developmental pathways are expected to covary positively because variation in these developmental pathways (i.e. genetic variation, resource allocation or developmental noise) will generate covariation at the phenotypic level (‘direct interaction’ sensu Klingenberg, 2008). Phenotypic correlations resulting from this type of interaction will be referred to as ‘developmental correlations’ hereafter. On the other hand, covariation between traits may result from a similar phenotypic response to environmental variation (i.e. phenotypic plasticity; Bradshaw, 1965), and traits showing similar reaction norms should be more strongly integrated than traits showing different reaction norms (Schlichting, 1986; Schlichting and Pigliucci, 1998). Hence, congruent reaction norms may result from the effects of environmental variation on shared developmental pathways (Donohue and Schmitt, 1999), but may also result from a similar response to environmental variation from traits with more or less independent developmental pathways (‘parallel variation’ sensu Klingenberg, 2008). In the following, correlations generated by phenotypic plasticity and measured among environments are referred to as ‘environmental correlations’. Phenotypic correlations will therefore result from the combination of developmental and environmental correlations. This view is similar to the one developed by Searle (1961) on genetic and environmental correlations. However, because developmental correlations may involve localized epigenetic effects (Zelditch and Swiderski, 2011), genetic and developmental correlations are not necessarily synonymous. Importantly, directions of developmental and environmental correlations are not necessarily congruent. While developmental correlations between homologous or structurally adjacent traits are likely to be mostly positive, norms of reaction in the opposite direction may occur and generate negative environmental correlations among traits (Fig. 1A). Phenotypic correlations will therefore depend on the direction and the relative magnitude of the covariance generated by either developmental or environmental variation (Fig. 1B). Under constant environmental conditions, developmental correlations should prevail and phenotypic integration should mainly reflect these correlational patterns. Under a varying environment, however, phenotypic integration will be strongly affected by phenotypic plasticity, and developmental correlations may be masked by environmental correlations (see Fig. 1B for further explanations). Consequently, the decoupling of the phenotypic variation between floral and vegetative traits inferred from patterns of phenotypic correlations may depend on the degree of environmental variation, or on the type of environmental factor varying, with different factors being expected to generate different reaction norms.

These considerations suggest that distinguishing between environmental and developmental correlations may be important when studying the decoupling of phenotypic variation. Indeed, failure to identify non-congruent correlation patterns may seriously undermine the interpretation of studies testing the Berg hypothesis. To illustrate this idea, we analysed patterns of variation and covariation in leaf size and flower size in two populations of Campanula rotundifolia from contrasting environments that were exposed to different temperature treatments. Temperature is a particularly important factor for plants, affecting growth, morphology and life histories (Larcher, 2003). In the face of the compelling evidence of the ongoing changes in global climate, understanding temperature-induced phenotypic plasticity seems particularly important especially in arctic and alpine environments where temperature changes are the most pronounced (Maxwell, 1992; Stendel et al., 2008; Gottfried et al., 2012). By analysing correlations between flower size and leaf size within and among temperature treatments, we expect to be able to distinguish between developmental and environmental correlations and test whether the decoupling of the phenotypic variation between these two series of traits depends on environmental variation.

**MATERIALS AND METHODS**

**Study species**

Campanula rotundifolia L. (Campanulaceae) is a circumpolar perennial herb spanning large altitudinal gradients (Giblin, 2005). The flowers of C. rotundifolia are bell-shaped, protandrous and predominantly outcrossing even though they are not self-incompatible (Nyman, 1991). Pollination biology including the role of secondary pollen presentation has been well documented (Nyman, 1992; Cresswell and Robertson, 1994). The main pollinators are bees and bumble-bees, at lower and higher altitudes, respectively (Bingham and Orthner, 1998). This altitudinal change in pollinator predominance is probably caused by the ability of bumble-bees to tolerate lower average temperatures at higher altitudes (Bishop and Armbruster, 1999; Bingham and Ranker, 2000). Flower size and shape in C. rotundifolia are expected to be important for pollination accuracy. Indeed, in species with secondary pollen presentation (i.e. pollen placement by the anther onto another floral structure which in turn places the pollen on the pollinator; Nyman, 1992), or species where pollinators have to crawl into the inner surface of the corolla to reach the reward, we expect a tight fit between the pollinator size and the corolla size to ensure pollen transfer efficiency (Fenster et al., 2004; Armbruster et al., 2009; but see Kobayashi et al., 1999). Earlier observations on different populations of C. rotundifolia suggested a decoupling of the phenotypic variation in flower size and plant size, because high-altitude populations displayed lower plant height but larger flowers (Turesson, 1925; Shelter, 1982; Maad et al., 2013). Furthermore, the increase in flower size with a decreasing temperature (J. Maad, Uppsala University, Sweden, and W. S. Armbruster, University of Portsmouth, UK, unpubl. res.) suggests that, in this species, part of the decoupling of the phenotypic variation is generated by temperature-induced reaction norms in opposite directions in vegetative and floral traits.
FIG. 1. Schematic representation of how developmental and environmental sources of variation generate covariation among traits and how these combine to generate phenotypic correlation. In (A), we first represent how positive and negative correlations can be generated by congruent (left) or non-congruent (right) response to variation. Environmental correlations will be generated by phenotypic response to environmental variation (phenotypic plasticity), while developmental correlations will be generated by variation affecting the development of the traits (genetic variation, developmental noise). In the central panel, trait 2 is canalized against different sources of variation (genetic or environmental canalization). In this case, we expect no correlation between the two traits. Note also that developmental correlations are expected to be mostly positive, but negative developmental correlation may occur if the developmental pathways of two traits include a trade-off (e.g., Tomkins et al. 2005). In (B), we represent how environmental and developmental correlations will combine to generate phenotypic correlation between the two traits. In cases 1, 5 and 9, both types of correlation are congruent, environmental variation generating the same type of phenotypic variation as genetic variation or developmental noise [direct interaction sensu Klingenberg (2008) in case 1; developmental and environmental trade-off in case 9]. Consequently, phenotypic variation follows the direction of both environmental and developmental correlations. In cases 4 and 6, despite the two traits being developmentally independent, the environment generates some correlation between them, and the overall phenotypic correlation follows the pattern of environmental correlation (parallel variation sensu Klingenberg, 2008). In cases 2 and 8, despite the environmental canalization of trait 2, the developmental correlation between the two traits may generate some phenotypic correlations. We are not aware of any example of this particular scenario. In cases 3 and 7, developmental and environmental correlations are in opposite directions, and the phenotypic outcome is uncertain and depends on the relative amplitude of the two sources of variation. On the right-hand side of the matrix (C), we represent two possible scenarios. In both graphs, circles represent individuals raised in one of two environments (the grey and black circles representing individuals raised in environment 1 or 2). The solid lines represent developmental correlations, while the dotted lines represent environmental correlations. In the upper graph, the environmental variation is strong and the phenotypic correlation is primarily determined by this environmental correlation. In the lower graph, the limited environmental variation generates only a weak negative environmental correlation between the two traits. The two sources of covariance tend to cancel each other out, leading to an apparent absence of phenotypic correlation.
Experimental design

Second greenhouse-generation seeds were grown under two different temperature treatments. At flowering, we recorded traits reflecting the vegetative size of the plants, including leaf area, and traits reflecting the size of the flower that is assumed to affect the fit with the pollinator. We then analysed patterns of variation and covariance between flower size and leaf size, within and among environments.

The seeds used in this experiment were derived from individuals collected at two different sites in the Dovre mountain region (Norway). One population was located at 510 m a.s.l (low-altitude population, 62°32’N, 09°36’E), while the other population was located at 1100 m a.s.l (high-altitude population, 62°31’N, 09°40’E); these populations are referred to as populations G and H, respectively, in Maad et al. (2013). In 2005, individuals collected in the wild and maintained in the greenhouse (NTNU, Trondheim) were crossed in a partial diallel experiment in order to estimate genetic variance of floral traits (J. Maad, Uppsala University, Sweden, and W. S. Armbruster, University of Portsmouth, UK, unpubl. res.). The F1 generation was grown under greenhouse conditions and crossed to produce new seeds to be used in the present experiment. Thirty genetic families per population were chosen randomly at the beginning of the experiment in November 2009. For each of the 30 families in each population, approx. 200 seeds were sown in ten individual pots (20 seeds per pot) in early November 2009 and kept at 19 °C to ensure germination. Following germination, seedlings were thinned in January 2010 to make sure that each pot contained only one plant. In all pots, we kept the largest seedling close to the centre of the pot. We subsequently placed half of the pots from each family in a room at an average temperature of approx. 12 °C, and the other half in a room at an average temperature of approx. 19 °C (Supplementary Data Fig. S1). The position of each pot on one of the four tables in each room was randomly assigned. We applied a light:dark cycle of 20 h:4 h, resembling the average summer conditions in mid-Norway. For practical reasons, the temperature treatment was confounded with the room. However, the two rooms used in this study were contiguous in the greenhouse and strictly similar with respect to the light environment, the supplementary lighting being provided by two high-pressure 400 W sodium lamps over each table in each room. We also took great care in maintaining plants under similar moisture conditions in the two rooms. Consequently, although the treatment truly corresponds to the combined effects of the temperature treatment and the rooms, we refer to it as temperature treatment hereafter. It is also important to note that the difference in temperature between the two treatments may represent an extreme situation compared with the differences normally encountered by natural populations. However, because our primary aim was to compare environment-induced correlations between vegetative and reproductive traits with developmental correlations, we chose treatment levels that were expected to maximize the effect size at the expense of the ecological realism.

Out of the 600 pots (2 treatments × 2 populations × 30 families × 5 replicates), germination failed in 48 pots (12 in the low-altitude population and 36 in the high-altitude population). Overall, 294 plants produced flowers, 42 and 98 from the low-altitude population, and 44 and 110 from the high-altitude population, in the cold and warm environment, respectively (Supplementary Data Table S1). For logistic reasons, the experiment was terminated in early June 2010 on day 208 after sowing.

Measurements

From March 2010 onward, plants were monitored every second to third day to record the emergence of the first flower. Floral traits were measured on one inflorescence per plant in early pistillate phase in order to control for possible ontogenetic variation in flower size. Measurements of floral traits included three measurements of flower length [flower length (FL), corolla length (CL) and tube length (TL)] and two measurements of the flower diameter [corolla width (CW) and tube diameter (TD); Fig. 2]. All floral measurements were taken with a digital calliper with 0.01 mm precision by a single person (N.C.O.). To account for a possible trade-off between the number of flowers and flower size, we recorded the number of flowers and buds present at the time of the floral measurements. Because number of flowers is not a morphological trait affecting the fit with pollinators, it was not included among the floral traits analysed to test the Berg hypothesis.

We also measured several traits related to the vegetative size of the plant 3 weeks after the first flower opened. These measurements included rosette diameter (average of two diameters perpendicular to each other) and stalk height (height of the highest flower above the soil surface). The three largest leaves were collected and scanned, and we estimated their area using Image-J 1.43 software (http://rsb.info.nih.gov/ij/). Leaf area was square root transformed in order to provide a measure of leaf size on a scale (mm) similar to the floral measurements. After all measurements were completed, the total above-ground biomass was estimated by drying the plants for 48 h at 40 °C and weighing them to the nearest 0.001 g. This biomass is referred to as dry weight.

![Fig. 2. Drawings of the different floral traits measured (solid arrows). FL, flower length; CL, corolla length; CW, corolla width; TL, tube length; TD, tube diameter. Abbreviations: OFL, orthogonal flower length; and CD, corolla diameter, are measurements derived from FL and CW, respectively. OFL and CD were not used in the analysis.](image-url)
Statistical analyses

We first tested whether floral traits were less variable than vegetative traits by comparing the variational properties of the different traits. We estimated the amount of variation due to the temperature treatment and the population of origin by conducting a variance-component analysis on each trait separately. We used mixed-effects models where temperature, population and family were entered as random factors to partition the variance between these different levels (Crawley, 2007, p. 638). We expressed the different components of the variance as mean-square scaled variances in order to compare the variance across traits. Mean-square scaled variances are equivalent to square coefficient of variation but have the advantage of being additive.

Leaf size was estimated as the mean of the three leaf area measurements. To estimate the overall flower size, we ran a principal component analysis (PCA) on the floral traits. For morphological traits, the first axis of the principal component analysis (PC1) essentially captures size variation (Wagner, 1984). We therefore defined flower size as the scores on the first axis of the PCA on floral traits. The first axis of the PCA comprised 69% of the total variance in floral traits (Supplementary Data Table S2). We then tested the effects of temperature and population on flowering time and both size variables using mixed-effects models with population and temperature as fixed factors and family as a random factor. Model selection was conducted on models fitted with maximum likelihood (ML), while parameter estimates were obtained from the best models fitted with restricted maximum likelihood (REML). Because none of the floral traits was negatively affected by the number of flowers [model on flower size with flower number as predictor variable, Akaike information criterion (AIC) = −457.5; without flower number, AIC = −464.2], we did not correct our estimates of flower size for variation in the number of flowers.

It is not possible to disentangle environmental from developmental correlations entirely because developmental correlations will always be present between the two size measurements. By analysing the phenotypic correlations within and among treatments, we intended to obtain estimates of the developmental correlations within treatment, i.e. when the effect of the environmental variation on covariance was minimal. Among-treatment correlations, on the other hand, provided estimates of the sum of the developmental and environmental correlations when environmental variation was expected to represent the primary source of trait covariance.

Patterns of covariation between leaf size and flower size were estimated using Pearson correlation coefficients between the two variables, within and among treatments. However, in order to account fully for the structure of our data (family nested in populations) and obtain unbiased parameter estimates and P-values for the covariance between leaf size and flower size, we used mixed-effects models where flower size was regressed on leaf size with population as a fixed factor and family as a random factor. Models were conducted on the whole data set (i.e. including the among-treatment variation) and within each treatment separately. Graphical inspections of the residuals from all the models revealed that these were normally distributed. All analyses were computed using R version 2.11.0 (R Development Core Team, 2010).

Because our experimental design included several individuals per family, we should have been able to estimate genetic correlations among traits. However, due to germination and flowering failures, the final design was unbalanced and several families were represented by only one individual. Because this strongly jeopardized any analysis of the genetic correlation (Lynch and Walsh, 1997), we decided to focus here on the phenotypic correlations only.

RESULTS

Variational properties of vegetative and floral traits

Variational properties of vegetative and floral traits differed markedly. Vegetative traits, including leaf size, were much more variable than floral traits as demonstrated by their higher square coefficients of variation (Table 1). Additionally, while the temperature treatment generated on average 57% (range 50–75%) of the phenotypic variation in vegetative traits, with 61% for leaf size, it only generated an average of 18% (range 14–28%) of the phenotypic variation in floral traits (Table 1). Variation in floral traits was essentially expressed at the among-individual level, the differences between populations and temperature treatments accounting for an equal proportion of the phenotypic variance.

Effects of temperature and population of origin on flowering time, flower size and leaf size

Plants flowered on average 28.3 ± 3.6 d earlier in the warm environment, and the plants from the high-altitude population flowered on average 8.0 ± 3.4 d earlier than those from the low-altitude population (Table 2). Similarly, the temperature treatment and the population of origin had an additive effect on leaf size, leaves being larger in the warm environment and in the plants from the low-altitude population (Table 2; Fig. 3). In contrast, flowers from the high-altitude population were larger than those from the low-altitude population, and an increasing temperature negatively affected flower size, this latter effect being more marked in the high-altitude population (Table 2; Fig. 3).

Correlation between leaf size and flower size within and among environments

Due to reaction norms in opposite directions, correlations between flower size and leaf size among treatments tended to be negative or absent (Fig. 4). Within treatment, however, leaf size and flower size were positively correlated in the cold environment, but independent from each other in the warm environment (Fig. 4). These patterns were confirmed statistically when we regressed flower size on leaf size, accounting for the structure of the data using mixed-effects models (Table 3).
Table 1. Components of the phenotypic variance in vegetative and floral traits

<table>
<thead>
<tr>
<th>Trait</th>
<th>Mean ± s.e.</th>
<th>Temperature</th>
<th>Population</th>
<th>Family</th>
<th>Residual</th>
<th>CV²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetative traits</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stalk height (mm)</td>
<td>225.00 ± 54.2</td>
<td>9.89</td>
<td>0.63</td>
<td>0.61</td>
<td>5.96</td>
<td></td>
</tr>
<tr>
<td>Rosette diameter (mm)</td>
<td>85.66 ± 30.51</td>
<td>19.94</td>
<td>0.00</td>
<td>0.57</td>
<td>6.27</td>
<td></td>
</tr>
<tr>
<td>Dry weight (mg)</td>
<td>615.10 ± 387.6</td>
<td>33.85</td>
<td>1.63</td>
<td>0.93</td>
<td>18.91</td>
<td></td>
</tr>
<tr>
<td>Leaf size (mm)</td>
<td>3.57 ± 0.81</td>
<td>8.23</td>
<td>0.23</td>
<td>0.00</td>
<td>5.03</td>
<td></td>
</tr>
<tr>
<td>Floral traits</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flower length (mm)</td>
<td>24.08 ± 0.91</td>
<td>0.27</td>
<td>0.43</td>
<td>0.15</td>
<td>1.01</td>
<td></td>
</tr>
<tr>
<td>Corolla length (mm)</td>
<td>21.44 ± 0.97</td>
<td>0.41</td>
<td>0.57</td>
<td>0.15</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>Tube length (mm)</td>
<td>15.26 ± 0.88</td>
<td>0.47</td>
<td>0.57</td>
<td>0.15</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>Corolla width (mm)</td>
<td>20.44 ± 1.10</td>
<td>0.39</td>
<td>0.15</td>
<td>0.14</td>
<td>1.68</td>
<td></td>
</tr>
<tr>
<td>Tube diameter (mm)</td>
<td>14.65 ± 0.79</td>
<td>0.64</td>
<td>0.00</td>
<td>0.32</td>
<td>1.37</td>
<td></td>
</tr>
<tr>
<td>Ln (no.of flowers)</td>
<td>1.25 ± 0.39</td>
<td>14.47</td>
<td>1.56</td>
<td>0.00</td>
<td>13.21</td>
<td></td>
</tr>
</tbody>
</table>

For each trait, the different variance components are reported as mean-square scaled variances (σ² / s²) on the top line, and percentage of the total variance on the bottom line. The reported means correspond to the estimates of the fixed effect in mixed-effects models. These were used to scale the variance components. The residual variance component corresponds to the variance among full-sibs (n = 294). Total square coefficients of variation are presented in the last column.

Table 2. Effects of treatment and population on flowering time, flower size and leaf size

<table>
<thead>
<tr>
<th>Fixed effects</th>
<th>AIC</th>
<th>AIC weights</th>
<th>Low-altitude and cold treatment</th>
<th>Temperature effect: warm treatment</th>
<th>Population effect: high altitude</th>
<th>Interaction effect: warm treatment × high altitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flowering date ~Pop × Temp</td>
<td>3076.04</td>
<td>0.257</td>
<td>188.53 + 4.23</td>
<td>−26.68 ± 5.17</td>
<td>−5.99 ± 5.85</td>
<td>−3.16 ± 7.23</td>
</tr>
<tr>
<td>Flowering date ~Pop + Temp</td>
<td>3074.23</td>
<td>0.635</td>
<td>189.61 ± 3.42</td>
<td>−28.30 ± 3.60</td>
<td>−8.06 ± 3.42</td>
<td></td>
</tr>
<tr>
<td>Flowering date ~Pop</td>
<td>3122.27</td>
<td>2.3 x 10⁻¹¹</td>
<td>1.6 ± 10⁻¹¹</td>
<td>0.384</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flowering date ~Temp</td>
<td>3077.76</td>
<td>0.109</td>
<td>1.6 ± 10⁻¹¹</td>
<td>0.384</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flowering date ~1</td>
<td>3123.09</td>
<td>0.242</td>
<td>1.6 ± 10⁻¹¹</td>
<td>0.384</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf size ~Pop × Temp</td>
<td>793.7</td>
<td>0.242</td>
<td>2.948 ± 0.122</td>
<td>1.580 ± 0.110</td>
<td>−0.304 ± 0.126</td>
<td></td>
</tr>
<tr>
<td>Leaf size ~Pop</td>
<td>947.3</td>
<td>9.71 x 10⁻³⁵</td>
<td>1.580 ± 0.110</td>
<td>−0.304 ± 0.126</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf size ~Temp</td>
<td>795.5</td>
<td>0.098</td>
<td>1.580 ± 0.110</td>
<td>−0.304 ± 0.126</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf size ~1</td>
<td>950.5</td>
<td>2.17 x 10⁻³⁵</td>
<td>1.580 ± 0.110</td>
<td>−0.304 ± 0.126</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flower size ~Pop × Temp</td>
<td>1042.64</td>
<td>0.776</td>
<td>0.060 ± 0.280</td>
<td>−1.022 ± 0.284</td>
<td>2.114 ± 0.381</td>
<td>−0.812 ± 0.384</td>
</tr>
<tr>
<td>Flower size ~Pop + Temp</td>
<td>1045.13</td>
<td>0.224</td>
<td>0.060 ± 0.280</td>
<td>−1.022 ± 0.284</td>
<td>2.114 ± 0.381</td>
<td>−0.812 ± 0.384</td>
</tr>
<tr>
<td>Flower size ~Pop</td>
<td>1094.95</td>
<td>3.4 x 10⁻¹²</td>
<td>0.060 ± 0.280</td>
<td>−1.022 ± 0.284</td>
<td>2.114 ± 0.381</td>
<td>−0.812 ± 0.384</td>
</tr>
<tr>
<td>Flower size ~Temp</td>
<td>1070.82</td>
<td>5.9 x 10⁻⁷</td>
<td>0.060 ± 0.280</td>
<td>−1.022 ± 0.284</td>
<td>2.114 ± 0.381</td>
<td>−0.812 ± 0.384</td>
</tr>
<tr>
<td>Flower size ~1</td>
<td>1121.22</td>
<td>6.7 x 10⁻¹⁸</td>
<td>0.060 ± 0.280</td>
<td>−1.022 ± 0.284</td>
<td>2.114 ± 0.381</td>
<td>−0.812 ± 0.384</td>
</tr>
</tbody>
</table>

Estimates (± s.e.) obtained from the models fitted with REML are presented for the best model or the two best models when ΔAIC between competing models was < 2 AIC units. All models were mixed-effects linear models where family was included as a random effect. Fixed factors were population of origin (Pop) and temperature treatment (Temp). Estimates are given for a trait measured in the low-altitude population in the cold environment. The temperature effect shows how the trait changes when plants from the low-altitude population are grown in the warm environment. The population effect shows how plants from the high-altitude population perform under cold treatment compared with the plants from the low-altitude population. The interaction effect shows how the trait in the plants from the high-altitude population changes from a cold to warm environment compared with the change observed in plants from the low-altitude population. The most parsimonious models (lower AIC and lowest number of parameters) are presented in bold. Models are presented following the R syntax: response variable ~ predictor variables.

**DISCUSSION**

We hypothesized that the superimposition of the different sources of covariation that generate phenotypic correlations may hamper our capacity to assess the degree to which variation in vegetative and floral traits is decoupled in flowering plants with specialized pollination. We further suggested that manipulating the environment and analysing phenotypic correlations within and among environmental treatments should enable us to distinguish between environmental and developmental correlations. This approach should provide a better...
insight into the mechanisms producing phenotypic correlations and should help understanding if and how phenotypic variation is decoupled between the two types of traits. We illustrated this idea by analysing correlations between leaf size and flower size in *C. rotundifolia*, a bee-pollinated perennial herb, grown at two different temperatures.

Flower size was less variable than leaf size and less sensitive to the temperature treatment. This result supports previous observations showing that floral traits are generally more canalized than vegetative traits against environmental variation (Fenster, 1991; Diggle, 1992; Waitt and Levin, 1993, 1998; Cresswell, 1998; Armbruster *et al.*, 1999; Wolfe and Mazer, 2005; Hansen *et al.*, 2007; Pe´labon *et al.*, 2011), possibly due to the stabilizing selection generated by pollen transfer efficiency (Conner and Sterling, 1995; Cresswell, 1998, 2000; Hodgins and Barrett, 2008; Rosas-Guerrero *et al.*, 2010). Nevertheless, flower size responded to the temperature treatment with a reaction norm opposite to the one observed in leaf size. Flower size decreased with an increasing temperature, while leaf size increased. Interestingly, these patterns of phenotypic plasticity were mimicking patterns of variation generated by differences in altitude among populations. Plants from the low-altitude population that experienced a warmer environment grew larger leaves than the plants from the high-altitude population, but developed smaller flowers in both experimental treatments. Because these plants were second greenhouse generation, we interpreted these differences as resulting from genetic differences among populations.

The decrease in plant size with altitude (or latitude) has been generally interpreted as resulting from the negative effect of low temperature on plant growth (Larcher, 2003). Our results show that such an effect of altitude in *C. rotundifolia* is generated by the combination of phenotypic plasticity and genetic differences among populations, a pattern also observed by Olsson and A˚gren (2002) in *Lythrum salicaria*, Byars *et al.* (2007) in *Poa hiemata*, and Gonzalo-Turpin and Hazard (2009) in *Festuca eskia*. Similarly, while the increase in flower size with altitude (Tureson, 1925; Shetler, 1982; Galen *et al.*, 1987; Scobel and Scott, 2002; Fabbro and Körner, 2004; Maad *et al.*, 2013; but see Totland, 2001, 2004) has been generally interpreted as an adaptive response to different selection pressures (i.e. local adaptation), our results show that flower size variation also results from phenotypic plasticity in response to

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**FIG. 3.** Effects of the temperature treatment on leaf size (leaf area in mm) and flower size (PC1 scores) in both populations (low- and high-altitude, as indicated in the key). Means (± s.e.) are estimates from mixed-effects models.

**FIG. 4.** Within- and among-treatment phenotypic correlations between leaf size (leaf area in mm) and flower size (PC1 scores) in each population (cold and warm environments, as indicated in the key). Dotted lines represent the regression across environment while the solid lines represent the regressions within environment. Among-treatment correlation coefficients (95% confidence interval) are \( r = -0.21 \) (−0.36 to −0.05) and \( r = -0.09 \) (−0.26 to 0.08), for the high- and low-altitude population, respectively. Within each treatment, the correlation coefficients are: in the cold environment, \( r = 0.34 \) (0.05 to 0.58) and \( r = 0.26 \) (−0.08 to 0.54); and in the warm environment, \( r = 0.12 \) (−0.08 to 0.30) and \( r = 0.03 \) (−0.17 to 0.23), for the high- and low-altitude population, respectively.
temperature variation. Similar variations in flower size have been observed for other abiotic factors such as water availability (Galen, 1999; Carroll et al., 2001; Herrera, 2005; Caruso, 2006), soil nutrients (Delesalle and Mazer, 1996; Frazee and Marquis, 2004) or temperature (Vogler et al., 1999; Catley et al., 2002; (J. Maad, Uppsala University, Sweden, and W. S. Armbruster, University of Portsmouth, UK, unpubl. res.). Note also that our results do not support the hypothesis of a trade-off between flower size and flower number mediated by altitude, a result also observed in the congeneric C. rapunculoides (Volger et al., 1999).

Reaction norms in response to the temperature treatment for flower size and leaf size were in opposite directions, inducing a negative environmental correlation between the two series of traits in one population, and the decoupling of the variation in the other population. When plants were grown under similar conditions, however, positive correlations between the two size measurements were observed for both populations in the cold environment, but not in the warm environment. These results support the idea that, in C. rotundifolia, the decoupling of the phenotypic variation between vegetative and floral traits depends on the relative magnitude of the different sources of variation generating phenotypic correlations. Under limited variation in temperature, developmental correlations (correlations measured within treatment in our study) prevail, and positive correlations are observed between vegetative and floral traits, suggesting an incomplete decoupling of the phenotypic variation between the two types of trait. When temperature variation increases, developmental correlations are gradually overshadowed by the effect of phenotypic plasticity that generates negative environmental correlations. Consequently, conclusions about the decoupling of the phenotypic variation between flower size and leaf size depend on the relative contribution of environmental correlations to the overall phenotypic correlation, this contribution depending on the level of environmental variation. Such a scenario provides a possible explanation for the environmental dependency of phenotypic correlations often observed in plants (Schlichting, 1989; Donohue and Schmitt, 1999; Volger et al., 1999; Brock and Weinig, 2007) and for the inconsistent results of the studies testing the Berg hypothesis.

The Berg hypothesis has often been interpreted in terms of phenotypic integration and modularity (Armbruster et al., 2004). These two variational properties of complex organisms are considered to result from genetic integration (via pleiotropy) and parcellation, respectively (Cheverud, 1996; Wagner, 1996; Wagner and Altenberg, 1996). Although the role of parcellation of genetic effects in the modularity and the decoupling of the phenotypic variation between vegetative and floral traits has been demonstrated in Arabidopsis thaliana (Jaeger et al., 2005), our results also show that both environmental canalization and trait- or module-specific phenotypic plasticity can contribute to the decoupling of phenotypic variation. Environmental canalization of flower size (i.e. buffering of the floral traits against environmental variation) has long been recognized and confirmed by numerous studies, including this one, showing the relative consistency of floral traits compared with vegetative traits in the face of environmental variation. The second mechanism, consisting of reaction norms in opposite directions in leaf size and flower size, has not been recognized as a mechanism generating decoupling of the phenotypic variation. It remains unknown whether reaction norms in opposite directions evolved as the result of selection for decoupled variation or simply because of independent evolution of adaptive plasticity in flower size and plant size. In our case, the second scenario seems more likely due to the apparent disconnection between the mechanisms involved in the phenotypic response to temperature variation of the two types of traits (but see Chapin et al., 1993). Indeed, while the phenotypic response of flower size to temperature variation

### Table 3. Among- and within-treatment relationship between flower size and leaf size

<table>
<thead>
<tr>
<th>Fixed effects</th>
<th>AIC</th>
<th>AIC weights</th>
<th>Intercept</th>
<th>Leaf size</th>
<th>Population effect: high altitude</th>
<th>Interaction effect: leaf size × altitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among environments</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Flower size ~ leaf size × Pop</td>
<td>1092.76</td>
<td>0.434</td>
<td>0.036 ± 0.527</td>
<td>-0.205 ± 0.116</td>
<td>2.248 ± 0.720</td>
<td>-0.205 ± 0.169</td>
</tr>
<tr>
<td>Flower size ~ leaf size + Pop</td>
<td>1092.76</td>
<td>0.562</td>
<td>0.036 ± 0.527</td>
<td>-0.205 ± 0.116</td>
<td>2.248 ± 0.720</td>
<td>-0.205 ± 0.169</td>
</tr>
<tr>
<td>Flower size ~ leaf size</td>
<td>1113.76</td>
<td>1.19 × 10^-5</td>
<td>-0.205 ± 0.116</td>
<td>2.248 ± 0.720</td>
<td>-0.205 ± 0.169</td>
<td></td>
</tr>
<tr>
<td>Flower size ~ Pop</td>
<td>1102.23</td>
<td>0.004</td>
<td>-0.205 ± 0.116</td>
<td>2.248 ± 0.720</td>
<td>-0.205 ± 0.169</td>
<td></td>
</tr>
<tr>
<td>Flowering date ~ leaf size</td>
<td>1128.46</td>
<td>7.67 × 10^-9</td>
<td>-0.205 ± 0.116</td>
<td>2.248 ± 0.720</td>
<td>-0.205 ± 0.169</td>
<td></td>
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<tr>
<td>Cold environment</td>
<td></td>
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<tr>
<td>Flower size ~ leaf size × Pop</td>
<td>314.67</td>
<td>0.343</td>
<td>-1.977 ± 1.336</td>
<td>0.636 ± 0.444</td>
<td>0.925 ± 1.801</td>
<td>0.536 ± 0.627</td>
</tr>
<tr>
<td>Flower size ~ leaf size + Pop</td>
<td>313.46</td>
<td>0.627</td>
<td>-2.716 ± 0.957</td>
<td>0.894 ± 0.310</td>
<td>2.395 ± 0.469</td>
<td></td>
</tr>
<tr>
<td>Flower size ~ leaf size</td>
<td>331.96</td>
<td>6.0 × 10^-5</td>
<td>-2.716 ± 0.957</td>
<td>0.894 ± 0.310</td>
<td>2.395 ± 0.469</td>
<td></td>
</tr>
<tr>
<td>Flower size ~ Pop</td>
<td>315.55</td>
<td>0.030</td>
<td>-0.526 ± 1.336</td>
<td>0.636 ± 0.444</td>
<td>0.925 ± 1.801</td>
<td>0.536 ± 0.627</td>
</tr>
<tr>
<td>Flowering date ~ leaf size</td>
<td>334.59</td>
<td>1.6 × 10^-5</td>
<td>-2.716 ± 0.957</td>
<td>0.894 ± 0.310</td>
<td>2.395 ± 0.469</td>
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<tr>
<td>Warm environment</td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Flower size ~ leaf size × Pop</td>
<td>742.60</td>
<td>0.132</td>
<td>-1.492 ± 0.526</td>
<td>0.091 ± 0.108</td>
<td>1.324 ± 0.265</td>
<td></td>
</tr>
<tr>
<td>Flower size ~ leaf size + Pop</td>
<td>740.95</td>
<td>0.302</td>
<td>-1.492 ± 0.526</td>
<td>0.091 ± 0.108</td>
<td>1.324 ± 0.265</td>
<td></td>
</tr>
<tr>
<td>Flower size ~ leaf size</td>
<td>760.44</td>
<td>1.8 × 10^-5</td>
<td>-1.492 ± 0.526</td>
<td>0.091 ± 0.108</td>
<td>1.324 ± 0.265</td>
<td></td>
</tr>
<tr>
<td>Flower size ~ Pop</td>
<td>739.69</td>
<td>0.566</td>
<td>-1.074 ± 0.189</td>
<td>1.293 ± 0.263</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flowering date ~ leaf size</td>
<td>758.49</td>
<td>4.7 × 10^-5</td>
<td>-1.074 ± 0.189</td>
<td>1.293 ± 0.263</td>
<td></td>
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</tbody>
</table>

These are the results of mixed-effect models where flower size is regressed on leaf size with population as fixed factor and family as a random factor. Estimates (+ s.e.) were obtained from models fitted with REML. We present here estimates for the best or the two best models when ΔAIC between competing models was < 2 AIC units. The most parsimonious models (lower AIC and lowest number of parameters) are presented in bold.
mimics the expected adaptive response to size variation or rarification of pollinators with altitude, size variation of the vegetative parts essentially reflects the effect of temperature on growth (note that the variation in plant size due to local adaptation is limited compared with the temperature-induced variation).

Overall, these results show that phenotypic correlations among traits may depend on the environment, but also on the degree of environmental variation. While providing a possible explanation for the inconsistencies encountered among studies testing the Berg hypothesis, these results also underline the importance of distinguishing the different sources of covariation if we are to understand the evolution of morphological integration and modularity in flowering plants.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: variation in the temperature of the two rooms during the experiment. Table S1: descriptive statistics of the recorded traits in each population and each environment. Table S2: results of the principal component analysis on the floral traits.

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LITERATURE CITED


