

Flower-level developmental plasticity to nutrient availability in *Datura stramonium*: implications for the mating system

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● **Background and Aims** Studies of phenotypic plasticity in plants have mainly focused on (1) the effect of environmental variation on whole-plant traits related to the number of modules rather than on (2) the phenotypic consequences of environmental variation in traits of individual modules. Since environmental and developmental factors can produce changes in traits related to the mating system, this study used the second approach to investigate whether within-individual variation in herkogamy-related traits is affected by the environment during plant development in two populations of *Datura stramonium*, an annual herb with a hypothesized persistent mixed mating system, and to determine which morphological traits may promote self-fertilization.

● **Methods** Full-sib families of two Mexican populations of *D. stramonium*, with contrasting ecological histories, were grown under low, mid and high nutrient availability to investigate the effects of genetic, environmental and within-plant flower position on flower size, corolla, stamen and pistil lengths, and herkogamy.

● **Key Results** Populations showed differences in familial variation, plasticity and familial differences in plasticity in most floral traits analysed. In one population (Ticumán), the effect of flower position on trait variation varied among families, whereas in the other (Pedregal) the effect of flower position interacted with the nutrient environment. Flower size varied with the position of flowers, but in the opposite direction between populations in low nutrients; a systematic within-plant trend of reduction in flower size, pistil length and herkogamy with flower position increased the probability of self-fertilization in the Pedregal population.

● **Conclusions** Besides genetic variation in floral traits between and within populations, environmental variation affects phenotypic floral trait values at the whole-plant level, as well as among flower positions. The interaction between flower position and nutrient environment can affect the plant's mating system, and this differs between populations. Thus, reductions in herkogamy with flower positions may be expected in environments with either low pollinator abundance or low nutrients.

Key words: *Datura stramonium*, flower-level developmental reaction norms, nutrient availability, within-individual variation, herkogamy, population variation, probability of self- and cross-fertilized flowers, complete selfing, mating system, reproductive assurance.

INTRODUCTION

The property of a given genotype to produce different phenotypes in response to distinct environmental conditions is a ubiquitous plant character called phenotypic plasticity (Pigliucci, 2001). However, modular organisms, such as plants, whose modules develop sequentially during the plant's ontogeny, face repeated opportunities for small-scale spatial and temporal environmental variation that may affect the module phenotype in a single individual (Winn, 1996). This view assumes that organs in repetitive modules are prone to alter their development in an autonomous way according to programmed developmental responses to changes in the environment during growth (Herrera, 2009). These instances have been referred to as 'developmental organ-level phenotypic plasticity' (Herrera, 2009; 'intra-individual plasticity' *sensu* Kawamura, 2010).

Plasticity can be expressed in whole-plant traits, such as growth, size and fecundity (e.g. Pigliucci and Schlichting, 1995; Pigliucci *et al.*, 1997) as well as in the traits of reiterated structures such as leaves, flowers and fruits (Goodspeed and Clausen, 1915; Schmalhausen, 1949; Sultan, 1987; reviewed in Herrera, 2009). Until recently, phenotypic plasticity studies have mainly focused on the analysis of environmental variation on whole-plant traits related to the number of modules rather than on phenotypic consequences of environmental variation on the traits of individual modules (Herrera, 2009; cf. growth and some reproductive traits, Pigliucci and Schlichting, 1995; Pigliucci, 1997; Pigliucci *et al.*, 1997). This modular nature of plasticity (de Kroon *et al.*, 2005) has striking consequences for plants since it could generate sub-individual variation affecting the evolutionary trajectory of organ traits by setting upper limits in the response to selection (i.e. constraining it), and opens up

the opportunity for selection by animals on plant-level variability determining the size of the realized phenotypic space at the individual and population levels (Herrera, 2009).

The role of environmental factors in the expression of vegetative traits in plants is well established (Pigliucci, 2001), in contrast to flowers that have been traditionally considered the least plastic traits at the intra-specific level (Sinnott, 1921), shaped by developmental homeostasis (reviewed by Fenster and Galloway, 1997) and strong stabilizing selection (Armbruster *et al.*, 2004). However, some floral traits are subject to marked modifications under stress by both internal and external conditions during development (e.g. flower size; Goodspeed and Clausen, 1915; Schlichting and Levin, 1984; reviewed in Herrera, 2009). In plants that produce flowers sequentially along branches, the order of flower production may have a strong influence on some traits such as herkogamy (Barrett and Harder, 1992; Vallejo-Marin and Barrett, 2009) but not on others (Vogler *et al.*, 1999; Bissell and Diggle, 2008; reviewed in Diggle, 2003). Herkogamy, the stigma–anther separation, has been the focus of in-depth research (reviewed in Barrett *et al.*, 2009) given its role as a major determinant of mating patterns in plant populations (Darwin, 1862; Webb and Lloyd, 1986). The expression of phenotypic plasticity in herkogamy can modify the frequency of self- and cross-fertilized flowers in self-pollinated species (Vallejo-Marin and Barrett, 2009). This modified rate of self- and cross-fertilization may help to match current environmental conditions. An increase in self-fertilization has been predicted in more stressful environments, where either biotic (e.g. pollinator abundance, Darwin, 1878) or abiotic conditions limit cross-pollination (e.g. dry season and an abundance of ephemeral habitats, Rick *et al.*, 1978; Holtsford and Ellstrand, 1992; Elle and Hare, 2002; Moeller and Geber, 2005, reviewed in Levin 2010).

Datura stramonium L. (Solanaceae) is a predominantly self-fertilizing annual herb that has heritable variation in herkogamy that is positively correlated with the outcrossing rate (Motten and Stone, 2000). However, herkogamy can exhibit a remarkable pattern of continuous within-individual variation (cf. *Eichornia paniculata* marked bimodal pattern, Barrett and Harder, 1992) and this has been attributed to family-level inbreeding depression history (Stone and Motten, 2002). The effect of environmental factors on this variation has received less attention, and the search for genotype by environment interactions has been related to among-site variation rather than to specific environmental stressors (e.g. Motten and Stone, 2000). If flowers of *D. stramonium* produce a continuous variation in herkogamy that affects the relationship between selfed vs. outcrossed flowers (Motten and Stone, 2000), it is of interest to determine whether these contrasting floral phenotypes are produced as a systematic within-plant trend of variation. Here, our aim was to determine the effect of specific factors influencing within-plant variation in floral traits of *D. stramonium* and discuss its implications for mating system evolution.

We present results of a study of genetic variation in developmental organ-level reaction norms. A strong increase in plant biomass and total alkaloid content related to soil nitrogen addition has been documented in *D. stramonium* (Weaver and Warwick, 1984). We examined the pattern of developmental trajectories among six sequential flower positions (i.e. successive metamers, Fig. 1), and its plasticity to three different soil

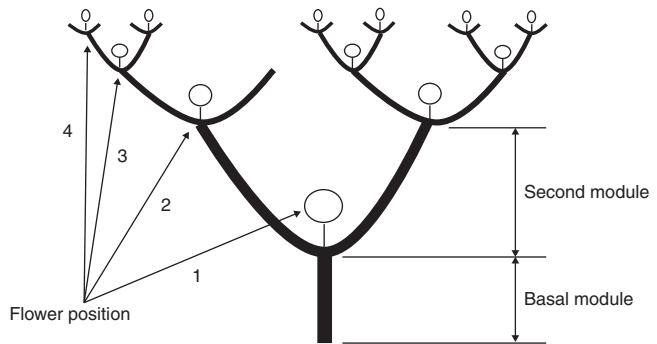


FIG. 1. Leeuwenberg's architectural model of *Datura stramonium*. The model consists of a sympodial succession of equivalent sympodial units (metamers), each of which is orthotropic and determinate in its growth. Then, the apex is expected to transform into one flower in each node. A module refers to a portion of an axis (metamer) made up of a single terminal meristem which corresponds to a sympodial unit. Thus, every flower position represents the traits expressed at each sympodial unit, resulting in the sequence of within-individual floral variation observed; in the figure, there is a sequential decrease in flower size and metamer length for the first four positions.

nutrient availabilities in two populations that differ in pollinator abundance and soil fertility. Our study addressed three specific questions. (1) How much genetic variation for floral trait within-plant variation and their plasticity exists in two populations of *D. stramonium*? An answer to this question would provide us with some empirical estimates of the potential for the evolution of the shape of organ-level developmental trajectories in heterogeneous environments, (2) Does stress (low nutrient availability) increase the probability of self-pollinated flowers? (3) What floral traits promote an increased self-fertilization?

MATERIALS AND METHODS

Study species and populations

Datura stramonium is a colonizing annual plant widely distributed around the world. Although it is found in all types of soil, it prefers rich soils (Weaver and Warwick, 1984), rapidly assimilating nitrogen in the form of nitrate or ammonium (Lewis and Probyn, 1978; Platt and Rand, 1982). The species produces hermaphroditic self-compatible flowers, with tubular corollas and copious quantities of nectar. The flowers last only one night (Motten and Antonovics, 1992). The stamens and the style are inserted inside the corolla, with the stamen filaments adnate to the corolla tube in the proximal middle part (Motten and Stone, 2000). Herkogamy is positively correlated with the outcrossing rate in *D. stramonium* (Motten and Stone, 2000). Flowers having the stigma at or below the level of the anthers produce only selfed seeds. In flowers with the stigma exerted beyond the level of the anthers (i.e. anther–stigma separation >3.5 mm), outcrossing increases in proportion to the degree of the exertion (Motten and Stone, 2000). The average estimated outcrossing rate is low ($t = 1.3\%$), but variation in t has been observed (range: 0–18%; Motten and Antonovics, 1992). Narrow-sense heritability ranges from 0.20 to 0.31 in a population derived from a full diallel cross using eight inbred lines (that include a natural occurring polymorphism for hypocotyl and flower colour) in different environments (Motten and Stone, 2000).

Variation in herkogamy attributable to dominance variance appears to be exhibited in the richest environments, with exerted stigmas being recessive (Motten and Stone, 2000). Flower visitors are hawkmoths, honey-bees and bumble-bees (Sharma, 1972; Grant and Grant, 1983; Motten and Antonovics, 1992). The species is considered a weed in some countries, but in México it is a colonizing (ruderal) plant, most commonly encountered in disturbed habitats (Núñez-Farfán and Dirzo, 1994).

In *D. stramonium*, in contrast to many plants where the adult phase begins when sexuality is first apparent, maturity and sexuality are not necessarily synonymous. In this species, the position of flowers may be used to establish an architectural category to which a plant belongs, so that sexuality is also important in the vegetative growth dynamics of the plant. This property is due to its architectural model called Leeuwenberg (Hallé *et al.*, 1978), in which branching produces equivalent orthotropic modules, each with deterministic growth culminating in the production of a terminal flower (Fig. 1). An important feature of the model is the decrease in length and primary width of successive modules and leaf size; thus, the first module is the longest, with the largest leaves (Hallé *et al.*, 1978). We use the term module to refer to sequential, often semi-autonomous structural and functional sub-units of plants (*sensu de Kroon et al.*, 2005), which are produced during development (White, 1979; Preston and Ackerly, 2004) but not necessarily at the same time. Then, a module refers to a portion of an axis (metamer) made up of a single terminal meristem which corresponds to a sympodial unit (Bell, 1991). Thus, every flower position represents the traits expressed at each sympodial unit, resulting in the sequence of within-individual floral variation observed (Fig. 1).

Seeds from two populations (natural progeny) of *D. stramonium* from central Mexico were collected: the Ticumán population in the state of Morelos, and the Pedregal de San Angel Ecological Reserve population, south of Mexico City. These populations differ in climate, vegetation and type of soils (Valverde *et al.*, 2001). The Ticumán population is pollinated by several hawkmoth species (genus *Sphinx*, A. López, pers. obs.; of 241 marked flowers, 163 flowers were visited in 150 min), and the rate of outcrossing among plants varies between 0 and 80%, with an average of 18% (Cuevas, 1996). In contrast, the Pedregal population is incidentally pollinated by honey-bees but in some seasons pollinators are very scarce (J. Núñez-Farfán and A. López Velázquez, pers. obs.; from 200 marked flowers, one flower was visited in 120 min in the nearest locality to Pedregal). Previous studies suggest that the Ticumán population possess greater genetic variance in quantitative traits than the Pedregal population (Núñez-Farfán and Dirzo, 1994; Fornoni *et al.*, 2003). In contrast, the Pedregal population is highly inbred and self-compatible (91.8%, $n = 98$ flowers; Núñez-Farfán *et al.*, 1996). Moreover, in plants of this population grown in the greenhouse, herkogamy accounted for only 1% of the variance in seed number per fruit ($r^2 = 0.013$; $F = 1.4$; $P < 0.05$; $n = 476$; Fornoni and Núñez-Farfán, 2000).

Cultivation of plants and experimental treatments

Full-sib families of each field maternal progeny were derived from one generation of selfing in the greenhouse. Seeds were

sown in pots (1.5 L) and kept at a 12:12 h (light/dark) photoperiod; the mean temperature was 28/23 °C. Due to low germination in some families, only five and ten families from Ticumán and Pedregal were used, respectively. When cotyledons were fully expanded, seedlings were transplanted to pots, with a mixture of sand, soil and turface (fritted clay).

When plants were 2 weeks old, 12 replicates of each family were randomly assigned to one of three nutrient levels, which were supplied four times at 10 d intervals: 'low' (no nutrients added to the soil mixture), 'medium' (4 g L⁻¹ of water of 20–20–20 NPK solution added), 'high' (6 g L⁻¹ of water of 20–20–20 NPK solution added). Pots were arranged in a complete randomized design in a common garden at Harvard University, Cambridge, MA, USA. Pots were sunk into the soil to limit fertilizer loss.

Plant traits

Random seedling mortality reduced initial sample sizes from $n = 12$ plants per genotype. For 52 and 112 plants (254 and 563 flowers; not all plants produced flowers in all positions, see below) from Ticumán and Pedregal populations, respectively, three floral traits were measured with a calliper to the nearest 0.1 mm on six sequential flower positions along the plant: (1) corolla length, from the base of the calyx to the top of one lobe; (2) stamen length, from the base of the ovary to the top of the anthers; and (3) pistil length, from the base of the ovary to the top of the stigma. Herkogamy was estimated by subtracting the height of the stamen from the height of the pistil. Positive herkogamy implies that the stigma is exerted above the level of anthers and negative herkogamy implies the opposite (Schoen, 1982). Our aim here was not to compare plants at the same chronological age, which might vary to a great extent in our system because more than one flower is produced at different times in each sequential developmental stage. Hence, we did not use comparisons at a common point in time because they are important in relation to real-time processes such as reproductive output in relation to the length of the growing season but at a common developmental stage (Coleman *et al.*, 1994), represented by the metamer number as an index (flower position). This index reveals the inherent growth strategy of the *D. stramonium* architectural model defining both the way the plant elaborates its form and the resulting sequence of activity of the endogenous morphogenetic processes of the organism, resulting in the intra-individual floral variation observed.

Statistical analysis

We assessed within-plant variation in floral characters by means of two analysis of covariance (ANCOVA) models. The first tested for genetic and environmental effects on whole-plant traits related to the number of modules; this analysis was intended to test the differences between populations. The second model tested for genetic and environmental effects, within populations, on the traits of individual modules (i.e. the positional change in the measured traits) or the shape of the developmental trajectories.

After checking for normality and homoscedasticity, only herkogamy did not meet the analysis of variance (ANOVA)

assumptions of equal variance. Then, we used the pistil/stamen length ratio instead of length subtraction (see ‘Plant traits’, above). Results and significance levels were similar using both measures, so descriptive statistics and ANOVA results of subtraction data are presented. Since trait values were standardized (centred on the mean, dividing by the standard deviation) prior to ANOVAs, the mean squares for each trait–factor combination are directly comparable across traits, enabling interpretation of the relative importance of each factor in the analysis for explaining the phenotypic variation (Pigliucci and Kolodynska, 2006). We do not report Bonferroni-like corrections for multiple tests, often applied to maintain the overall probability of committing type I error, because these increase the probability of type II error (Moran, 2003; Banta *et al.*, 2010). Instead, we reported the ‘native’ *P*-values (e.g. Pigliucci and Kolodynska, 2006). Population pattern reaction norms of floral traits were plotted against the three levels of nutrient availability using the least squares means adjusted for the covariate effect in the full model.

Genetic and environmental effects on whole-plant traits

A full mixed-model ANOVA was conducted to investigate the relative importance of (1) flower position (used as a covariate); (2) population (genetic variation among populations); (3) family, nested within population (genetic variation at the family level); (4) treatment (presence of average phenotypic plasticity regardless of specific populations or families); (5) treatment by population interaction (genetic variation for plasticity among populations); and (6) treatment by family interaction (genetic variation for plasticity among families, within populations). All main effects were considered fixed, except for family and its interactions, which were considered random.

Genetic and environmental effects on the traits of individual modules

We performed a detailed analysis of the reiterated floral traits throughout the six sequential flower positions along the plant, and the interaction of these within-plant trajectories with family and nutrient effects; individual analyses were conducted for each population. An ANCOVA was used according to the model: $y = \text{flower position (covariate), family, treatment, family} \times \text{treatment, flower} \times \text{family, flower} \times \text{treatment}$, where the interaction terms involving flower position were intended to investigate the variation of within-plant trajectories at the family level (i.e. the flower \times family term) or at the nutrient level (i.e. plasticity of within-plant trajectories; flower \times treatment term). The three-way interaction was never significant and was excluded. The independence of the trait expression in each module of the plant motivated our use of an ANCOVA instead of repeated measures ANOVA (a model more suitable for time-dependent measurements such as for growth traits; e.g. Pigliucci and Schlichting, 1995; Pigliucci *et al.*, 1997). Regression analysis of estimates of sequential floral parameters has also been used (e.g. Barrett and Harder, 1992; Vogler *et al.*, 1999; Bissell and Diggle, 2008). We also used the genotypic averages of flower positions and measurements of flower traits to plot the architectural developmental trajectories for each

genotype in the three environments. We used a ‘character state approach’ (i.e. using the mean values for each flower position, namely the character state; Pigliucci 2001) to plot architectural developmental trajectories with the aim of inspecting the patterns.

Flower length and phenotypic integration effects across environments

To ascertain whether flower length is a function of flower position, a principal component analysis (PCA) with Varimax rotation was applied to the correlation matrix of four floral measurements for the entire data set. Varimax rotation is a method for orthogonal rotation which results in high loadings for fewer variables; the rest will be near zero (Hair *et al.*, 1998). We extracted factors and scores of floral traits on those factors: the first factor explained the most variance, which had high loadings for all floral traits except herkogamy. The scores for each factor reflect the weight and direction of the contribution of each individual plant’s combined trait values to that component, and these can be analysed in the same manner as the traits themselves (Pigliucci *et al.*, 1997; Kristjánsson, 2002; Engelmann and Schlichting, 2005; Bissell and Diggle, 2008). The scores were all normally distributed and were used as dependent variables in the two ANCOVA models used to evaluate the other floral traits measured (see above).

An additional ANCOVA was used to evaluate differences between populations (main factor) in the slope of size factor on flower position (covariate) in each environment; a significant population by flower position interaction will be interpreted as a change in the slope of the regression line of the size factor on flower position for the two populations (i.e. test of parallelism, e.g. Gianoli, 2004; Gonzalez and Gianoli, 2004).

As a measure of phenotypic integration and to assess the integrated response of floral traits to the effects of nutrient environments, we repeated the PCA for each population/environment matrix. Salient loadings of traits on the first factor at all nutrient environments are interpreted as evidence of strong phenotypic integration (e.g. Bissell and Diggle, 2008). We included traits in the interpretation of a factor if they had a factor of pattern coefficient ≥ 0.40 (i.e. a salient loading, which is the cut-off most often used in PCA, Harman, 1976; Gorsuch, 1983; Bissell and Diggle, 2008). That is, only traits that load consistently across environments on the first factor represent a suite of correlated characters maintained by co-ordinated development (Bissell and Diggle, 2008). Additionally, we performed classical paired comparisons at the phenotypic level for all traits, among correlation environmental matrices for each population and among populations for each environment (Appendix; Supplementary Data Tables S2 and S3).

Modified rate of self- and cross-fertilization

Within-individual variation in contrasting floral phenotypes was evaluated using logistic regression (Barrett and Harder, 1992; Vallejo-Marín and Barrett, 2009). Flowers were classified as contributing to self- (herkogamy < 3.5 mm) or cross-fertilization (herkogamy ≥ 3.5 mm), and coded with one and zero before the analysis, respectively. The threshold of 3.5 mm

TABLE 1. ANOVA of individual floral traits of two populations of *Datura stramonium* grown under three levels of nutrient availability

Trait	Flower (d.f. = 1)		Population (d.f. = 1)		Family (d.f. = 13)		Treatment (d.f. = 2)		Treatment by population (d.f. = 2)		Treatment by family (d.f. = 26)		Error (d.f. = 771)
	MS	<i>P</i>	MS	<i>P</i>	MS	<i>P</i>	MS	<i>P</i>	MS	<i>P</i>	MS	<i>P</i>	MS
Corolla length	0.43	0.42	174.04	<0.0001	4.38	0.0034	8.23	0.0037	2.50	0.14	1.30	0.0034	0.67
Stamen length	0.63	0.35	156.52	<0.0001	3.28	0.0118	4.02	0.0400	0.85	0.48	1.19	0.0206	0.71
Pistil length	5.56	0.0037	76.69	0.0151	10.99	<0.0001	16.50	<0.0001	1.34	0.28	1.05	0.0295	0.66
Herkogamy	6.99	0.0008	0.39	0.8750	17.18	<0.0001	17.41	<0.0001	0.52	0.56	0.92	0.05	0.61
Flower size (factor 1)	0.36	0.47	179.85	<0.0001	2.15	0.1152	4.90	0.0263	1.41	0.32	1.27	0.0080	0.70

The family term and its interactions are random and nested within populations.
Probability values in bold were considered not significant ($P > 0.05$).
Flower indicates flower position.

was chosen because flowers with stigmas protruding above this level showed continuous variation and a direct influence on the extent of outcrossing in *D. stramonium* (Motten and Stone, 2000; see ‘Study species’ above). The model was fitted using the module GLM with a binomial error, and a logit link function (JMP, Version 7. SAS Institute Inc., Cary, NC, USA) for each population. Statistical significance of the family term is interpreted as evidence of genetic variation in herkogamy, significance of the treatment effect as an indication of environmental effects, and the interaction as evidence of genetic variation for plasticity (Vallejo-Marín and Barrett, 2009). We used flower position as a covariate in the full model, and interaction terms between categorical effects (i.e. family and treatment) and flower position were also included as potential predictors in the logistic regression model (Barrett and Harder, 1992). If any interaction term was significant, we further explored the metameric influence (i.e. position-dependent floral trait expression) on the probability of producing selfed flowers for each nutrient environment using the module Nominal Logistic (JMP, Version 7).

RESULTS

Genetic and environmental effects on whole-plant traits

The first ANOVA model (Table 1; Fig. 2) revealed a significant effect of flower position on pistil length and herkogamy (median values in Supplementary Data Table S1). Populations were significantly different for all traits except herkogamy. Flowers were larger, and had greater corolla, stamen and pistil lengths in the Ticumán population (Fig. 2). Genetic variation among families within populations was significant for all traits except flower size. The experimental increment in nutrient availability had a significant effect for all traits (Fig. 2). Nutrient addition affected flower size (longer lengths for all floral traits). The Ticumán population showed less phenotypic plasticity between the medium and high nutrient availability for all floral traits except herkogamy (Fig. 2). However, genetic differences in plasticity among populations were not significant for any trait (Table 1, treatment by population effect). A significant treatment by family interaction indicates genetic variation for plasticity within populations for all floral traits (Table 1; herkogamy showed marginal significance).

It is interesting to note that for most characters, a large fraction of phenotypic variance is accounted for by genetic differentiation among populations, followed by nutrient availability

and family main effects (Table 1). The exception to this pattern was herkogamy, which had little variance associated with population differentiation. These results indicate that genetic differences in plasticity, even when significant, contribute relatively little to phenotypic variation across the nutrient settings examined. From the perspective of whole-plant trait variation examined with this ANOVA model, there is a significant amount of variation explained by flower position for herkogamy and pistil length [Table 1, mean square (MS) of flower effect].

Genetic and environmental effects on the traits of individual modules

Analyses by population reveal that the Ticumán population has significant among-family genetic variation in traits, whereas the Pedregal population does not. In contrast, Pedregal shows both much greater plasticity and environment-dependent genetic variation (i.e. genetic variation for plasticity; Table 2, Fig. 3).

The effect of flower position was significant for herkogamy and pistil length in the Pedregal population, and corolla length in the Ticumán population (Table 2). A significant flower position by family interaction for all traits except stamen length reveals genetic variation for position-dependent floral trait expression in the Ticumán population. Almost all traits except herkogamy showed a significant flower position by treatment interaction (highlighting the modification of developmental trajectories by the nutrient environment) in the Pedregal population (Table 2). In the Ticumán population, both corolla and flower size showed significant flower position by treatment interaction; stamen length showed a marginally significant effect, whereas pistil length and herkogamy did not (Table 2).

The inspection of developmental trajectories showed a more homeostatic development for the Pedregal families for all traits in the low nutrient environment (Fig. 3A–D). Populations showed opposite within-plant trends in this environment, with all traits except herkogamy appearing to increase with flower position for the Ticumán population, but decreasing for the Pedregal population (Fig. 3A–D; see below). Populations did not show opposite trends in mid and high nutrient environments (Fig. 3E–L). In these environments, an increase in phenotypic variance, for almost all traits, was evident in the Pedregal population. However, familial variation and developmental stability for herkogamy is maintained across environments in this population. In the high-nutrient environment, the corolla-dependent

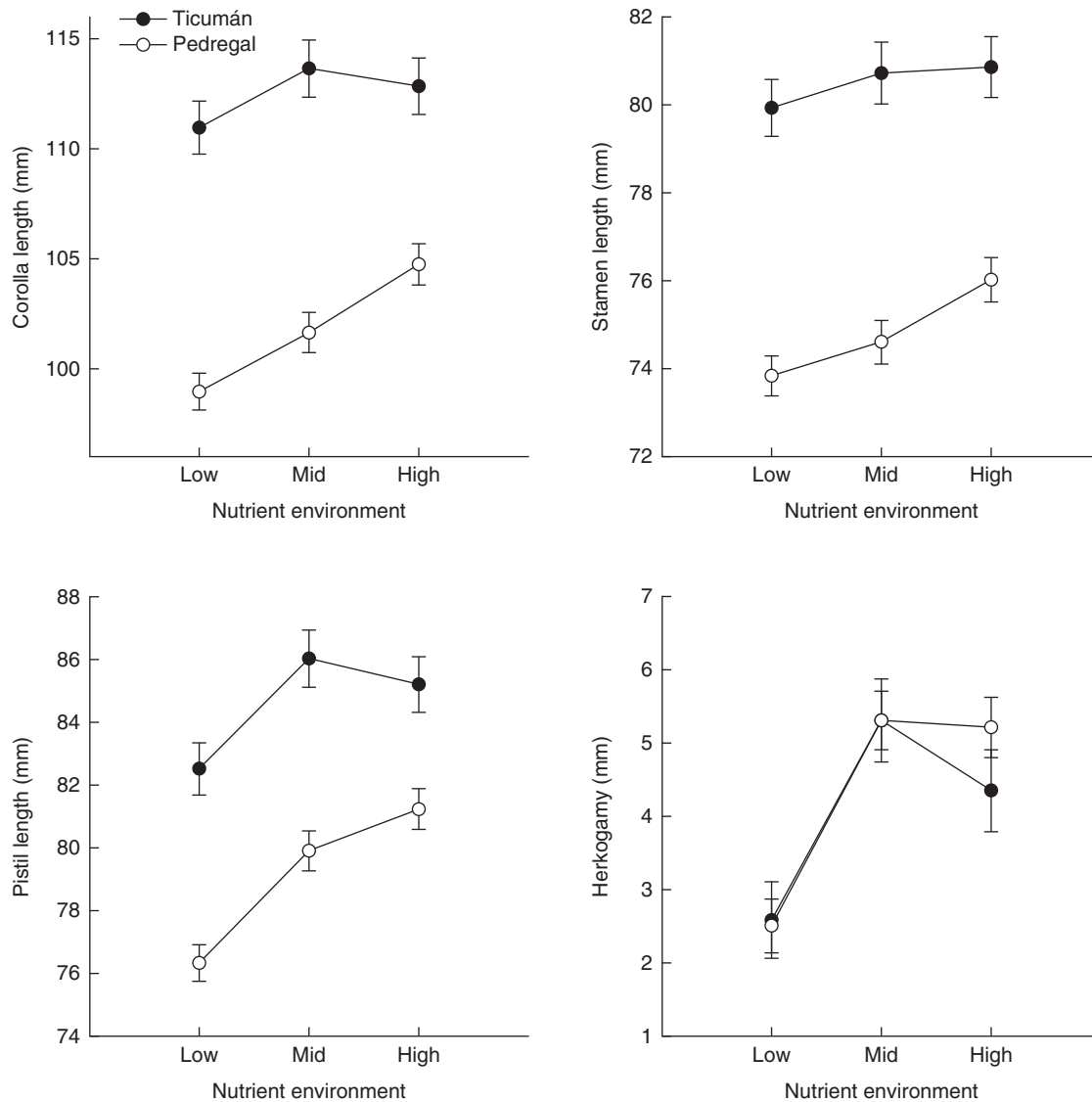


Fig. 2. Reaction norms of floral traits in response to nutrient availability of Ticumán and Pedregal populations of *Datura stramonium*. Mean values (\pm s.e.) are depicted.

variation in stamen and pistil length was evident for the Pedregal population that showed a very low value for corolla, stamen and pistil length in the fourth flower position (Fig. 3I–K); a similar result was observed for the fifth position in low nutrients (Fig. 3B–D). Notwithstanding, the herkogamy value in these flower positions did not drop as would be expected, highlighting the strong control of herkogamy in this population (Fig. 3L). Conversely, one family in the Ticumán population showed a decrease in the value of corolla length that was associated not with the stamen length but with pistil length (Fig. 3I–K). This resulted in lower herkogamy values in this family for the second flower position (Fig. 3L).

In the Pedregal population, the largest fraction of phenotypic variation was not accounted for by the genetic differentiation among families, but by the main effect of nutrient availability, followed by flower position, flower position by treatment and treatment by family interaction effects; flower position by

family interaction explained little phenotypic variance. This implies that environmentally and architecturally phenotypic variation played a more important role than either its interaction or the genetic differentiation for flower position. Conversely, in the Ticumán population, the greatest fraction of phenotypic variation was associated not with the main effect of nutrient treatments but with genetic differentiation among families, followed by flower position by family and flower position by treatment interactions. The main effect of flower position explained little phenotypic variance (Table 2).

Flower size (factor 1) differed between populations and treatments, being smaller in the Pedregal and in the low-nutrient environment for both populations (Table 1; Fig. 4A). The significant flower position \times treatment interaction in flower size (both populations) and herkogamy-related traits (corolla, stamen and pistil length in Pedregal) led us to explore meta-merismic variation for these traits in each environment (Table 2).

TABLE 2. ANCOVA of individual traits within two populations of *Datura stramonium* grown at three levels of nutrients availability

Population, trait	Flower		Family		Treatment		Treatment by family		Flower by family		Flower by treatment		Error
	MS	P	MS	P	MS	P	MS	P	MS	P	MS	P	MS
Pedregal	(d.f. = 1)		(d.f. = 9)		(d.f. = 2)		(d.f. = 18)		(d.f. = 9)		(d.f. = 2)		(d.f. = 521)
Corolla length	2.36	0.06	1.01	0.74	13.1	0.0013	1.58	0.0015	0.36	0.85	2.15	0.0426	0.68
Stamen length	2.5	0.61	2.04	0.21	6.02	0.0178	1.35	0.0133	0.37	0.86	3.72	0.0055	0.71
Pistil length	5.95	0.0014	0.61	0.74	17.77	<0.0001	0.94	0.0463	0.66	0.33	3.03	0.0054	0.57
Herkogamy	3.62	0.0087	1.54	0.15	15.84	<0.0001	0.89	0.0350	0.86	0.10	0.24	0.63	0.52
Flower size (factor 1)	2.29	0.07	1.15	0.64	8.04	0.0091	1.51	0.0042	0.37	0.86	3.22	0.0108	0.63
Ticumán	(d.f. = 1)		(d.f. = 4)		(d.f. = 2)		(d.f. = 8)		(d.f. = 4)		(d.f. = 2)		(d.f. = 232)
Corolla length	2.35	0.0465	11.94	0.0001	0.96	0.22	0.53	0.51	1.48	0.0418	4.33	0.0008	0.59
Stamen length	1.68	0.12	5.50	0.0042	0.39	0.57	0.65	0.48	0.98	0.22	2.05	0.05	0.68
Pistil length	0.32	0.52	31.38	<0.0001	3.72	0.09	1.15	0.16	2.38	0.0170	1.33	0.18	0.77
Herkogamy	0.33	0.50	48.26	<0.0001	5.11	0.0301	0.97	0.23	5.45	<0.0001	0.38	0.60	0.73
Flower size (factor 1)	2.22	0.06	4.15	0.0071	0.40	0.52	0.57	0.51	1.34	0.08	3.09	0.0082	0.63

The family term is random.

Probability values in bold were considered not significant ($P > 0.05$).

Flower indicates flower position.

Opposite directional trends in flower size were observed only in the low-nutrient environment (where flowers were smaller): using mean family values [Ticumán, slope = 0.18 ± 0.07 (mean \pm s.e.), $F_{1, 28} = 6.50$, $P = 0.0166$, $R^2 = 0.19$; Pedregal, slope = -0.12 ± 0.03 , $F_{1, 54} = 22.61$, $P < 0.0001$, $R^2 = 0.28$] (Fig. 4B). Differences between populations were significant (ANCOVA, $F_{1, 86} = 24.26$, $P < 0.0001$, $R^2 = 0.67$; test of parallelism on family mean values) (Fig. 4B). This highlights the results of developmental trajectory plots observed for corolla, stamen and pistil traits in this environment (see above), the characters with higher loadings for flower size (i.e. factor 1 of the PCA with Varimax rotation; see the Materials and Methods). Corolla showed an opposite within-plant trend of variation only in the low-nutrient environment: using mean family values (Ticumán, slope = 2.01 ± 0.80 , $F_{1, 28} = 6.30$, $P = 0.0181$, $R^2 = 0.18$; Pedregal, slope = -1.029 ± 0.28 , $F_{1, 58} = 13.25$, $P = 0.0006$, $R^2 = 0.19$). Differences between populations were significant (ANCOVA, $F_{1, 86} = 19.44$, $P < 0.0001$, $R^2 = 0.62$; test of parallelism on family mean values). A within-plant trend of variation was observed in stamen and pistil lengths only in the low-nutrient environment for the Pedregal population: stamen (slope = -0.72 ± 0.15 , $F_{1, 58} = 23.84$, $P < 0.0001$, $R^2 = 0.29$); pistil (slope = -1.06 ± 0.15 , $F_{1, 58} = 28.56$, $P < 0.0001$, $R^2 = 0.33$).

Phenotypic integration effects across environments

The integrated response of floral traits to the effects of nutrient environments showed that corolla, stamen and pistil load consistently and with no appreciable differences across environments in both Ticumán and Pedregal populations (i.e. the salient loadings were >0.40 and were constant across environments) (Table 3).

Ratio of self- and cross-fertilization

Flower position in the Pedregal population had a strong influence on the probability of producing self-fertilized flowers [log

ratio test (LRT) of flower effect; slope = 0.18 ± 0.06 , $\chi^2_{1, 521} = 7.76$, $P = 0.0053$]. Flowers in the low-nutrient environment were smaller and more likely to self (LRT of treatment effect; $\chi^2_{2, 521} = 37.53$, $P < 0.0001$, proportions of selfed flowers = 0.57 ± 0.04 , 0.28 ± 0.03 and 0.30 ± 0.04 , for low- mid- and high-nutrient environments, respectively), implying an environmentally influenced (i.e. plastic) rate of self- and cross-fertilization. Genetic variation at the family level was significant (LRT of family effect; $\chi^2_{9, 521} = 23.51$, $P = 0.0051$). Interaction effects were not significant in the Pedregal population. In the Ticumán population, only the family effect was significant (LRT of family effect; $\chi^2_{4, 232} = 84.54$, $P < 0.0001$).

We further explored the metameric influence on the probability of producing selfed flowers in the Pedregal population for each environment using the nominal logistic. Results indicated that flower position only affects the production of selfed flowers in the low-nutrient environment: as flower position increases, the probability of producing selfed flowers increases (Fig. 4C; slope = -0.31 , $\chi^2_{1, 192} = 12.44$, $P = 0.0004$).

DISCUSSION

The main aim of our study was to find a within-plant trend of variation for floral traits. Irrespective of the causes of variation in these trends, they have striking consequences for the mating system and its evolution in different abiotic environments. The specific mechanisms that govern automatic self-pollination are the most significant aspects of the evolution of selfing (Barrett and Harder, 1992; Barrett et al., 2009). One challenging question, regarding the causal nature of within-plant variation, is whether contrasting floral phenotypes of a plant are produced randomly in relation to development, or whether positional effects are involved (Diggle, 2014) and affected by different environmental conditions (Barrett et al., 2009). In this sense, we were unable to test for effects attributable to flower position alone, which makes a more complex experimental design necessary. Very often this design implies an additional treatment where flowers are prevented from producing fruits and

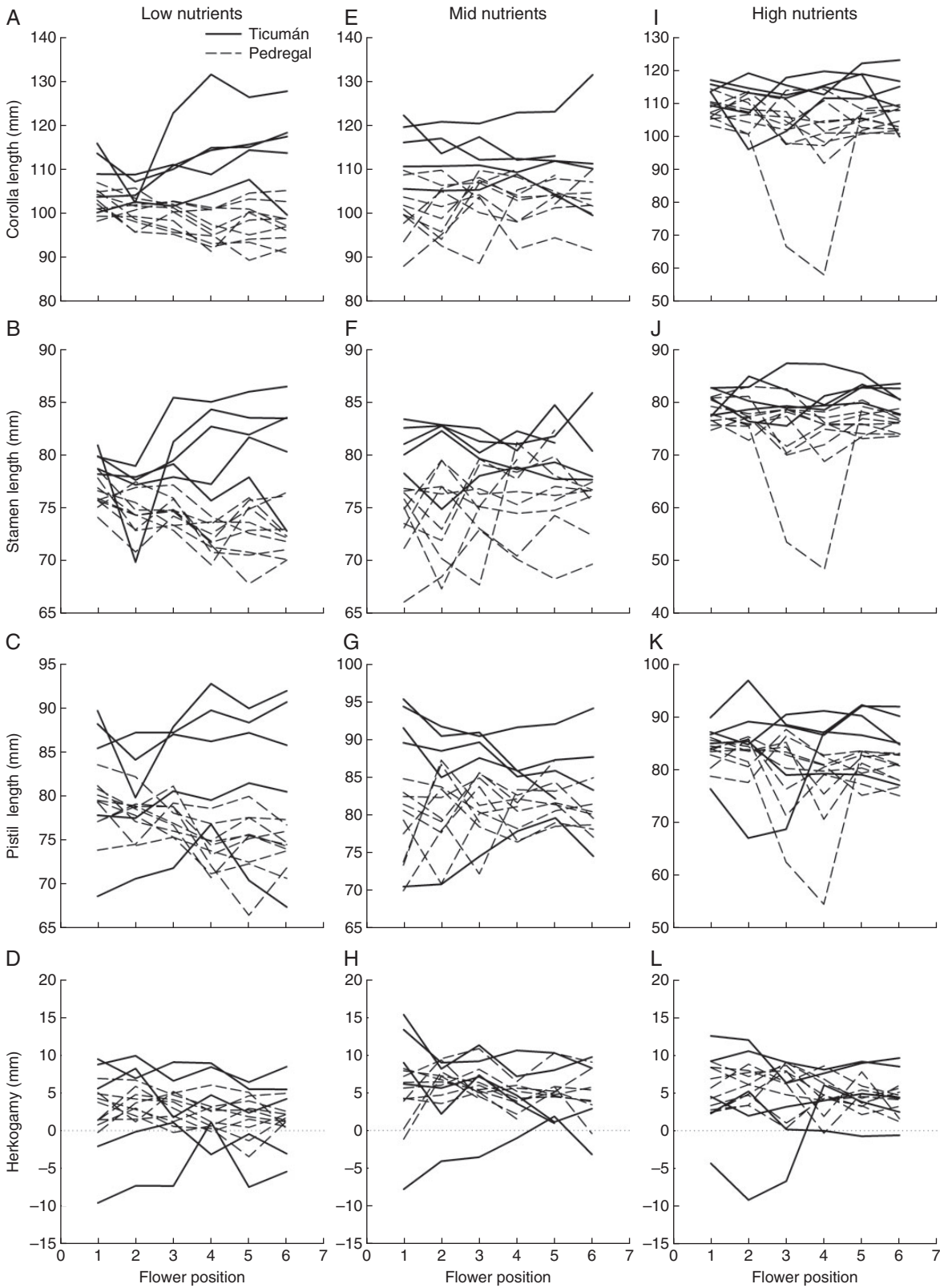


Fig. 3. Flower-level developmental reaction norms for full-sib families of two populations of *Datura stramonium* grown in three nutrient environments. Genotypes from Ticumán and Pedregal populations are plotted. The zero level of herkogamy is indicated by the reference lines in (D), (H) and (L).

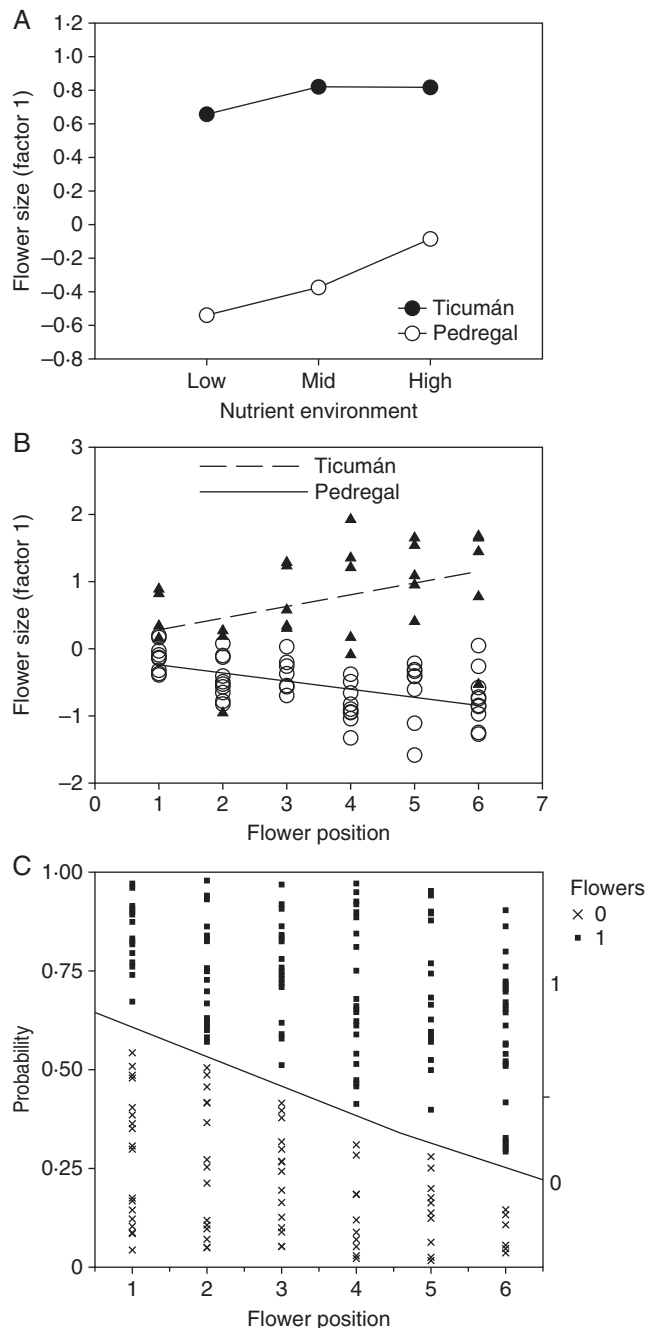


FIG. 4. Environmental and within-plant variation in flower size in two populations of *Datura stramonium*. (A) Population reaction norms to nutrient availability. (B) Among-population ($F_{1, 86} = 24.26$, $P < 0.0001$, $R^2 = 0.67$, test of parallelism) differentiation (using mean family values) in systematic trends of within-plant variation in the low-nutrient environment. (C) Probability of producing self-fertilized flowers (squared symbols) in the low-nutrient environment for the Pedregal population using nominal logistic regression ($\chi^2_{1, 192} = 12.44$, $P = 0.0004$, full model). The line represents a sequential decrease in the probability of producing outcross flowers. 1 = selfed flowers, 0 = outcrossing flowers.

compared at equivalent positions of flowers allowed to set fruit (as in our design) (Diggle, 1997, 2003).

In *Datura stramonium*, the effect of flower position on herkogamy variation has not yet been measured, since the analysis

TABLE 3. Principal component analysis for length factor in two populations of *Datura stramonium* growing in three levels of nutrient availability

Trait	Ticumán			Pedregal		
	Low	Medium	High	Low	Medium	High
Corolla	0.96	0.870	0.89	0.90	0.96	0.94
Stamen	0.98	0.930	0.94	0.97	0.98	0.98
Pistil	0.74	0.620	0.66	0.73	0.80	0.85
Herkogamy	-0.03	0.052	0.12	0.05	-0.01	0.05
% variance	60.85	50.41	53.59	56.91	62.85	64.30

of within-plant variation makes an intensive sampling necessary. Also, because not all flowers of a plant display the same phenotype, this might explain an absence of correlation between flower size and herkogamy in this species (e.g. Motten and Antonovics, 1992; Motten and Stone, 2000). However, it is well known that flower size is often affected by changing environmental conditions (Coleman *et al.*, 1994; Pigliucci, 2001), resulting in reductions in herkogamy (Fishman *et al.*, 2002). In *D. stramonium* we found that the ability to alter flower herkogamy is not independent of environmentally and architecturally induced flower size (cf. *Eichornia paniculata*; Vallejo-Marín and Barrett, 2009). Furthermore, we have shown that populations of *D. stramonium* with different ecological histories may differ in genetic variation for within-plant trajectories, and how the environment alters these. Examination of these trajectories revealed that whole-plant phenotypic plasticity is driven by different within-plant trajectories between populations. In the following, we will discuss our results in terms of changes in nutrient availability, stressing the differences between the two populations examined.

Genetic and environmental effects on flower-level developmental trajectories

Our results show that *D. stramonium* displays a high degree of among-population differentiation. More genetic variation was detected for the Ticumán population, in contrast to the low level of genetic variation in the Pedregal population. Phenotypic plasticity was higher in the Pedregal population and it had significant genetic variation. In contrast, the Ticumán population is less plastic, and genetic variation for plasticity is low, except for herkogamy that showed a marked pattern of plasticity (Table 2; Fig. 2). Flower length was a major determinant of differences between populations; flowers are larger in Ticumán but all other traits followed this pattern. Contrary to the expectations of a consistent correspondence between sexual organs and pollinator positioning (i.e. uniform morphological expression of floral traits) necessary to ensure effective pollen transfer (Lloyd and Web, 1992), floral traits in *D. stramonium* can respond to abiotic environments by means of a variable morphological expression.

The Ticumán population had greater genetic variation both at the whole-plant trait and at the flower position levels; major developmental variability in all environments across flower positions was detected (Fig. 3; see the perturbation of each family line with flower position). Failure to produce organ phenotypes

that are closely consistent with those expected from a pre-determined developmental and morphogenetic plan may contribute to within-plant variation in the characteristics of reiterated structures (Herrera, 2009). In this population, there was not a systematic within-plant trend of variation in the flower positions for all floral traits in all environments. In contrast to the Ticumán population, in the Pedregal population there was a modification of developmental trajectories by the environment and a directional trend of flower length and herkogamy in the low-nutrient environment.

In this study, the Ticumán population interacts with taxonomically diverse assemblages of mutualists that could differ in their preferences for a given floral trait; this would explain the genetic variation for flower position in this population where mutualists are more heterogeneous in their preferences, and the non-genetic variation for flower position of Pedregal where mutualists are most alike or their abundance is very low. It has been shown that populations that present different pollinator assemblages differ in the average flower phenotype (Johnson, 1997; Johnson and Steiner, 1997; Nattero and Cocucci, 2007). However, in other cases, population differentiation in flower characters could be due to factors related to geographic differences among localities or genetic drift (Dominguez et al., 1998; Boyd, 2000; Mascó et al., 2004).

Flower length and integration across environments

Pedregal flower length was influenced strongly by nutrient availability: flowers in low-nutrient environments were smaller in contrast to larger flowers in a high-nutrient environment (Table 2, Fig. 4A). This plasticity was not evident in the Ticumán population. Notwithstanding, population differentiation in mean flower length was marked, and the Ticumán population expressed the largest flower length. Developmental trajectories of this character showed a clear trend in the low-nutrient environment (Fig. 4B); the Pedregal population decreased its flower length with flower position as expected for the architectural pattern of *D. stramonium*, where a decrease in the length and primary width of successive modules is correlated with the size of leaves and hence with the magnitude of the carbon contributions to growth of organs placed in those modules (Hallé et al., 1978; Herrera, 2009). Reproductive sinks are mainly supplied by their local source leaves (Marshall, 1996). Conversely, the Ticumán population showed a trend of increasing flower size with flower position. These opposite trends exemplify how different developmental trajectories determine mean whole-plant responses. Clearly, an increase in size in the Ticumán population favoured higher developmental variability, whereas a decrease in flower size in the Pedregal population favours lower developmental variability that is correlated with a tendency to reduce herkogamy (slope = -0.34 ± 0.15 , $F_{1, 58} = 5.04$, $P = 0.0286$, $R^2 = 0.08$), and hence with an increase in the probability of producing selfing flowers (Fig. 4C). Increases in flower size related to greater plant size due to increases in nutrient availability have been reported more often (Kagaya et al., 2009).

Phenotypic and genetic correlations among floral traits are expected to occur because of shared developmental pathways

(Krizek and Fletcher, 2005) and strong stabilizing selection for floral integration (Armbruster et al., 2004). In this study, flower size (length factor) was correlated with all traits except herkogamy; this suite of correlated characters is maintained in the two populations regardless of environmental variation, and they participate in a common function that responds as a whole to flower position (mostly in the low-nutrient environment) (Table 3; Fig. 4B).

We interpret the maintenance of this suite as evidence of common developmental regulation of correlated characters (e.g. Bissell and Diggle, 2008). Since herkogamy was not correlated with length factor, the influence of size is clearly accomplished by means of its correlation with pistil and stamens, which showed a strong association with corolla (Fig. 3). This positive association of pistil and flower size suggests that a reduction in herkogamy is associated with smaller flowers in the Pedregal population. Also, despite the strong correlation of floral parts (i.e. corolla, pistil and stamen lengths), their plastic responses are not equal. In fact, slopes for within-plant trends differ for stamen and pistil lengths, with sequential reduction in pistil length being greater than that for stamens (see Results 'Genetic and environmental effects on the traits of individual modules'). Thus, pistil height reduction in the low-nutrient environment drives a reduction in herkogamy and increases the probability of self-fertilization (cf. flower and flower by treatment interaction in Table 2). Other studies have shown the opposite for *D. stramonium* (Motten and Antonovics, 1992; Motten and Stone, 2000) and *E. paniculata* (Barrett et al., 2009), i.e. no correlation between an estimate of flower size (i.e. corolla or perianth) and herkogamy. However, a more precise estimate of flower size should have the length of all floral organs involved (factor 1 or length factor in Bissell and Diggle, 2008), since, from a morphometric point of view, it is the correlation of these characters that properly measures flower size. Notwithstanding, the latter could not be the case, at least for *E. paniculata*, because during the early stages of the establishment of selfing, the association between perianth and herkogamy does not occur and the stigma-anther separation can be altered independently of changes in flower size (Vallejo-Marín and Barrett, 2009). In fact, the association between herkogamy and perianth size occurs in other groups where reductions in herkogamy are often associated with the evolution of small flowers (e.g. *Mimulus*; Fishman et al., 2002). The perianth seems a good estimator of flower size in these groups.

Implications for the mating system

Phenotypic plasticity is a strategy by which individual plants may adjust to environmental heterogeneity (Bradshaw and Hardwick, 1989). Heterogeneity in nutrient availability could be a major regulator of the population dynamics of some herbaceous plant species (Lewis and Probyn, 1978; Thompson, 1994). Depending on the timing of germination and on their proximity to the parent, seedlings of short-lived plant species can be susceptible to the effect of nutrient depletion provided by the litter of the mother plant, and therefore can find themselves in a rich- or in a poor-nutrient environment, which induces major phenotypic changes in terms not only of plant size

but of architecture as well (Thompson, 1994). The Pedregal population locality has volcanic thin soils, with a very irregular topography, which creates heterogeneity in the deposition of soil materials as nutrients (Cano-Santana and Meave, 1996). Therefore, phenotypic plasticity could be selected in the Pedregal population to cope with these heterogeneous soil conditions. Variation in the rate of self- and cross-fertilized flowers in this population resulting from plasticity could allow individuals to match current environmental conditions. The increase in self-fertilization in the Pedregal population could be favoured in low-nutrient environments where low abundance of pollinators favours the decrease of cross-pollination. Other studies have reported an increase of selfing in stressful environments (Stebbins, 1957; Elle and Hare, 2002; Elle, 2004; Moeller and Geber, 2005; Vallejo-Marin and Barrett, 2009).

The potential for evolutionary change in the mating system of extensively self-fertilized species such as *D. stramonium* may depend in part on the relative fitness of selfed and outcrossed progeny under different environmental conditions (Motten and Antonovics, 1992). If increased selfing results in mostly homozygous lines in this species, most individuals in a population will thus be largely purged of deleterious recessive alleles and, as a result, the level of inbreeding depression will be decreased (Lande and Schemske, 1985; Yahara, 1992). If this is true for *D. stramonium*, two scenarios could be considered: (1) that outcrossing in a population would eventually be eliminated (Fisher, 1941; Jain, 1976; Wells, 1979; Charlesworth, 1980); or (2) that some level of outcrossing might be favoured if subsequent rounds of outcrossing progeny of homozygous lines generate a very large amount of genetic variability and novelty (Allard *et al.*, 1968; Motten and Antonovics, 1992).

Within-individual variation may constitute an adaptive strategy to produce variable progeny, in which individuals produce more than one functional class of reproductive organs (Lloyd, 1984; Herrera, 2009). Given the low levels of outcrossing reported in *D. stramonium*, the species has been hypothesized to represent a persistent mixed mating system rather than a transition to complete selfing (Motten and Antonovics, 1992; Cuevas, 1996; Motten and Stone, 2000). However, the former may occur in populations where long periods of increased pollinator abundance favour the increase of a non-systematic within-plant trend of variation (cf. fig. 6.4b in Herrera, 2009), such as the Ticumán population, which showed genetic variation for almost all traits in relation to flower position. This could lead to adaptive levels of within-plant variance, as it is supported by predictions from genetic models of the adaptive consequences of selection on environmental components of phenotypic variance (Bull, 1987; Zhang, 2005). Conversely, a transition to complete selfing instead of a mixed strategy may be possible in *D. stramonium* if more stressful environments where either biotic (e.g. low pollinator abundance) or abiotic conditions (e.g. nutrient availability causing a reduced flower size) favour the fixation of a systematic within-plant trend of variation that provide reproductive assurance (Darwin, 1878), such as in the Pedregal population. Future field studies are required to investigate in depth whether within-plant variation of herkogamy-related traits in *D. stramonium* has the potential for evolutionary change of the mating system under different ecological conditions and hence if it has any adaptive value.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. **Table S1:** median values (first to third quartile) of selected floral traits based on mean family values of six sequential flower positions in 817 flowers of two populations of *Datura stramonium* grown in low, mid and high nutrient availability. **Table S2:** phenotypic correlation coefficients between floral traits, and flower integration indexes for three nutrient environments in two populations of *Datura stramonium*. **Table S3:** comparisons of integration pattern among nutrient environments both within and among populations. Each value corresponds to the correlation coefficient between correlation matrices.

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APPENDIX

To determine the level of association of the floral phenotype among environments, we calculated the associations between floral traits in each population with the Pearson product–moment correlation coefficient at the phenotypic level in each environment (Supplementary Data Table S2).

The phenotypic integration level of floral traits for each nutrient environment, in each population, was assessed using the variance of eigenvalues of the corresponding correlation matrix (Wagner, 1984). Because treatments differed in sample

size, corrected INT values were estimated for the purpose of comparison among nutrient environments and populations. Standard errors and confidence intervals of each INT were obtained by bootstrapping (Cheverud *et al.*, 1989; Herrera *et al.*, 2002). To compare the integration patterns among nutrient environments and populations, we performed pairwise comparisons among all correlation matrices using Mantel test (Baker and Wilkinson, 2003; Pérez *et al.*, 2007). Standard errors were obtained by bootstrapping (Supplementary Data Table S3).