

## RESEARCH PAPER

# A test of phenotypic selection on petal form in the wild carnation, *Dianthus inoxianus*

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Evolution; fitness; flower; Hawkmoth; petal size; pollination.

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**ABSTRACT**

Floral phenotypes are considered a product of pollinator-mediated selection, which also has the side effect of decreasing floral variation within species. Correlates of flower visibility and function were studied in a carnation species (*Dianthus inoxianus*), which has crepuscular anthesis and scent-based pollination by the hawkmoth *Hyles livornica*. We also assessed constancy of flower form in nature and in cultivation and, using fruit set as an estimate of plant relative fitness, tested whether the main pollinator exerted phenotypic selection on floral traits. Petal claw, which is roughly equivalent to the average depth at which an insect's proboscis must be inserted to reach nectar, was remarkably constant among wild plants (coefficient of variation 8%). In contrast, the area of the visible part of the petal, and the intensity of a coloured dot pattern on the petal was very variable (respectively CV = 34% and 102%). Cultivation in a common environment revealed significant variation among genotypes as regards petal area, degree of laciniation and extension of the dot pattern, but not petal claw length, which remained steady. Petal area, shape and colour did not affect relative fitness during the year of study, but plants with intermediate petal claws (*i.e.* floral tubes) set significantly more fruit. Results are compatible with low response of the main pollinator to variation in visual traits (petal area, laciniation, colour) and high responsiveness to variation in other aspects (tube length). Inconsistent phenotypic selection by pollinators may add to other causes of floral variation in the genus *Dianthus*, the causes of which are discussed.

**INTRODUCTION**

Flowers are regarded as the least variable organs of plants within a species, particularly so when compared to vegetative parts (Berg 1960; Armbruster *et al.* 1999). But this is not to say that floral phenotypes are totally invariant within a species, and in fact many studies have attested floral quantitative variation among conspecifics (Galen 1996; Cresswell 1998); admittedly, this variations may easily turn into an obstacle to reproduction and should be small (but see Herrera *et al.* 2001, 2002; Herrera 2004, 2005). In fact, pollinator-mediated selection is considered a major factor in shaping the floral phenotype and decreasing intraspecific floral variability (Alexandersson & Johnson 2002; Sletvold *et al.* 2010; Schiestl & Johnson 2013).

On the other hand, there are also instances of plants with unexpectedly high intraspecific floral variation. For example carnation (*Dianthus* spp.), particularly species such as *D. caryophyllus*, in which flowers of varying colours, shapes and scents are known. Researchers soon became interested in the genetics of floral variation in *Dianthus* (Geissman & Mehlquist 1947; Mehlquist & Geissman 1947), and this interest still remains, for example on the role of endopolyploidy and transposable elements (Itoh *et al.* 2002; Agulló-Antón *et al.* 2013; Chacón *et al.* 2013). Carnation is the second most important species on the worldwide market for cut flower (Zvi & Vainstein 2007), so studying floral variation in this genus has obvious economic importance because more knowledge can result

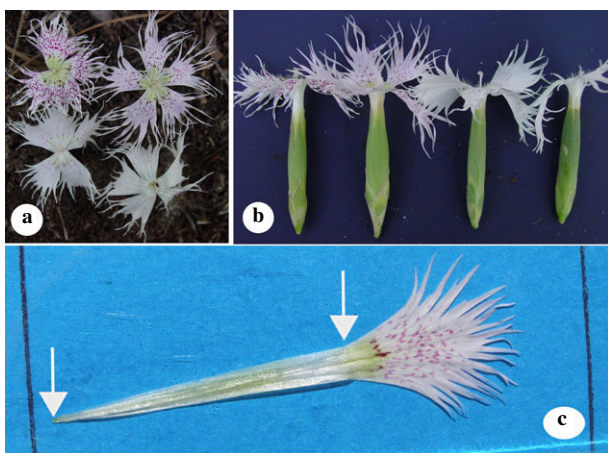
into better ornamental breeding. Moreover, the flowers of *Dianthus* are also good subjects to study phenotypic selection in the wild: there is evidence that pollinators can mould flowers through selection of some phenotypes over others (Cresswell 1998; Giles *et al.* 2006; Fenster *et al.* 2015), and that different traits may respond differently to selection (Herrera *et al.* 2002). Studying floral variations in *Dianthus* has the potential to improve our understanding of flower evolution in natural plant populations.

Studies on the reproduction of wild *Dianthus* populations have considered the effects of population fragmentation on seed set (Jennersten 1988); the efficiency of different butterfly pollinators (Erhardt 1990; Bloch *et al.* 2006); incidence of selfing (Erhardt *et al.* 1995); and the relationship between floral morphometrics and pollination syndromes (Jürgens 2006). The present study deals with floral variation and phenotypic selection in the southern Spanish endemic carnation *Dianthus inoxianus*, Gallego (Gallego 1986), the highest-order polyploid in the *D. broteri* polyploid complex (2 $\times$ , 4 $\times$ , 6 $\times$ , 12 $\times$ ), a rapidly diversifying group of Mediterranean carnations that is evolutionary young (0.9–2.1 million years; Valente *et al.* 2010). The different cytotypes of this complex rarely if ever coexist, and molecular data indicate that they do not exchange genes (Balao *et al.* 2009, 2010).

Endemic to a small littoral area of the Doñana National Park in south Spain, *D. inoxianus* is an allogamous perennial subshrub with thin branches, narrow (3-mm wide) leaves and a

tussock-like appearance. Plants may be up to 0.5-m tall in the vegetative phase, but they often remain much shorter (0.1–0.2 m) and hard to detect because of vertebrate herbivory. Plant conspicuousness increases considerably at the moment of inflorescence development, from May through August. The flowering stems bear strongly protandrous, sweet scented, white flowers (sometimes with pink spots) that open at night and secrete nectar at the bottom of the elongated calyx tube (Fig. 1). No fruit is set if insect access is prevented, suggesting that flowers do not self-pollinate (seemingly as a result of protandry; F. Balao, unpublished data). Seeds are most often passively dispersed when the wind shakes the capsules, or can also be occasionally collected by ants.

*Dianthus inoxianus* represents a relatively rare component of the xerophytic scrub formation, dominated by shrubby species of Cistaceae, Lamiaceae and Papilionidae, which is typical of fixed sand dunes in the region. It can be locally abundant on occasions, but has a restricted distribution area and is, in absolute terms, a rare species: total number of reproductive individuals has been estimated at <2000 (Balao *et al.* 2007). From a population genetics perspective, they constitute a single panmictic meta-population (Balao *et al.* 2010). *D. inoxianus* also represents an exceptional case in that it depends on a single pollinator species for reproduction, the long-tongued (30.5 mm in S Africa; Johnson & Liltved 1997; 26.2 mm, F. Balao, unpublished data) hawkmoth *Hyles livornica*. This species accounted for 75 of 88 visits to *D. inoxianus* flowers in 5 years of field observations (F. Balao, unpublished data). In contrast, biotically pollinated taxa in Mediterranean Spanish scrub most often form vast, nearly continuous populations, and have generalistic pollination systems with diverse pollinator guilds consisting of dozens of visitors in several insect orders (Herrera 1987, 1988). Several major and minor compounds in the scent of *D. inoxianus* elicit electrophysiological responses in the antennae of *Hyles livornica* (Balao *et al.* 2011a), which points to a tight relationship with its hawkmoth pollinator (although tightness is not reciprocal, since *H. livornica* is actually a rather generalist



**Fig. 1.** *Dianthus inoxianus* flowers and petals. (a) top view of flowers depicting colour and shape variability; (b) side view of the same flowers; (c) petal detached and flattened to be photographed and measured through image analysis. White arrows mark the elongated portion of the petal enclosed inside the calyx tube (*i.e.* CLAW), extending from the tip to the portion where it bends outwards to become the blade of the petal.

migrant that can forage on many other plant species across its distribution area; *e.g.* Johnson & Liltved 1997; Johnson *et al.* 2002). In this context, phenotypic selection by the main pollinator should be readily detectable. The main goals of the present study are to: (i) investigate phenotypic variation in correlates of flower function and visibility in *D. inoxianus*; (ii) assess trait constancy among conspecifics, both in nature and in cultivation; and (iii) test whether the main pollinator exerts any significant phenotypic selection on petal form. This will help to better understand reproductive isolation and evolution in the group of Mediterranean carnation species to which *D. inoxianus* belongs.

## MATERIAL AND METHODS

### Plant sampling and petal measurements

A low proportion (<10%) of *D. inoxianus* plants present female organs only (*i.e.* strictly speaking the species should be described as gynodioecious), but we restricted sampling to perfect-flowered plants. We collected one open flower in the male stage from each of 172 individuals across the whole area of distribution. Maximum distance among sampled plants was 35 km. Flowers were kept in numbered sealed plastic bags and transferred to the lab to perform measurements. One randomly chosen petal per flower was placed flat on a brightly coloured surface with a scale line drawn, and photographed with a digital camera at 1600 × 1200 resolution. Pictures were then processed with image analysis software (GIMP 2.8.10, <http://www.gimp.org/>) to determine four quantitative petal features presumably relevant to floral visibility and function (see Fig. 1): length of the petal part enclosed inside the calyx (CLAW, hereafter), which is equivalent to calyx tube length and determines the depth at which nectar is concealed; total area of the petal 'blade' (*i.e.* the part of the petal that is external to the calyx; AREA), a major determinant of overall visibility; area accounted for by laciniae relative to total blade area (LACINIA, a percentage); and area covered by pink-coloured dots relative to total blade area (DOTTING, a percentage). We considered these two percentages as enhancers or modifiers of flower visibility. Linear and surface measurements were first noted in pixels, and then converted to mm and mm<sup>2</sup>, the conversion consisting in multiplying pixel number by the scale factor derived from the reference line in the photographs. Descriptive statistics and phenotypic correlations among the studied traits were calculated with the STATISTICA package (version 6; StatSoft Inc., Tulsa, OK, USA). Variables were log (CLAW, AREA) or arc-sin square root-transformed (LACINIA, DOTTING) prior to analyses.

We tested the repeatability of image analysis by measuring two randomly chosen petals, instead of just one, in 20 flowers from 20 plants. The first and second measurements correlated very significantly ( $r = 0.998$ ;  $P < 0.001$ ) and did not differ much in relative terms (95% confidence interval 2.5–4.3%; mean difference 3.1%), so we considered the procedure acceptably repeatable. Furthermore, and even though the data we report below are based on a single flower per plant, we also checked within-plant floral variations in a subsample of 18 shrubs that had two randomly chosen flowers measured instead of one. In this subsample the study variables were analysed through repeated measures ANOVA with the first and second flower as replicates. Most often, no statistically significant vari-

ation was found among flowers within plants (for CLAW, AREA and DOTTING, respectively  $F = 2.45$ ,  $F = 0.826$ , and  $F = 1.54$ ;  $df = 1, 17$ ,  $ns$  in all cases). For LACINIA, however, weak but statistically significant within-plant variation was detected ( $F = 5.88$ ;  $df = 1, 17$ ,  $P = 0.03$ ).

### Floral variation in cultivated plants

Wild *D. inoxianus* exhibited considerable variation in petal form, but it was unclear which part of this variation was genetically determined or environmentally induced. In order to investigate among-plant variation while controlling for phenotypic plasticity, we measured petals in vegetatively propagated plants grown in a common environment. To achieve this, 100 randomly chosen wild plants were used as sources of cuttings. Each plant was >5 m away from its nearest neighbour, so it was assumed that it represented a different genotype. A previous genetic study (Balao *et al.* 2010) also failed to find vegetative propagation of plants in the field. Cuttings were given a root stimulant (CLONEX<sup>®</sup>; Growth Technology Ltd., Somerset, UK), inserted in numbered rooting containers with Perlite for 1 month, then transferred to individual pots with a rich substrate, and given adequate water and light for 2 years in greenhouse facilities of the University of Seville. Despite the favourable growing conditions, only 17 cuttings of six genotypes eventually flowered. They produced from one to four flowers per cutting, a total of 38 flowers. In these, one randomly chosen petal was picked, photographed and morphological variables determined following the method explained above. With these data we computed a general linear model MANOVA (STATISTICA package; Statsoft Inc., Tulsa, OK, USA) that tested the effect of genotype on petal form. The relatively low success of the cloning procedure, and the resulting small sample size precluded a more comprehensive analysis with additional factors (*e.g.* clone identity).

### Univariate selection differentials

To investigate the relationship between petal phenotype and relative reproductive success, we estimated percentage fruit set (*i.e.* ratio of flowers to fully-developed fruits) in 26 wild plants living in a relatively dense stand near the centre of the population (two to 32 flowers per plant,  $N = 481$  flowers). Plants were chosen at this particular area (rather than through the whole range of the species) in order to maximise the chances of pollinator visitation to flowers, and minimise environmental variability. Plant-based fruit set, divided by stand average, was then used as an estimate of relative fitness during that particular reproductive season, and in this particular area. Additionally, each plant had its petal phenotype characterised by measuring CLAW, AREA, LACINIA and DOTTING. Standardised linear and quadratic coefficients (*i.e.* selection differentials) were calculated for these traits using univariate linear regression models (Lande & Arnold 1983). Relative fitness was separately regressed on each standardised trait value (mean = 0 and variance = 1), and the slope of the regression used as an estimate of the strength of selection (Lande & Arnold 1983; Conner 2001; Kingsolver *et al.* 2010). The quadratic selection differential was estimated as twice the quadratic regression coefficient (Stinchcombe *et al.* 2008). The robustness of regression estimates was tested using 10,000 bootstrap iterations. Bootstraps and

**Table 1.** Major statistics for petal traits in wild *Dianthus inoxianus*, data based on  $N = 172$  flowers, one from each of 172 plants.

	traits			
	CLAW (mm)	AREA (mm <sup>2</sup> )	LACINIA (%)	DOTTING (%)
Mean (SE)	30.21 (0.18)	87.48 (2.29)	31.86 (0.52)	5.46 (0.42)
Coefficient of variation (%)	8	34	21	102
Skewness (g1)	0.32	1.46	0.34	1.51
Kurtosis (g2)	0.08	3.64	0.21	2.41

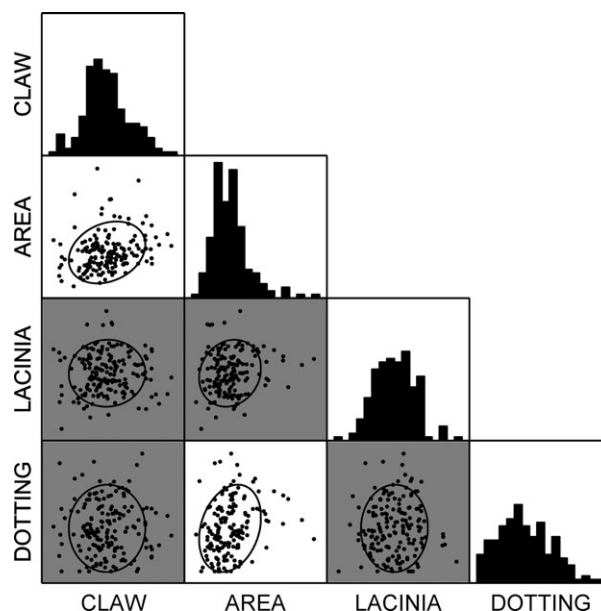
confidence intervals (BCa; bias-corrected and accelerated; Efron & Tibshirani 1986) were calculated using package *boot* in the R statistical software (R Foundation for Statistical Computing, Vienna, Austria).

## RESULTS

### Phenotypic correlations and variability in wild plants

As shown in Table 1, the average depth at which an insect's proboscis must be inserted to reach nectar in *D. inoxianus* (*i.e.* CLAW) is around 30 mm. This elongated – and hidden – portion of the petal is remarkably constant in length (coefficient of variation 8%) compared to the area of the broad visible part of the petal (CV = 34%) and, particularly, the extension of the dot pattern on petals (CV = 102%). Of all four traits examined, CLAW also had the smallest kurtosis and skewness.

Figure 2 depicts phenotypic correlations among the four floral traits. Except for CLAW–AREA ( $r = 0.25$ ,  $P < 0.01$ ) and DOTTING–AREA ( $r = 0.25$ ,  $P < 0.01$ ), correlations were most often not significant, suggesting that the studied traits varied with relative independence.



**Fig. 2.** Scatterplot matrix showing phenotypic correlations among petal characteristics in *Dianthus inoxianus*, with 90% confidence ellipses and frequency distributions for the variables (untransformed data). Correlations statistically not significant are shown in a grey background.  $N = 172$  plants.



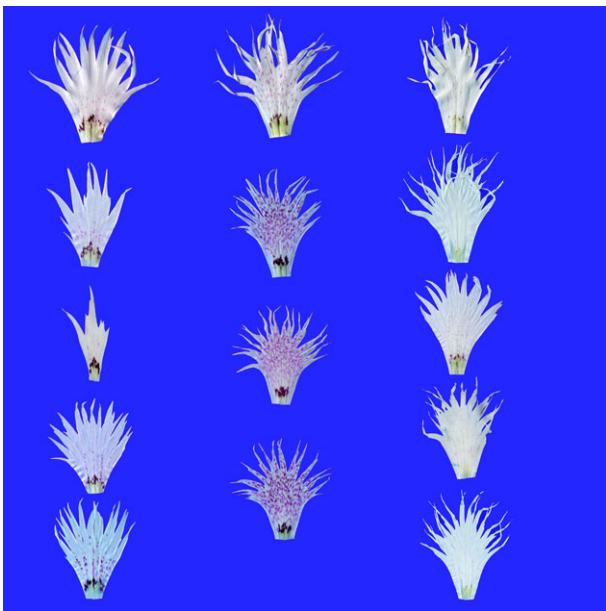
**Table 2.** Petal form as affected by genotype in cloned *D. inoxianus* plants. The General Linear Model MANOVA is based on six genotypes.

Multivariate test				
Wilks' Lambda	df	F	P	
0.017	20, 97	11.661	<0.001	
Univariate tests				
Source	MS	df	F	P
CLAW	0.63	5	0.31	ns
Error	2.01	32		
AREA	5742.89	5	3.36	0.01
Error	1707.57	32		
DOTTING	576.26	5	126.93	0.01
Error	4.54	32		
LACIN1A	17.94	5	5.69	<0.001
Error	3.82	32		

### Flower form in cultivated plants

An analysis of petal form as affected by genotype is shown in Table 2. Significant variations occurred among plants regarding AREA, DOTTING and LACINIA, but not CLAW. In other words, flower tube length did not vary to any significant extent among plants, whereas the shape and size of the visible portion of the petals often did.

Figure 3 presents a comparison of petal form in wild and cultivated plants with, for simplicity, only three floral phenotypes from the wild (top row) and their corresponding greenhouse-grown counterparts shown. On occasions, the size and degree of laciniation of the petals of a wild plant could be noticeably different from those of the corre-

**Fig. 3.** Petal variation in *Dianthus inoxianus*. One petal from each of three different wild shrubs are depicted in the top row, and below are their greenhouse-cultivated counterparts. Petals in a column are the same genotype.**Table 3.** Univariate linear ( $\beta'_i$ ) and quadratic ( $\gamma'_i$ ) selection coefficients of four petal traits in *Dianthus inoxianus*.  $BC_a$  is (bootstrapped) confidence interval at 95%.

Petal phenotypic trait	$\beta'_i$	$BC_a$	$\gamma'_i$	$BC_a$
CLAW	0.06	-0.15 to 0.35	<b>-0.46</b>	<b>-0.94 to -0.10</b>
AREA	-0.12	-0.36 to 0.12	0.18	-0.44 to 0.60
DOTTING	0.09	-0.18 to 0.31	0.00	-0.50 to 0.50
LACINIA	-0.12	-0.33 to 0.08	-0.02	-0.52 to 0.54

Coefficients statistically different from zero are in bold.

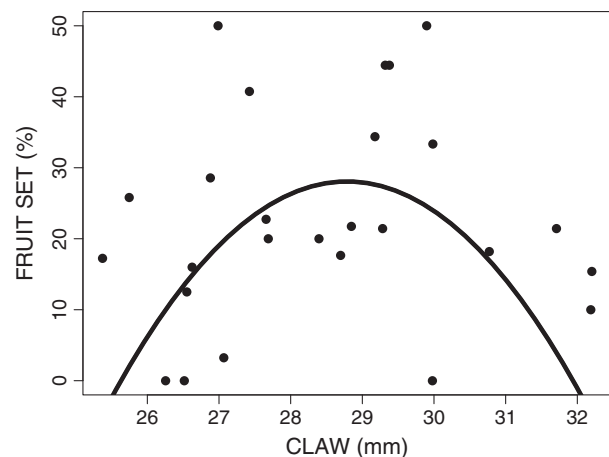
sponding cultivated clones (e.g. left column), and the same applied to intensity and extension of the dot pattern (middle column). Nevertheless, there were also examples (right column) of genotypes in which the wild shrub and the cultivated clones were very similar in form. Because of the small sample size, our data do not allow a proper analysis of floral form as affected by clone identity.

### Selection coefficients in wild plants

As shown in Table 3, AREA, LACINIA nor DOTTING did not have selection coefficients significantly different from zero. In sharp contrast, the petal attribute less variable among those studied (CLAW) indeed imposed a significant quadratic selection coefficient on relative fitness: plants with tubes shorter or longer than the mean (ca. 29 mm) faced significant relative fitness costs in that year. On average, fitness was highest in *D. inoxianus* with intermediate CLAW values (Fig. 4), at least for that particular reproductive season.

### DISCUSSION

The flowers of *D. inoxianus* are noticeably variable. Interestingly, however, the length of the hidden part of the petal, or claw (a direct correlate of flower tube length), was comparatively constant both among wild plants and cultivated clones. The same relationship is found in the similarly-structured flowers of *Silene dioica* (Caryophyllaceae), where Giles *et al.*

**Fig. 4.** Quadratic selection coefficient (solid line) for petal claw length (CLAW) in 26 *Dianthus inoxianus* plants.

(2006) reported relatively steady tube lengths. Furthermore, the extensive variations that exist among *D. inoxianus* in petal area or colour had no obvious consequences for fruit production (used here as a surrogate of fitness), whereas minute differences in floral tube length correlated significantly with relative fitness. This finding can be related to reports in some Papilionoid legumes, in which flower parts without direct involvement in pollen exchange (e.g. calyx size) are intraspecifically more variable than others connected directly to reproduction (e.g. keel petals; Herrera 2001b). Our results warn against uncritically attributing equivalent functional importance to every floral trait when studying the pollination biology of plants.

Floral tubes in *D. inoxianus* are both relatively long (30 mm) and steady (CV = 8%) when compared with co-occurring plant species. For example, in a sample of 58 southern Spanish taxa investigated by Herrera (1996), the length of the floral tube ranged from 4.2 to 29.6 mm, with intraspecific coefficients of variation between 10% and 25%. In this biogeographic context, *D. inoxianus* would be near or at the top of the range of floral tube lengths, and at the bottom of intraspecific tube length variation. This fits nicely with Fenster's (1991) hypothesis that tube length should be constant in taxa with deep tubular flowers (as a result of increased pollinator discrimination and reinforced phenotypic selection by the correspondingly species-poor pollinator array). Although pollinator-mediated selection on flower tube has been widely detected (Alexandersson & Johnson 2002; Anderson *et al.* 2014), Herrera's (1996) species sample did not show a significant general inverse relationship between absolute tube length and intraspecific variance. This could be attributed to the fact that his survey of Spanish species included a large proportion of small-flowered taxa (*i.e.* with corollas too small to be any use for pollinator discrimination), and hence phenotypic selection on tube length in his sample would be the exception, not the rule. Nevertheless, taxa with floral tubes long enough to effectively discriminate pollinators, like *D. inoxianus*, do confirm the predicted inverse relationship between tube length and intraspecific variation.

Coexistence of constant and unstable floral traits in *D. inoxianus* implies some lack of 'integration' of the floral phenotype (Conner & Via 1993; Armbruster & Schwaegerle 1996; Herrera 2001a; Armbruster *et al.* 2014). We hypothesise that this is a result of 'blindness'/low responsiveness of the major pollinator, the fast-flying sphingid *H. livornica*, toward visual traits (petal area, laciniation, colour) and high responsiveness to others (tube length, floral scent). Field observations that white- and pink-flowered plants are not discriminated by

*H. livornica* visitors support this view (F. Balao, unpublished data). In contrast, their antennae show electrophysiological responses when exposed to some components of *D. inoxianus* scent, which contains 68 volatile compounds (Balao *et al.* 2011a). Specifically, aliphatic 2-ketones, with a deterrent function in other plant species, seem to act as a cue to the main pollinator in this case (Balao *et al.* 2011a). This sphingid species seems very fastidious as regards floral scent, and because phenotypic selection might in fact be operating on floral scent volatile composition rather than on visual cues (as occurs in *Penstemon digitalis*; Parachnowitsch *et al.* 2012), individual variations of scent composition in *D. inoxianus* might be worth further study.

Continuous floral variation is a complex phenomenon with proximate causes that are both genetic and environmental, but decoupling of traits that are non-functional (or relatively unimportant) from highly selected flower traits is expected only under low developmental constraints (Cheverud 1984). We have shown that *D. inoxianus* floral traits are often uncorrelated with each other, suggesting they do not share developmental patterns. Furthermore, polyploidy causes shifts in the structure of the phenotypic covariance matrix (Balao *et al.* 2011b), which may still contribute to decouple variation among floral traits. In cultivated carnation species, on the other hand, petal size correlates positively with cell size and cell ploidy level (modified by endoreduplications of chromosomes and mixoploidy; Agulló-Antón *et al.* 2013). Additionally, flowers (of a given cultivar) collected during autumn are smaller than those collected during winter (Chacón *et al.* 2013). Activity of transposable elements has also been related to floral variegation in cultivated carnation species (Itoh *et al.* 2002). Most or all of these factors may also operate in wild *D. inoxianus* plants and deserve further study, particularly if there is mixoploidy in flower cells.

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