

Experimental evidence for the Berg hypothesis: vegetative traits are more sensitive than pollination traits to environmental variation

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Summary

1. In plants with specialized pollination, functionally important floral traits are expected to be under strong selection for accuracy. This may, however, conflict with a general tendency for size-related traits to covary. Previous studies have addressed this fundamental conflict by analysing natural variation across samples of structures, but here we compare the effects of experimentally induced environmental differences on variation in serially homologous pollination and vegetative traits.

2. We examined the effects of experimental variation in nutrient availability and total daily irradiance on two pairs of serially homologous traits in two populations of *Dalechampia scandens*: (i) The length of the floral bract blade and the length of the leaf blade, and (ii) the length of stipules associated with bracts and leaves. The first pair contrasts a floral trait that is likely to experience canalizing selection (bract blade) with a homologous vegetative trait that does not experience canalizing selection (leaf blade). The second contrasts homologous floral and vegetative traits that are likely to experience similar selective pressures. We also examined variational properties of two blossom traits that interact directly with pollinators: the area of the resin gland and the length of the styles.

3. Variation in the bract blades was decoupled from variation in the vegetative traits and followed the variational patterns of the two blossom traits that are functional in pollination. Stipules associated with bracts and leaves were affected similarly by the experimental treatments in a pattern characteristic of vegetative traits.

4. These results are consistent with the Berg hypothesis of decoupling and canalization of specialized floral structures and support the idea that the variability can evolve in response to selection on variation.

Key-words: Berg hypothesis, canalization, *Dalechampia scandens*, homology, insect pollination, modularity, integration, phenotypic plasticity

Introduction

Structurally adjacent or developmentally interrelated characters show strong tendencies to covary in size (Olson & Miller 1958). Nevertheless, plants with specialized animal pollination may often be under strong selection for accuracy of floral trait expression, because flower size variation is likely to be maladaptive when pollinators are more or

less fixed in size and behaviour (Armbruster *et al.* 2004, 2009a). Berg (1959, 1960) was among the first to suggest a pattern of modular variance, wherein specialized flowers are partly decoupled from phenotypic variation in vegetative parts as a result of canalizing selection generated by pollinators. Berg further suggested that the decoupling of the phenotypic variance between reproductive and vegetative traits creates correlation pleiades, i.e. groups of traits showing stronger phenotypic correlation among traits belonging to the same group than among traits belonging

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to different groups or pleiades. This perspective is consistent with the contemporary emphasis on modular partitioning of the variation along functional lines (Wagner 1996; Wagner & Altenberg 1996; Hansen 2006; Wagner, Pavlicev & Cheverud 2007) and is also central in the concept of phenotypic integration (Armbruster *et al.* 2004; Pigliucci & Preston 2004; Mitteroecker & Bookstein 2007; Klingenberg 2008; Hallgrímsson *et al.* 2009; Mitteroecker 2009).

One of the underlying assumptions of Berg's hypothesis is that trait variability, the capacity of a trait to vary (Wagner & Altenberg 1996), may be affected by selection on variation. Selection on variation, which depends on the convexity of the fitness function (Layzer 1980), should affect the organisms' or traits' sensitivity to genetic and environmental differences. This sensitivity, in turn, determines the organisms' or traits' variability. Traditionally, we define the mechanisms buffering genetic and environmental differences as genetic and environmental canalization, respectively (Wagner, Booth & Bagheri-Chaichian 1997; Flatt 2005). It is currently unclear to what extent selection for accuracy is able to mould genetic variation and variability in the face of pleiotropic constraints (Hansen 2006, 2010; Hallgrímsson *et al.* 2009; Pélabon *et al.* 2010). Nevertheless, there seems to be a consensus that canalizing selection (selection against variability) resulting from selection for accuracy should favour environmental canalization (Proulx & Phillips 2005; Zhang & Hill 2005), and indirectly, genetic canalization (Wagner, Booth & Bagheri-Chaichian 1997; de Visser *et al.* 2003; Rifkin *et al.* 2005).

The large number of factors that can affect the variability of traits may explain the varying support for Berg's hypothesis provided by empirical studies of variational and covariational patterns in vegetative and reproductive parts in flowering plants (Diggle 1992; Waitt & Levin 1993, 1998; Conner & Sterling 1995, 1996; Armbruster *et al.* 1999; Wolfe & Krstolic 1999; Magwene 2001; Herrera *et al.* 2002; Juenger *et al.* 2005; Brock & Weinig 2007; Hansen, Pélabon & Armbruster 2007; Perez-Barrales, Arroyo & Armbruster 2007; Chalcoff, Ezcurra & Aizen 2008; Ordano *et al.* 2008). Indeed, while reproductive traits generally show lower phenotypic variation than vegetative traits (Fenster 1991; Cresswell 1998; Herrera 2001; Chalcoff, Ezcurra & Aizen 2008), this pattern is not restricted to species with a specialized pollinator (Armbruster *et al.* 1999). Furthermore, it remains unclear whether within- and among-population variation in flower size is generated primarily by differences in abiotic factors or in pollinator fauna. Responses of flower size to variation in water or nutrient availability have been documented (Frazee & Marquis 1994; Galen 1999; Carroll, Palladry & Galen 2001; Herrera 2005; Caruso 2006), and covariation between floral and foliar traits in response to abiotic environmental changes has been observed in some insect-pollinated species (Lambrecht & Dawson 2007). In two studies, the strength of correlation between vegetative and reproductive traits depended on the environment (Waitt & Levin 1993; Brock

& Weinig 2007), suggesting that these correlations may remain hidden when environmental variation is limited, e.g. under greenhouse conditions or during a single season in natural habitat. Therefore, manipulative studies may be necessary to ensure sufficient power to detect unequivocal evidence of the decoupling of phenotypic variation between vegetative and reproductive traits.

Studying the evolution of variational properties may also present difficulties when traits differ in complexity or dimensionality (Dworkin 2005; Hallgrímsson *et al.* 2009). For example, Herrera, Arista & Ortiz (2008) showed that petal fusion (connation) can affect patterns of floral variation. Furthermore, traits have often been classified as reproductive or vegetative solely on the basis on whether or not they pertained to the flower, without adequate consideration of the type or strength of selection that may affect each particular trait (Diggle 1992; Ordano *et al.* 2008). While strong stabilizing selection is expected to result from the selection for accuracy on traits directly involved in the transfer of the pollen to and from the pollinator (Cresswell 2000; Armbruster *et al.* 2009a,b) selection on other floral traits with different functions may be weaker or even of a different nature, such as directional selection (Hodgins & Barrett 2008; Boberg & Ågren 2009; Sánchez-Lafuente & Parra 2009). In this context, comparing patterns of variation between homologous or adjacent structures with different functions should allow teasing apart the effects of selection on variation generated by the different functions from the effects of the development (similar developmental pathway and/or structural adjacency) when comparing variational properties (Young & Hallgrímsson 2005; Hansen, Pélabon & Armbruster 2007; Hallgrímsson *et al.* 2009).

In the present study, we compare the phenotypic response to artificially induced environmental variation on three blossom traits conjectured to be under canalizing selection with the corresponding response in one blossom trait and two vegetative traits not under such selection. We take advantage of the pseudanthial nature (cluster of flowers forming a blossom-like structure) of the reproductive units of *Dalechampia* vines to compare reproductive and vegetative traits with a high degree of homology, thereby comparing variational properties among traits with different functions while controlling for complexity, dimensionality and structural adjacency as in Hansen, Pélabon & Armbruster (2007). We increased the range of phenotypic variation in the different traits by manipulating the soil nutrients and the amount of light received daily by the plants. In order to test the generality of the observed pattern, environmental variation was imposed on experimental plants from two distant populations of *Dalechampia scandens*, originating from Mexico and Venezuela [likely two cryptic species, see Pélabon *et al.* (2004, 2005)]. Blossoms of these two populations possess reward-producing glands of very different size (average \pm SE area of the gland in the Mexican population: 26.41 ± 0.40 mm²; Venezuelan population: 16.97 ± 0.23 mm²) and are presumably pollinated by different species of bees (Armbruster 1985, 1988).

Materials and methods

STUDY SYSTEM AND EXPECTATIONS

Dalechampia scandens (Euphorbiaceae) is a Neotropical vine with functionally integrated pseudanthial inflorescences, or blossoms, which have specialized gland-like structures producing resin that attracts resin-collecting bees (Webster & Webster 1972; Armbruster 1984, 1996). *Dalechampia scandens* populations are pollinated by large resin-collecting female bees in the genera *Eulaema*, *Eufriesea*, *Euglossa* (Apidae: Euglossini) or smaller *Hypanthidium* (Megachilidae: Anthidiini) and/or worker *Trigona* (Apidae: Meliponini). Local populations of *D. scandens* usually show a degree of adaptation to one size class of bees (Armbruster 1985; Hansen, Armbruster & Antonsen 2000).

The *Dalechampia* blossom comprises one male and one female sub-inflorescence, each subtended by one showy involucre bract (Fig. 1). Bracts and leaves are serially homologous, the former being modified leaves, and they have very similar morphologies (Fig. 1). The derivation of the involucre bracts from leaves is indicated by occasional

developmental errors wherein leaf-like structures are produced at nodes where bracts belong and bract-like structures at short-shoot nodes where leaves belong. Furthermore, *Tragia* and *Plukenetia*, candidate sister genera of *Dalechampia*, have leaves rather than bracts at the base of their inflorescences. Thus, *Dalechampia* bracts allow us to compare the variational properties of two homologous structures under presumably different selective pressures. Indeed, when the flowers are receptive, the bracts open to advertise the flowers to pollinators by day and close to protect them by night (Armbruster 1997). During this period, the bracts are brightly coloured (white or light green in the study populations) and likely serve an advertising function, as shown in *D. scandens* where bract size positively influences pollinator attraction and pollen arrival rate (R. Pérez-Barrales, G.H. Bolstad, C. Pelabon, T.F. Hansen & W.S. Armbruster, unpublished data). Similar results have also been observed in *D. ipomeifolia* (Armbruster, Antonsen & Pélabon 2005). Before and after the receptive period, bracts are photosynthetic and are closed around the developing bud or fruits. The size of the upper and lower bract needs to be coordinated so that the bracts fit together tightly when closed for nocturnal protection of flowers. Bract size in *D. scandens* may also

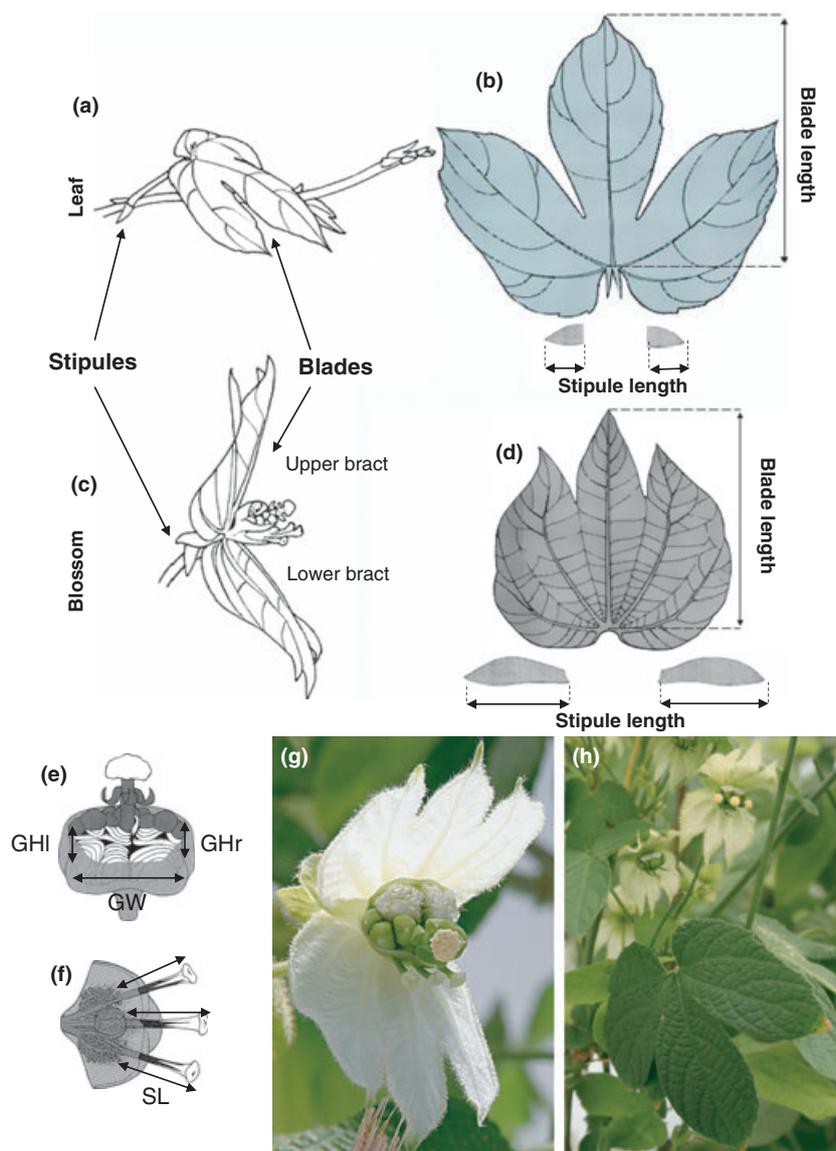


Fig. 1. Drawings representing the different traits measured on the leaf and the blossom of *Dalechampia scandens*. (a) Leaf on a shoot, (b) adaxial view of a leaf and leaf stipules, (c) lateral view of a blossom, (d) adaxial view of the upper bract and bract stipules, (e) cluster of male flowers showing the resin-producing gland, (f) cluster of female flowers showing the style length, (g) blossom with one male flower open (photo P.H. Olsen) (h) blossoms and leaves (photo C. Pélabon). The leaf (or leaf-like bract) has three parts: two stipules, a petiole and a blade. Leaf and bract stipules are located at the insertion of the petiole on the stem. The petioles of the bracts are, however, greatly reduced, the blades being virtually sessile. Hence the bract blades and respective stipules are inserted very near one another. Traits measure on the male cluster: GHI and GHR: gland height left and right, respectively, GW: gland width. Traits measured on the female cluster: SL style length, measured on the three styles.

be under stabilizing selection. In a recent study, Perez-Barrales *et al.* (unpublished data) found that stabilizing selection acted on bract size as the result of conflicting selection generated by pollinators and seed predators. The number of viable seeds produced increased with increasing bract size due to the increase in visitation rate and pollen load, up to a bract area of ca. 320 mm² (population mean = 341.02 ± 8.5 mm²), and then decreased due to increasing seed predation on larger blossoms. These selective pressures and diverse functions suggest that bracts, in contrast to leaves, may experience strong canalizing selection. Therefore, we expect the phenotypic variation in bract blades to be largely decoupled from variation in leaf blades and other vegetative traits, despite their close homology with leaves.

In order to test this hypothesis, we experimentally imposed environmental variation on plants from two different populations of *D. scandens*. We compared the variational properties of leaf blades and bract blades and their associate stipules, and two additional traits directly associated with pollination function, the area of the resin-producing gland and the average length of the styles (Fig. 1). Rather than simply distinguishing between vegetative (leaf) and reproductive (blossom) traits in developing expectations for the variational properties of the different traits, we based our expectations on hypothesized differences in selective pressures acting on the traits (as summarized in Fig. 2). For example, the two photosynthetic stipules associated with each involucre bract are not visible to pollinators visiting open blossoms, nor are they involved in any aspect of pollination or bract closure. Therefore, their size is presumably not under pollinator-mediated selection, and their variational properties should be similar to those of vegetative traits despite being part of the blossom. We also presume that pollinators do not select on leaf-blade or leaf-stipule traits, because the leaves are not physically close to the flowers and pollinators do not visit the leaves.

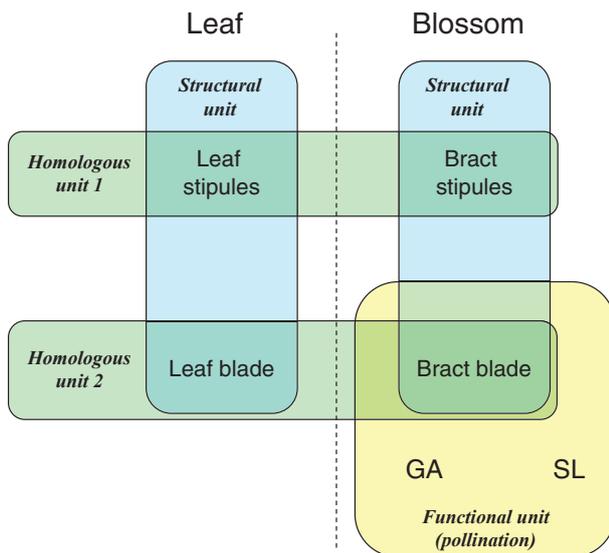


Fig. 2. Diagram explaining the nature of the comparisons performed in this study. We compare the variational properties of two pairs of serially homologous traits; Homologous unit 1: leaf and bract stipules; homologous unit 2: leaf and bract blades. This also allows us testing the effect of structural proximity when comparing variation between stipules and blade in leaf or bract (structural units). We also compare the patterns of variation among functionally related traits, i.e. blossom traits involved in the interaction with pollinators (functional unit).

On the other hand, style length affects the distance between the resin-producing gland and the stigma, and therefore affects the accuracy of the pollination function, which depends on the fit between the pollinator, the pollen-bearing (anther) and the pollen-receiving (stigma) structures (Armbruster 1988; Armbruster *et al.* 2004, 2009a). Size variation in the position of the stigma relative to the gland, which determines the pollinator's position on the blossom, should be maladaptive. Consequently, we expect style length to be less responsive to environmental variation than vegetative structures. The area of the resin-producing gland is a measure of the resin-secreting surface and correlates with the amount of resin produced by the blossom. It influences which species of bee will visit the blossoms, predicting the size of the largest visitors (Armbruster 1984, 1988). Variation in gland area (GA) within a population might attract different pollinator species, compromising the effectiveness of the population's pollination system (e.g. Armbruster 1985, 1996). Furthermore, a larger gland would also increase the variation in the pollinator placement on the blossom when collecting the resin and would decrease the precision of the fit between the male and female function (Armbruster *et al.* 2009a). For these reasons, gland area is also expected to be insensitive to environmental variation. We thus predict bract blades to be less affected by environmental variation than vegetative traits and to display patterns of phenotypic variation similar to the reproductive traits (gland area and style length) despite their morphological and developmental differences.

There is one additional level of homology available for comparison in this system. The two bracts in a blossom are homologous to two leaves at adjacent nodes on a shoot; the lower bract is homologous to the proximal leaf (closer to the base of the shoot), and the upper bract is homologous to the distal leaf [Fig. 1a; see Webster & Webster (1972)]. Because the position affects bract length [Hansen *et al.* (2003); and see results], it is possible to test whether this effect is present also in the homologous leaves or is instead unique to bracts.

EXPERIMENTAL DESIGN AND MEASUREMENTS

Individuals from the two populations used in this experiment were the second greenhouse generation derived from seeds collected originally in the state of Quintana Roo, Mexico (20°13'N, 87°26'W) and near Tovar, Venezuela (8°21'N, 71°46'W). Before being placed in the different treatments, all the individuals were grown for ca. 1 year under long days and weekly fertilization (see below). In early September 2006, 136 individuals from each population were placed in the experimental environments (34 individuals per population in each of the four environments) and left to grow until mid-December 2006, when measurements were made over a period of 1 month.

We varied the quantity of fertilizer and the daily amount of light received by the plants. In the high-nutrient treatment, plants were fertilized weekly, while plants from the low-nutrient treatment were fertilized every third week. The fertilizer was applied similarly to all groups by flooding the storage tables with 5-cm deep water containing fertilizer for 20 min. Plants were also watered daily using the same technique. We induced variation in total daily irradiance by varying the day length of the supplementary lighting because it was easier than varying the irradiance intensity for a large number of plants. Plants from the high-light treatment were exposed to a L : D cycle of supplementary lighting of 13 : 11, while plants from the low-light treatment were exposed to a L : D cycle of 8 : 16, the irradiance per unit of time being constant during the light period and similar in the two treatments. Because the experiment was conducted from late September to December 2006 in Trondheim Norway (63°24'N), the amount of additional natural light was very low and similar across all

treatments. The high-light, high-nutrient treatment corresponded to the normal conditions used for growing *Dalechampia* in the greenhouse. These experimental treatments reflected habitat variation encountered by the species in nature, because *D. scandens* ranges from recently disturbed, sunny, nutrient-rich sites (tree falls, roadsides) to the shaded edges of moist forest, and even shallow acid soils on granite outcrops, which must be limited in soil nutrients.

For practical reasons, the light treatment was confounded with the room in which the plants were grown (one room with high light, one room with low light), while the nutrient treatment was confounded with the table on which the plants were placed (one table with high nutrient and one with low nutrient in each room for each population). Although this experimental design prevented us from estimating the exact effect of each treatment, it did not represent a problem for our study because our primary aim was to compare induced phenotypic variation between vegetative and reproductive traits, and the source of the variation is then irrelevant. Nevertheless, the room effects are likely to be limited compared with the effects of the experimental treatments. Indeed, the replication of the nutrient treatment in the two rooms with different light regime produced similar effects on the leaf blades of both populations. For readability, we will therefore refer to the two treatments with the terms 'light' and 'nutrient'.

On each individual, we measured two blossoms and two leaves at adjacent nodes on two separate branches (four bracts and four leaves in total). In order to control for the ontogenetic variation in blossom size, we measured the blossoms on the day the first (terminal) male flower opened. Leaves take approximately 30 days to reach full size (Pélabon *et al.* 2006), and plants generally grow by sending long twining shoots in several directions. To measure fully-expanded leaves, plants were left unpruned for ca. 2 month so that mature leaves could be obtained. We measured leaves near the base of the shoot (but not the basal-most leaf). We measured the length of the leaf blade, leaf stipules, bract blade and bract stipules in order to compare the variation using the most homologous measurements (Fig. 1). The gland area is estimated as the width of the gland multiplied by the average heights of the left and right half gland (Fig. 1). However, in order to keep the same measurement units (i.e. mm) across the different traits, we conducted the analyses on the square root of the gland area. The style length corresponds to the average length of the three styles. All measurements were made by a single observer (C.P.) using a digital calliper (0.01 mm precision) and optical magnifier ($\times 5$). All structures except the resin-producing gland were carefully removed and flattened under an acetate sheet before measurement. The stipule length corresponds to the average length of the left and right stipules. Leaf and bract blades were measured with the adaxial surface of the organ facing upward.

STATISTICAL ANALYSES

In order to test whether the phenotypic variation of traits involved in the pollination process was decoupled from the phenotypic variation of traits not involved in this process, we first analysed the direction and the strength of the response of the different traits to the experimental treatments. We further tested the differences in environmental canalization between traits by comparing mean-scaled variance components across traits.

In the first set of analyses, although proximal and distal structures could be considered as repeated measures of the same trait, we observed systematic differences between these two types of structures (see results), and in some cases the magnitudes of these differences were affected by the treatments. For simplicity, we present the results for only the distal ones (results for the proximal structures are pre-

sented as Supporting Information). Furthermore, the phenotypic variation induced by the treatments depended on the population (statistically significant interaction between treatment and population, not shown). In order to present these effects as clearly as possible we present separate analyses for the two populations. We applied linear mixed-effects models to each trait, where light (low vs. high) and nutrient (low vs. high) treatments were entered as fixed factors, and the individual plant identity as random factor. We selected the best models using Akaike's Information Criterion (AIC) on models fitted with restricted maximum likelihood (REML). None of the residual distributions departed noticeably from normality.

In the variance-component analyses, we partitioned the variance into among-treatment, among-individual within-treatment and within-individual components. Therefore, we combined light and nutrient treatments into a single factor with four levels (2×2) to estimate the total amount of phenotypic variation induced by the environmental manipulation. In order to compare the level of variation between structures of different size, we mean-scaled the variance components. Mean-scaled variances [$\text{var}(x)/\bar{x}^2$] are equal to the square of the CV, but have the advantage of being additive. We obtained the confidence intervals of the mean-scaled variances by resampling the posterior distribution of the parameters of the variance-component analyses using Markov Chain Monte Carlo methods. All statistics were performed in R 2.5.1 (<http://www.r-project.org>).

Results

Proximal leaf blades were on average 10% and 15% smaller than the distal leaves in the Mexican and Venezuelan population, respectively (Fig. 3). In contrast, the proximal (lower) bract blades were, respectively, 14% and 9% longer than the distal (upper) bracts in the two populations (Fig. 3). Similarly to leaf blades, proximal leaf stipules were on average 13% smaller than the distal ones in both populations. Proximal bract stipules were, in a pattern opposite to the bract blade, 8% smaller than the distal ones in the Mexican population, while they were less than 1% different in the Venezuelan population (Fig. 3).

Variation in nutrient availability had a dramatic effect on the length of the leaf blades (Tables 1 and 2, Tables S1 and S2, Fig. 4). Leaf blades from plants grown in nutrient-rich environments were on average 46% and 30% longer than the ones produced in nutrient-poor environments in the Mexican and Venezuelan population, respectively. In contrast, the light treatment had a limited effect on this trait. Leaf blades produced under low light in the Mexican population were on average 8% longer than the ones produced under high light, independently of nutrient availability (Table 1, Table S1, Fig. 4a). Furthermore, the light treatment had no effect on the leaf blades in the Venezuelan population or on any other traits, neither in the Mexican nor in the Venezuelan population (Table 2 and Table S2, Fig. 4b).

The effect of fertilization on bract blades contrasted sharply with the effect on leaf blades. High nutrient availability weakly increased, 4%, the size of the bract blade in the Mexican population, and weakly decreased it, 9%, in the Venezuelan population (Fig. 4, Tables 1 and 2, Tables S1 and S2). The variance-component analyses further illustrated the difference between leaf and bract blades (Table 3, Table S3);

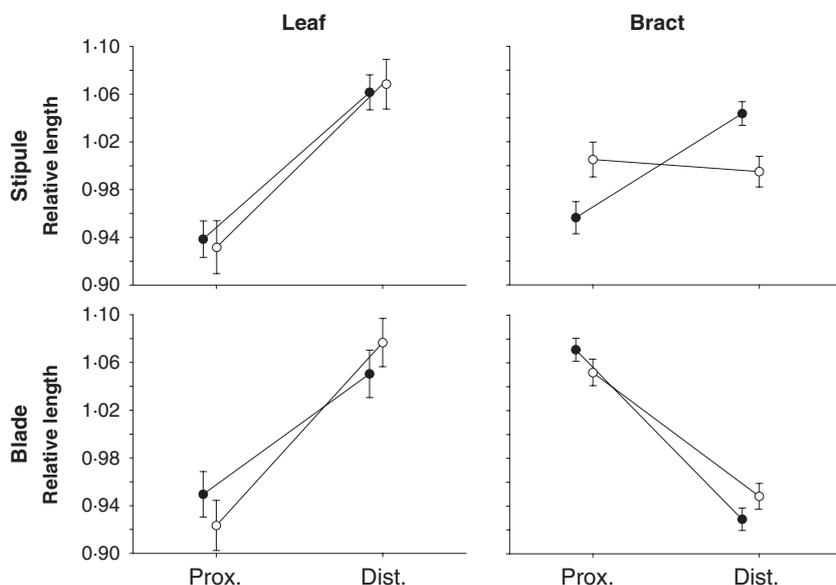


Fig. 3. Effect of the position (proximal vs. distal) on the relative length of leaf stipules, bract stipules, leaf blades, bract blades. The mean trait length (\pm SE) for each position was scaled by the grand mean for both types of traits in order to present the effect size on a similar scale (%) for all traits. Black symbols: Mexican population. Open symbols: Venezuelan population.

Table 1. Effects of the treatments on the different traits for the Mexican population

Trait	Fixed effects	AIC	Nutrient rich high light	Change to nutrient poor	Change to low light	Change to low light and nutrient poor
Leaf stipules	Full model	587.05	8.04 \pm 0.19	-0.54 \pm 0.30	-0.08 \pm 0.26	0.08 \pm 0.39
	Nutrient	583.14	8.00 \pm 0.13	-0.50 \pm 0.19		
Bract stipules	Full model	825.26	11.16 \pm 0.14	-0.80 \pm 0.20	-0.39 \pm 0.21	0.09 \pm 0.29
	Nutrient + light	823.37	11.14 \pm 0.12	-0.75 \pm 0.15	-0.34 \pm 0.15	
Leaf blade	Full model	2173.34	104.02 \pm 1.67	-35.05 \pm 2.36	4.38 \pm 2.38	2.87 \pm 3.36
	Nutrient + light	2172.09	103.31 \pm 1.45	-33.63 \pm 1.68	5.81 \pm 1.68	
Bract blade	Full model	1167.45	20.82 \pm 0.30	-0.65 \pm 0.42	-0.20 \pm 0.43	0.41 \pm 0.61
	Nutrient	1163.90	20.72 \pm 0.21	-0.45 \pm 0.30		
$\sqrt{\text{Gland area}}$	Constant	1164.17	20.49 \pm 0.15			
	Full model	506.30	5.22 \pm 0.08	-0.21 \pm 0.12	-0.08 \pm 0.12	0.28 \pm 0.17
	Nutrient	505.64	5.19 \pm 0.06	-0.07 \pm 0.09		
Style length	Constant	504.40	5.14 \pm 0.04			
	Full model	660.60	7.37 \pm 0.11	-0.39 \pm 0.15	-0.10 \pm 0.15	0.40 \pm 0.22
	Nutrient	660.80	7.32 \pm 0.08	-0.20 \pm 0.11		

Only the structures in distal position are considered here; the analyses for the proximal structures are presented in Table S1. Estimates (\pm SE) from the models fitted with REML are presented for the full model including light and nutrient with their interactions as fixed factor and plant identity as random factor, and for the best, or the two best models, when Δ AIC < 2 between competing models. Estimates are given in mm starting from a structure in distal position in the nutrient rich and high light treatment. The effect for nutrient and light show how the length of the structure changes when plants are grown under nutrient-poor and low-light environment.

Table 2. Effects of the treatments on the different traits for the Venezuelan population

Trait	Fixed effects	AIC	Nutrient rich high light	Change to nutrient poor	Change to low light	Change to low light and nutrient poor
Leaf stipules	Full model	884.05	10.97 \pm 0.36	-0.89 \pm 0.55	0.27 \pm 0.53	-1.44 \pm 0.77
Bract stipules	Full model	737.83	9.04 \pm 0.18	-0.49 \pm 0.25	-0.31 \pm 0.27	0.37 \pm 0.38
	Nutrient	735.18	8.91 \pm 0.14	-0.33 \pm 0.19		
Leaf blade	Full model	2063.58	96.38 \pm 1.91	-28.75 \pm 2.73	-0.51 \pm 2.89	4.34 \pm 4.00
	Nutrient	2061.59	96.16 \pm 1.43	-26.62 \pm 1.98		
Bract blade	Full model	1010.21	19.00 \pm 0.36	1.33 \pm 0.51	-0.57 \pm 0.54	0.76 \pm 0.75
	Nutrient	1007.45	18.75 \pm 0.27	1.66 \pm 0.37		
$\sqrt{\text{Gland area}}$	Full model	223.27	4.12 \pm 0.06	0.13 \pm 0.08	-0.47 \pm 0.09	0.41 \pm 0.12
Style length	Full model	647.52	7.89 \pm 0.16	0.38 \pm 0.23	-0.05 \pm 0.25	0.26 \pm 0.34
	Nutrient	637.44	7.87 \pm 0.12	0.51 \pm 0.17		

Only the structures in distal position are considered here; the analyses for the proximal structures are presented in Table S2. See caption of Table 1 for details.

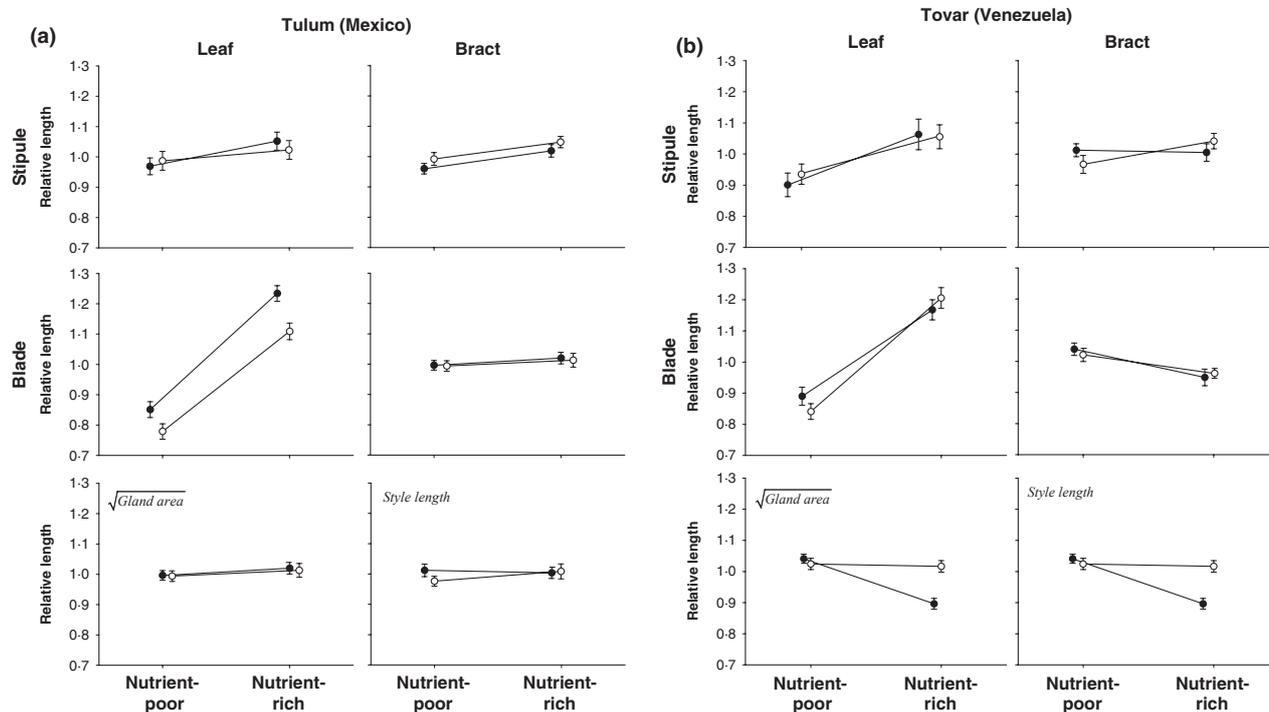


Fig. 4. Effects of the different treatments on the relative length of leaf stipules, bract stipules, leaf blades, bract blades, the gland area and style length in the Mexican (a) and Venezuelan (b) populations. For the stipules and blades, only the distal structures are considered here. Open symbols: High-light treatment. Black symbols: Low-light treatment. Mean trait length (\pm SE) in each treatment was scaled by the overall trait mean in order to present the effect size on a similar scale for all the traits.

most of the variance in the leaf blade length was generated by the treatments, with more than 67% and 51% of the variance resulting from changes in light and nutrient availability in the Mexican and Venezuelan populations, respectively. In contrast, less than 1% of the phenotypic variance in bract blade was generated by the experimental treatments in the Mexican population, while 18% was generated in the Venezuelan population.

Stipules were only weakly affected by the treatments (Tables 1 and 2, Tables S1 and S2, Fig. 4); most of the variance in leaf and bract stipules was observed at the within-individual level while the variation generated by the treatments ranged from 1% to 15% of the total variance (Table 3, Table S3). The high within-individual variance may be either due to poor developmental stability or due to measurement error. Because part of the measurement error can have resulted from the removal of the stipules from the plant, this measurement error cannot be estimated. Nevertheless, the effects of the treatments on both types of stipules were in the same direction as the effects on leaf blades.

The gland area and the style length showed the same pattern as the bract blades (Fig. 4, Tables 1 and 2), confirming the variational decoupling of pollination traits from vegetative traits. The lower mean-scaled variances of these traits (Table 3) also support stronger canalization of traits involved in pollination. As for bract blades, the levels of phenotypic variance induced by the treatments in the style length and gland area were population dependent. In the

Mexican population, the traits involved in pollination were essentially not affected by the light and fertilization treatments (Tables 1 and 3, Fig. 4). In the Venezuelan population, however, the blossom traits involved in pollination displayed some variation due to the fertilization treatments in interaction with the light treatment, but in a direction opposite to that observed in vegetative traits, i.e. with the largest structures observed under nutrient-poor environment (Fig. 4b). Although relatively limited in magnitude, this effect was consistent across traits involved in pollination and represented from 6% (in style length) to 32% (in gland area) of the total phenotypic variance (Table 3).

Discussion

All pollination traits displayed less phenotypic variation than traits not involved in pollination, confirming our previous observations in *D. scandens* under normal greenhouse conditions (Hansen, Pélabon & Armbruster 2007). However, we also found that the degree of environmental canalization of the pollination traits varied between populations. While the length of the bract blade, the style length and the gland area were nearly invariant in the Mexican population, these traits were weakly but consistently affected by the fertilization treatment in the Venezuelan population. Unexpectedly, these changes were in opposite direction as the changes observed in vegetative traits; larger structures were produced under the nutrient-poor conditions.

Table 3. Variance-component analysis and mean-scaled variances

Trait	Level of variation	Tulum (Mexico)		Tovar (Venezuela)	
		Mean-scaled variance, % (95% CI)	Percentage of total variance	Mean-scaled variance, % (95% CI)	Percentage of total variance
Leaf stipules	Among treatment	0.08 (0.00; 2.13)	3.07	0.92 (0.15; 11.39)	14.90
	Among individual in treatment	0.33 (0.00; 0.53)	13.00	1.99 (0.00; 4.05)	32.17
	Within individual	2.15 (2.04; 3.19)	83.93	3.27 (2.59; 6.26)	52.93
	Sum	2.56	100	6.18	100
Bract stipules	Among treatment	0.19 (0.04; 2.20)	13.80	0.02 (0.00; 0.68)	1.10
	Among individual in treatment	0.09 (0.00; 0.29)	6.54	0.67 (0.29; 1.22)	32.61
	Within individual	1.08 (0.95; 1.39)	79.66	1.37 (1.07; 1.87)	66.29
	Sum	1.36	100	2.06	100
Leaf blade	Among treatment	3.99 (1.04; 39.61)	67.17	3.39 (0.88; 37.36)	51.42
	Among individual in treatment	0.00 (0.00; 0.00)	0.00	0.40 (0.00; 0.88)	6.07
	Within individual	1.95 (1.66; 2.34)	32.83	2.80 (2.51; 3.84)	42.51
	Sum	5.94	100	6.59	100
Bract blade	Among treatment	0.00 (0.00; 0.00)	0.00	0.29 (0.57; 0.99)	17.88
	Among individual in treatment	0.31 (0.00; 0.40)	26.23	0.61 (0.36; 0.96)	37.45
	Within individual	0.86 (0.74; 1.23)	73.77	0.73 (0.57; 0.99)	44.67
	Sum	1.17	100	1.63	100
$\sqrt{\text{Gland area}}$	Among treatment	0.01 (0.00; 0.09)	0.43	0.44 (0.00; 0.60)	31.65
	Among individual in treatment	0.27 (0.00; 0.55)	17.98	0.25 (0.00; 1.02)	18.22
	Within individual	1.21 (1.09; 1.77)	81.59	0.70 (1.68; 2.80)	50.13
	Sum	1.49	100	1.39	100
Style length	Among treatment	0.05 (0.00; 1.06)	3.80	0.10 (0.00; 0.00)	5.58
	Among individual in treatment	0.18 (0.00; 0.54)	13.31	0.90 (0.64; 1.43)	51.29
	Within individual	1.13 (1.01; 1.63)	82.89	0.76 (0.58; 1.03)	43.13
	Sum	1.36	100	1.76	100

Components of the variance are expressed in percent. Mean-scaled variances are calculated as the components of the variance divided by the mean square. Confidence intervals (95% CI) were obtained by resampling method (see main text for details). Only the structures in distal position are considered here; analyses for the proximal structures are presented in Table S3).

RESPONSES TO LIGHT AND NUTRIENT TREATMENTS

Responses of vegetative traits to differences in daily irradiance and fertilization for the most part conform to the expected effects for such factors. Plants grown in the nutrient-limited environments produced smaller structures, while leaves produced under low light were larger. It is unclear, however, whether the phenotypic plasticity in leaf size associated with variation in daily irradiance reflects response mechanisms similar as in sun vs. shade leaf (Sleeman *et al.* 2002; Steinger, Roy & Stanton 2003; Avramov, Pemac & Tucic 2007), or instead reflects photoperiodic response (Adams & Langton 2005; Cookson, Chenu & Garnier 2007). Our data are more consistent with a sun–shade mechanism because larger leaves are generally produced under low daily irradiance, while smaller leaves are produced under short-day conditions (Adams & Langton 2005).

Although increasing nutrient availability generally enhances seed production via an increase in the number of flowers (Campbell & Halama 1993; Nagy & Proctor 1997; Muñoz *et al.* 2005; but see Heer & Körner 2002; Burkle & Irwin 2009), the effects of fertilization on flower size in insect-pollinated plants are rather unpredictable. For example, no effect was reported in *Linum lewisii*, while only a weak positive effect, compared with the effect on vegetative traits, was reported on *Ipomopsis aggregata* (Burkle & Irwin 2009).

Frazer & Marquis (1994) is the only report of decreasing in flower size with an increasing level of nutrient availability, but this effect was reversed under water stress. It is therefore difficult to interpret the observed increase in flower size in the nutrient-limited environment in the Venezuelan population. One can hypothesize that plants react to a sudden decrease in nutrient availability by increasing their reproductive effort, as suggested by the terminal-investment hypothesis (Bell 1980). Although an increasing size of the blossom in *D. scandens* does not increase the number of seed produced (there is a fixed number of nine ovules per blossom), it can still increase the quality of seeds if this size increase is correlated with an augmentation of the resources allocated to the developing fruits. Alternatively, larger bracts may increase the visitation rate and therefore the pollen load that, in turn, will favour pollen competition and possibly the genetic quality of the seeds produced (Mulcahy 1979; Armbruster & Rogers 2004; Lankinen & Armbruster 2007). The observed response may also result from a non-adaptive (even maladaptive) overcompensation in the resource allocation to the blossom under nutrient-limited environment.

The absence of phenotypic variation induced by the light treatment on bract blades is not entirely surprising. First, the effect of this treatment on the leaf blade was more limited than the effect of the nutrient treatment, therefore reducing the power to detect such an effect on smaller structures.

Furthermore, two studies that have experimentally tested the effects of light quality or quantity on the morphology of floral traits reported limited and sometimes unpredictable responses (Weinig 2002; Brock & Weinig 2007). On the other hand, *Dalechampia* bracts are photosynthetic for a large part of their life. Because photosynthates produced by photosynthetic tissues of the reproductive organs can contribute a substantial part of the carbon requirement for reproduction (Bazzaz & Carlson 1979; see Aschan & Pfantz 2003 for review), one could expect bracts to react to variation in light quality or quantity in a similar way as leaves do. Our study shows that this is not the case, suggesting therefore that bract blades are also canalized against variation in daily irradiance.

COMPARING VEGETATIVE AND REPRODUCTIVE TRAITS

From the few studies that have compared vegetative and reproductive traits under experimentally induced environmental variation (Frazee & Marquis 1994; Dorken & Barrett 2004; Mal & Lovett-Doust 2005; Caruso 2006; Brock & Weinig 2007; Burkle & Irwin 2009), it appears that reproductive traits are generally less variable than vegetative traits, whatever the environmental factor manipulated. Furthermore, responses of floral traits tend to be population- or genotype-specific, and cannot be predicted from the effect of the different treatments on vegetative traits, thus supporting the idea of decoupled phenotypic variation between vegetative and reproductive traits. Further comparison of the level of variation between vegetative and reproductive traits in these studies is, however, difficult due to the heterogeneity of the traits studied. Traits assessed include different types of measurement such as length, area or volumes, differences between lengths (herkogamy), diameter, concentration (nectar), number of structures and mass, measured on different scale types such as ratio, log-ratio or interval scale, which seriously compromise comparison. While controlling for homology, trait complexity and dimensionality, we found that phenotypic variation of the bract blades was lower and largely decoupled from the phenotypic variation of the leaf blades, and similar to the variation of traits directly involved in pollination. These observations support the idea that variational properties can be moulded by canalizing selection as hypothesized by Berg (1959, 1960).

Because bracts are not directly involved in pollen transfer, we do not expect the canalizing selection to result from the fit with the pollinator. There are, however, three different processes that can independently or simultaneously produce canalizing selection on the bracts. First, bracts can indirectly respond to canalizing selection on traits with which they are genetically or phenotypically correlated. In one of our study population, bract length was genetically correlated with several traits directly involved with the pollen transfer to and from the pollinator [genetic correlations with gland area, style length and gland-anther distance were 0.56, 0.43 and 0.63, respectively; Hansen *et al.* (2003)]. Alternatively, the protective function of the bracts is likely to impose correlated selection on the upper and lower bract

to be tightly fit together around the male and female flowers. Because these flower parts have relatively constant size, this may result in indirect canalizing selection. Finally, stabilizing selection could result from the conflicting selection created by the advertising function towards pollinators on the one hand, and the greater attraction of herbivores or seed predators by larger bracts on the other hand (Perez-Barrales *et al.* unpublished data).

Leaf and bract stipules displayed similar phenotypic responses to the environmental variation. Although this might not be surprising considering the homology between the two traits and their protective role for the shoot primordium and leaf or inflorescence buds when small, differences occur between these two structures. Leaf stipules are inserted at the base of the petiole and separated from the leaf blade. Furthermore, the growth and senescence of the leaf stipules are disconnected from the leaf blade; stipules often die before the leaf. Bract stipules, however, are more closely related to the bract blade since the petiole is almost absent in the blossom. This physical adjacency between stipule and blade in the bract also results in a more synchronized ontogenetic development and both senesce together and pull back away from the fruits just before capsule dehiscence. Our results suggest that the decoupling of the variation between vegetative and reproductive traits overrules structural proximity and involves reorganization of the developmental pathway. This last hypothesis is also supported by the reorganization in the development of serial structures between bracts and leaves, as illustrated by the opposite effect of the position (proximal vs. distal) on leaf-blade and bract-blade sizes.

Overall, these results confirm the decoupling of the variational properties of traits involved in pollination from other traits that are not. They further show that different function can overrule patterns of variation due to similar developmental pathways (between homologous traits) and structural adjacency. Finally, they are consistent with the idea that the variational property of a trait can evolve in response to selection on variation (Waddington 1957; Wagner & Altenberg 1996; de Visser *et al.* 2003; Wagner 2005; Hansen 2006, 2010; Wagner, Pavlicev & Cheverud 2007; Pélabon *et al.* 2010).

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Supporting Information

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

Table S1. Effects of the treatments on different traits for the Mexican population, for the structures in proximal position.

Table S2. Effects of the treatments on the different traits for the Venezuelan population, for the structures in proximal position.

Table S3. Variance-component analysis and mean-scaled variances for the structure in proximal position.

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