



## Differences in pollinator faunas may generate geographic differences in floral morphology and integration in *Narcissus papyraceus* (Amaryllidaceae)

Rocío Pérez-Barrales, Juan Arroyo and W. Scott Armbruster

R. Pérez-Barrales and J. Arroyo (arroyo@us.es), Depto de Biología Vegetal y Ecología, Univ. de Sevilla, Apartado 1095, ES-41080 Sevilla, Spain. RPB and W. S. Armbruster, School of Biological Sciences, Univ. of Portsmouth, Portsmouth, UK, PO1 2DY. WSA also at: Inst. of Arctic Biology, Univ. of Alaska, Fairbanks, AK 99775, USA and Dept of Biology, NTNU, NO-7491, Trondheim, Norway.

Pollinators may generate selective pressures that affect covariation patterns of multiple traits as well as the mean values of single floral morphological traits. Berg predicted that flowers pollinated by animals whose morphology closely matches the flower's shape will be phenotypically more integrated (tighter correlation of flower traits) than will flowers pollinated by animals not closely fitting the floral morphology. We tested this hypothesis by comparing, in the Strait of Gibraltar region (south Spain, northern Morocco), populations of *Narcissus papyraceus* that have geographical differences in pollinator faunas. Long-tongued, nectar-feeding moths dominate the pollinator faunas of those populations close to the Strait of Gibraltar, whereas short-tongued, pollen-feeding syrphid flies dominate in peripheral populations farther from the Strait. Populations pollinated by moths and flies differed in the mean values of several floral traits, consistent with the evolution of regional pollination ecotypes. Populations pollinated by moths showed stronger intercorrelation (floral integration) than populations pollinated by hoverflies. Moth-pollinated populations also showed less variation in flower traits than vegetative traits, and this difference was stronger than in fly-pollinated populations. Thus, the pattern of differences in the phenotypic architecture of the *Narcissus* flowers is consistent with the hypothesis that populations have responded to different selective pressures generated by different pollinators. These data also supported most of the specific predictions of Berg's hypotheses about integration and modularity.

Interactions between plants and their pollinators have been the subject of scientific interest since Darwin (1862) first explained the origins of floral form in terms of adaptive evolution. Soon thereafter it became widely accepted that most plant species are tightly adapted to pollination by one or a few pollen vectors. However, many studies, employing a variety of approaches (descriptive, observational and experimental) and levels (micro- and macroevolutionary), have shown that the situation is actually more complex, with many plants being pollinated by numerous kinds of animals (Waser et al. 1996, reviewed by Fenster et al. 2004). The situation is further complicated by variation among populations in the main pollinators, which sometimes leads to morphological differentiation or the formation

of pollination ecotypes (Armbruster 1985, Robertson and Wyatt 1990; see discussion in Thompson 2006).

Following Grant's (1949) seminal work, many studies of pollination biology focused on specialized, apparently co-evolved relationships. Most of these studies have been conducted at the species level using phylogenetically controlled analyses (Armbruster and Baldwin 1998, Armbruster et al. 2002, Beardsley et al. 2003, Pérez-Barrales et al. 2006). These studies draw conclusions about natural selection under the implicit assumption that the patterns seen at the species level are generated primarily by processes operating at the population level, although some authors have raised doubts about the validity of this assumption (Simons 2002). A few studies on the evolution of pollination

systems have measured natural selection (Schemske and Bradshaw 1999, Armbruster et al. 2005, reviewed by Fenster et al. 2004). In these studies, flower traits are usually treated as independent variables affecting components of plant fitness. Such studies generally focus on trait means and variances (Endler 1986).

Floral features do not, however, usually vary independent; instead, they often form ensembles of intercorrelated traits, as a result of pleiotropy, linkage, or developmental effects. This was perspicaciously noted by Berg (1959, 1960), who hypothesised that plants with tight relationships with one or a few similar pollinators should be under strong selection for stable floral morphology despite environment and genetic variation that might affect other traits (i.e. 'correlation pleiades' of Terentjev 1931, 'modularity' of Wagner 1996). Other authors have extended this argument to predict patterns of covariation of floral traits. Specifically, those traits that interact functionally in the pollination process should covary more tightly than those that do not (the "integration hypothesis", Conner and Via 1993), and those species with more specific pollination relationships (tighter fit with fewer species of pollinators) should exhibit floral traits that covary more tightly (Armbruster et al. 1999). Some authors have tested one of both of these hypotheses at the species level (Conner and Via 1993, Conner and Sterling 1995, 1996, Wait and Levin 1998, Armbruster et al. 1999, 2004), with generally supporting results. By contrast, results of the few studies at the population level have failed to support these hypotheses (Herrera 2001, Herrera et al. 2002; but see Waitt and Levin 1993).

According to Berg (1959, 1960) and subsequent authors (Conner and Via 1993, Conner and Sterling 1996, Armbruster et al. 1999), the nature and strength of the selective pressures exerted by pollinators will influence the degree of modularity (independence from vegetative traits) and integration of floral traits (correlations inter alia) (C. M. Herrera 2002, J. Herrera 2005). This general statement was partitioned by Armbruster et al. (1999) into several explicit predictions, which we summarize here and apply to the population level: (1) flowers should be modular; i.e. correlations between flower and vegetative traits should be lower than correlations between flower traits, and this difference should be greater in populations where flower shapes fit closely with pollinators; (2) floral traits will be more tightly intercorrelated than vegetative traits (i.e. exhibit greater phenotypic integration; Murren 2002), and this difference will be greater in populations with flowers that fit closely with pollinators; (3) independent of correlation strength, flower traits will be "buffered" from variation in vegetative traits in populations with flowers that fit closely with pollinators ("buffering" is shown by shallower regression slope; a slope of 1 would

indicate a completely coupled, isometric relationship between flower and vegetative traits); and (4) variation of flower traits (as measured by the coefficient of variation, CV) will be lower in populations that have flowers that fit closely with pollinators. When discussing the evolutionary significance of these patterns, Berg (1960) assumed that phenotypic correlations reflect genetic correlations; indeed this has been shown to often be the case (Waitt and Levin 1998).

*Narcissus papyraceus* (Amaryllidaceae) provides an excellent study system for testing hypotheses on pollinator-mediated selection. This species has flowers with long tubes and is style dimorphic (with long -L- and short -S- styles). The populations show a variable morph ratio, from isoplethy (L=S) to L-monomorphism (Arroyo et al. 2002). Preliminary observations by these authors suggest that monomorphic and dimorphic populations are pollinated by different insects (hoverflies and hawkmoths, respectively), which could be responsible for the variation in morph ratio. Despite extensive work on pollinator effects in the maintenance of style polymorphism in *Narcissus* (Arroyo and Dafni 1995, Baker et al. 2000a, 2000b, Arroyo et al. 2002, Thompson et al. 2003, Barrett et al. 2004), the effects of pollinator-mediated selection on the covariation of perianth traits has not been evaluated. If different pollinator faunas visit flowers of geographically isolated populations of *N. papyraceus*, then we can use this system to test the above predictions of Berg's hypothesis (1959, 1960) at the population level. The rationale behind this argument arises from the fact that this species has long-tubed flowers, with upper stamens well exposed (Fig. 1), and nectar hidden in the tube base. Winter-blooming *N. papyraceus* have no abundant, sympatric, co-flowering species, and thus it is almost the only available source of nectar for insects in this season. Hence nectar-feeding moths depend strongly on these flowers. Moths need to handle the flower to access the nectar and might thus exert stronger selective pressures on the whole flower. Hoverflies, by contrast, visit flowers of *N. papyraceus* only to forage pollen (their tongues are too short to access the nectar). Accessible pollen is located in exerted upper stamen whorl. Therefore, hoverflies may exert less selective pressure on the whole floral structure, although it may be strong on particular traits related to showiness and pollen delivery. Several studies have shown that insects handling flowers in different manner can generate very different selective pressures, and that this variation in selection can be observed among populations within species (Schemske and Horvitz 1984, Galen 1989, Campbell et al. 1996).

Thus, according to the Berg's (1959, 1960) and Conner and Via's (1993) hypotheses, flower traits of populations of *N. papyraceus* pollinated by moths should be more strongly decoupled from vegetative

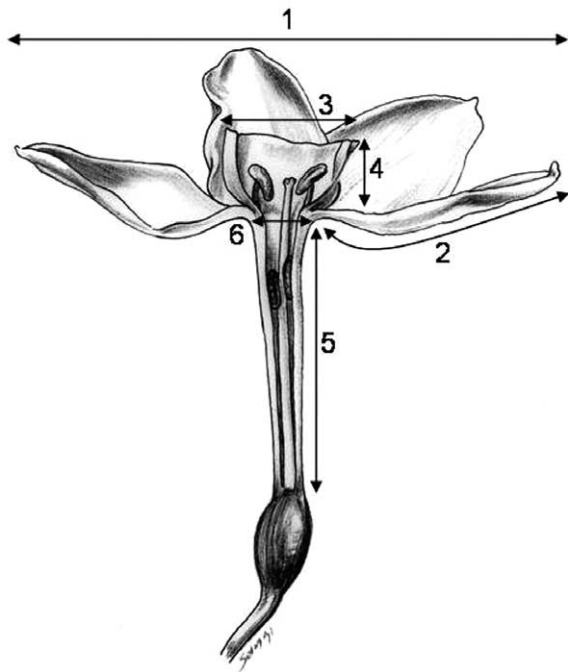


Fig. 1. Flower section of *Narcissus papyraceus*, only a long-styled morph (L), and two out three stamens per whorl are depicted. Measured flower traits: (1) flower width, (2) length of outer tepal, (3 and 4) corona width and length, (5 and 6) length and width of flower tube.

traits and more tightly inter-correlated than those of populations pollinated by hoverflies, assuming that flies do not generate selective pressures on the traits they do not interact with, such as flower tube length and width. In addition to trait correlations, it is also possible that each pollinator fauna exerts strong selection on particular flower traits directly associated to its behaviour in the flower. This directional selection would generate differences in floral-trait means in populations visited by different pollinators (Armbruster 1985, Robertson and Wyatt 1990; but see Herrera 1996 for a critical account). Generalist pollen feeders that cannot access the nectar, such as hoverflies, may also exert selective pressures, but these will act only on those few traits with which the flies interact, e.g. traits affecting attractiveness and accessibility of the androecium (Dilley et al. 2000). An advantage of the population approach to testing predictions of the Berg hypothesis is that the comparison largely controls for otherwise confounding effects of morphology, development, life-history, and phylogeny, which plague species level studies.

In this study we test if there are differences among populations in both trait means and, as Berg (1959, 1960) hypothesized, correlations. We first ascertain the occurrence of different guilds of pollinators in separate populations of *N. papyraceus*. Then, we search for

patterns of variation in flower and vegetative traits among those populations that can be accounted for by differences in the pollinator fauna. In particular, we address the following questions: (1) are flowers of isoplethic populations indeed visited mostly by long-tongued insects (moths) and flowers of L-monomorphic populations mostly visited by hoverflies? If so, (2) do flower traits show differences in mean values, as a possible result of divergent directional selection? and (3) are there differences among populations in patterns of modularity and integration of floral and vegetative traits?

## Material and methods

### Study organism and population sampling

*Narcissus papyraceus* (Amaryllidaceae) is a winter-blooming bulb-producing geophyte common in lowland, seasonally wet, deep soils across the Mediterranean Basin. The largest and most frequent populations occur in the Strait of Gibraltar region (southwestern Iberian Peninsula and northwestern Morocco), where this study was carried out. The perianth consists of a long, narrow tube (14.6 mm long, 4.4 mm outer width, averaged over 1165 flowers from 10 populations), six flat tepals and a wide cup-shaped corona (Fig. 1). This species is style-dimorphic and styles are either short (S) or long (L) but stamens are of similar height in both morphs, a polymorphism related to heterostyly (Lloyd and Webb 1992). Plants are self incompatible but, unlike most heterostylous species, they are within-morph compatible. Flowers need the assistance of pollinators to set fruits and seeds (see Arroyo et al. 2002 for details on the reproductive biology of the species). This species shows a distinct geographic pattern in the style-morph ratio across the Strait of Gibraltar. While populations close to the Strait are isoplethic (i.e. equal morph ratios), the S morph is less frequent towards peripheral areas, populations becoming L-biased, and then L-monomorphic farther away from the Strait (Arroyo et al. 2002). These authors suggested that this geographical pattern might result from natural selection generated by different pollinator faunas. Nectar-feeding insects with long proboscides (mostly nocturnal moths), which can reach the nectar at the bottom of the long-tubed flowers, pollinate effectively both S and L morphs in dimorphic populations close to the Strait of Gibraltar. In contrast, inland populations (mostly L-monomorphic) are thought to be mostly visited by pollen-feeding insects with short proboscides (mostly hoverflies) whose tongues would not be able reach the stigma of S-morph flowers.

Sixteen populations were selected based on results of a previous study (Arroyo et al. 2002). Only isoplethic and monomorphic populations were included in the

sampling (see Table 1 for geographic location, population size and morph ratio). Virtually all isoplethic populations known from the study area were included in the survey, since they are few and restricted to coastal localities on both sides of the Strait of Gibraltar. By contrast, monomorphic populations occur over a wider geographic range (Arroyo et al. 2002).

Eleven to 19 plants were collected from each of four isoplethic and six monomorphic populations for measurement of both flower and vegetative morphology on each plant (Table 1). Detailed measurements of flower morphology were made on a sample of one flower per plant ( $n=100$  randomly selected plants per population) in 12 of the 16 populations (Table 1). All floral measurements were made on the uppermost flower of the inflorescence, which is the first to open, to avoid confounding effects due to flower position. Given the reported strong vegetative reproduction, all the plants sampled were at least one meter apart, to avoid multiple samples from clones.

### Pollinator observations

We selected two isoplethic populations (no. 1 and 2) from the Iberian side of the Strait, and two monomorphic populations farther north (no. 4 and 7; Table 1). We located several  $10\text{ m}^2$  plots in patches with high flowering density (20–50 plants per plot). Censuses were performed during several days in January 1998, and December 2002 and 2003 in isoplethic populations and in February and March 1988, January 2003 and 2004, and February and March 2003 in monomorphic populations (Table 2). Insect observations were made between 0930 and 1700 (solar time), in periods of 15 min, with rest intervals of 10 min after three consecutive periods, and changing of observation plot after each resting interval. Observations were made over 6–7 days each season at flowering peak of the species. Total time effort of observation was 33 h 5 min in isoplethic populations and 30 h 15 min in monomorphic populations. The total observation time was used to calculate averaged insect visit rate to plots per hour. We did not estimate visit rate to individual flowers or plants due to the very low visit rate in this winter blooming species.

We counted visits by insects, ascertained whether they touched the sex organs of the flower, and captured representative specimens as vouchers. We identified insects in the field to morphospecies, which could correspond to more than one taxonomic species, as it was found by later identification by specialists. We also performed sunset and nocturnal censuses, from 1730 h to 2000 h, using a red light to avoid alteration of insect behaviour. Nocturnal insects were captured by means of white light traps: two nights (1700 to 0800) in winter

2002–03 and 2003–04 in population 1 (isoplethic) and 4 (monomorphic). We considered potential pollinators to be only those insects that bore pollen grains of *N. papyraceus* attached to their bodies, as determined by careful examination under a stereomicroscope ( $50\times$ ). All insects observed or captured were classified in two types: long or short proboscis (i.e. longer or shorter than the floral tube length, respectively; 14.8 mm in isoplethic and 14.5 mm in monomorphic populations). For this purpose we used the information supplied by V. González (2004, pers. comm.) on proboscis length.

### Measurements

All flower measurements were made with digital calipers with 0.1 mm precision. These were: (1) flower width, (2) length of outer tepal, (3) width and (4) height of corona, (5) width and (6) height of flower tube, from the point where it joins to the ovary (Fig. 1). Measurements made on vegetative traits were: bulb circumference, number of bulbils (as a result of vegetative reproduction), length and width of leaves, sheath length, number of flower stalks, length and width of stalk section at its middle height, spathe length, and number of flowers in the inflorescence. All vegetative variables were measured to the nearest 1 mm, except leaf width and stalk section, which were made to the nearest 0.1 mm. Flower measurements were made without prior knowledge of the expected pattern of trait covariation.

### Statistical analyses

#### *Flower morphology*

Differences in floral-trait means among populations and between population types (isoplethic and monomorphic) were tested by a nested ANOVA separately for each trait, and also by MANOVAs for all floral traits together. In isoplethic populations one-way ANOVAs were previously performed to search for within-population, between-morph differences in each of the floral traits. For the analyses concerning only floral traits, data used were those from the 12 populations that were sampled intensively ( $100\text{ flowers population}^{-1}$ ). Because of repeated hypothesis testing, we applied Bonferroni correction to maintain an experiment-wise error  $\alpha$ -level of 0.05.

#### *Patterns of variation and covariation of flower and vegetative traits*

For each population studied, we determined the Pearson correlation coefficients ( $r$ ), the regression coefficients ( $b$ ) for all pairs of floral and vegetative traits, and between pairs of floral traits and vegetative traits separately. These two measures of covariation, although closely related, capture slightly different

Table 1. Populations of *Narcissus papyraceus* sampled for flower and vegetative traits. \* Populations where samples for flower and vegetative traits were taken together (the same plants); + Populations where additional samples for flower morphology were taken.

Population	Number	Coordinates	Approx. population size	Sample size for flower traits		Sample size for vegetative traits		% of L-morph
				L	S	L	S	
Spain: Cádiz, Tarifa, Bolonia* +	1	36°7'N, 5°44'W	1 000 000	50	50	8	8	50
Spain: Cádiz, Los Barrios, Ahojiz-La Granja* +	2	36°13'N, 5°35'W	250 000	50	50	7	9	50
Spain: Cádiz, Algeciras, El Palancar*	3	36°5'N, 5°33'W	5000	–	–	8	7	50
Spain: Sevilla, Aznalcázar +	4	37°16'N, 6°14'W	1000	98	–	–	–	100
Spain: Sevilla: Pruna – Algámitas*	5	37°1'N, 5°10'W	250	–	–	13	–	100
Spain: Córdoba, Carcabuey, Valdecañas +	6	37°28'N, 4°21'W	1000	100	–	–	–	100
Spain: Huelva, Hinojos, arroyo La Parrilla* +	7	37°12'N, 6°26'W	1000	100	–	16	–	100
Spain: Huelva, Cartaya, El Rompido*	8	37°13'N, 7°7'W	1000	–	–	16	–	98.6
Spain: Huelva, Villanueva de los Castillejos* +	9	37°29'N, 7°15'W	1000	99	1	11	–	100
Spain: Huelva, Almonte, El Rocío +	10	37°9'N, 6°27'W	1000	97	3	–	–	98.5
Spain: Málaga, San Pedro de Alcántara +	11	36°30'N, 4°59'W	500	67	1	–	–	100
Portugal: Algarve, Messines-Alte* +	12	37°15'N, 8°15'W	1000	100	–	15	1	100
Portugal: Algarve, Tavira +	13	37°10'N, 7°39'W	500	100	–	–	–	100
Portugal: Algarve, San Brass – Sta Catarina*	14	37°9'N, 7°49'W	100	–	–	17	–	100
Morocco: Tangier-Tetouan, R'gaia +	15	35°41'N, 5°43'W	100	41	59	–	–	50
Morocco: Tangier-Tetouan, Oued Lediane* +	16	35°50'N, 5°38'W	5000	57	43	8	8	50

Table 2. Diurnal and nocturnal flower visitors of *Narcissus papyraceus*. Values in column 2 and 3 indicate total number of effective insect visits to flowers in selected plots (i.e. touching any flower sex organ) during the mentioned period. ST, short-tongued insects; LT, long-tongued insects (see Methods for tongue classes).

Flower visitors	Isolethic populations	Monomorphic populations
Diurnal	Total time effort: 33 h 5 min	Total time effort: 30 h 15 min
Diptera		
<i>Eristalis</i> sp. (ST)	82	1037
Other Syrphidae (ST)	46	88
Other Diptera (ST)	8	10
Bombylidae (LT)	9	3
Hymenoptera		
Wasps (ST)	0	1
<i>Bombus</i> spp. (LT)	12	2
<i>Anthophora</i> spp. (LT)	4	256
Other medium-large bees (LT)	3	30
<i>Apis mellifera</i> (ST)	5	5
Lepidoptera		
Butterflies (LT)	39	82
<i>Macroglossum stellatarum</i> (LT)	0	2
Other moths (LT)	0	1
Total number of visits	208	1517
Nocturnal		
Censuses:	Total time effort 8 h 15 min	Total time effort: 2 h 30 min
Moths (LT)	28	6
Captures (light trap):	Total time effort: 60 h	Total time effort: 60 h
Moths (LT)	27	0

aspects of modularity or integration (Armbruster et al. 1999). Variability of traits was measured by their coefficient of variation (CV). Differences between isoplethic and monomorphic populations in mean correlations, regression and coefficients of variation were tested by means of two-way ANOVAs, with population type (isoplethic and monomorphic) and type of comparison (inter-floral, inter-vegetative and floral-vegetative) as fixed factors. Replicates were mean population values for each statistic ( $r$ ,  $b$ , CV) and type of trait. Because population mean values of correlation and regression coefficients are based on values that are not fully independent, we estimated significance of differences between means, as analogue to  $t$ -statistic, and their confidence intervals by the first percentile method, by means of bootstrapping ( $n=20000$  permutations in each test, see Manly 1998 for details).

#### **Patterns of standardized phenotypic integration**

Among the several available methods for estimating phenotypic integration, those of Wagner (1984) and Cheverud et al. (1989) provide a standard direct measure, taking into account the influence of different sample sizes, and the contrast against a null correlation matrix, with no covariation of traits, which allows comparisons with other studies. Following these authors, phenotypic integration was estimated using eigenvalues of a correlation matrix through a principal component analysis (PCA). The magnitude of pheno-

typic integration is represented by the integration index (the variance of the eigenvalues of the correlation matrix of each population). Because sample size varied among populations, we used the corrected integration index, by subtracting the expected value of integration under the assumption of random covariation (random integration = no. of characters  $- 1$ /no. of plants; Wagner 1984, Herrera et al. 2002). The integration index was expressed as percentage of the maximum possible value, which is the number of traits considered (Herrera et al. 2002). To test for differences in the integration index, we performed a two-way ANOVA, with type of trait (floral, vegetative) and population type (isoplethic, monomorphic) as fixed factors. Given the same lack of independence noted above, the significance of differences between means and confidence intervals of integration index were estimated by bootstrapping as described above ( $n=20\ 000$  permutations).

Those analyses involving direct comparison between floral and vegetative traits were based on data from the 10 populations in which whole-plant sampling was carried out. Prior to analysis, all data from the whole plants were log transformed, because the magnitudes and variances of the variables differed greatly (e.g. leaf length and flower size). For analysis of data from intensive sampling of flowers, data were not transformed, since they approached normality and homoscedasticity, and they were analysed separately from vegetative data.

Statistical analyses were performed with Statistica ver. 6.0 (Anonymous 2001), except procedures that involved bootstrapping estimation, which were performed with Data Pilot ver. 1.03 (Anonymous 2003).

## Results

### Pollinators

All insects observed visiting the flowers and those captured and authenticated as probable pollinators (i.e. bearing *Narcissus* pollen) are included in Table 2. Diurnal visitors were classified into two groups: long- and short-tongued insects. We calculated the visitation rate to flowers of these two groups. In general, visitation rate was much higher in monomorphic than in isoplethic populations. In monomorphic populations, short-tongued pollinators visited flowers at a much higher rate (37.5 visits  $h^{-1}$  to a plot) than long-tongued ones (12.4 visits  $h^{-1}$ ). In isoplethic populations, these rates were 4.3 and 2.0 visits  $h^{-1}$  respectively. Therefore the proportion of short-tongued to long-tongued insects was higher in monomorphic (3.3:1) than in isoplethic (2.1:1) populations. Most long-tongued insects observed in monomorphic populations were pierid butterflies, which were only observed in a single year (1998). The vast majority of short-tongued insects were hoverflies (Syrphidae; Table 2), which were observed collecting pollen from upper stamens of *N. papyraceus* in all flowering seasons. The estimated proportion of short- to long-tongued insects is probably conservative for monomorphic populations, since it included some bees (e.g. *Anthophora* spp.) within the latter category. These bees, which were relatively frequent in monomorphic populations (Table 2), could rarely, if ever, reach the nectar at the tube base with their tongues; hence many of their visits were to effectively rewardless flowers or for pollen collection. This may account for their low floral constancy to *N. papyraceus*. A similar explanation may apply for *Bombus* in isoplethic populations (Table 2). Most bumblebees were in fact nectar robbers and were not included as legitimate pollinators.

Despite the smaller effort devoted to observations of crepuscular and nocturnal insects, and considering the drawbacks of this approach, we found a strikingly different pattern to the daylight one. Nocturnal insects were important pollinators in isoplethic populations, but not in monomorphic ones. Methodological difficulties in observations were in part overcome by using light traps. Captures confirmed these findings: we found moths bearing *Narcissus* pollen at isoplethic sites, but did not catch any moth at monomorphic sites (Table 2).

### Flower morphology: single traits

Means of flower traits, taken together, differed significantly between monomorphic and isoplethic populations (Wilk's lambda=0.7825,  $F_{6, 1148}=53.18$ ,  $p < 0.0001$ ) and among populations (Wilk's lambda = 0.2847,  $F_{60, 6019.785}=27.19$ ,  $p < 0.0001$ ). When looking at single traits separately, a mixed pattern was detected. While there were significant differences between populations for all six flower traits considered ( $F=17.79-70.99$ ,  $DF=10, 1153$ ,  $p < 0.0001$  in all cases), only four traits showed significant differences between monomorphic and isoplethic populations (tepal length:  $F=36.81$ ,  $p < 0.0001$ ; corona width:  $F=39.19$ ,  $p < 0.0001$ ; tube length:  $F=13.69$ ,  $p=0.0002$ ; and tube width:  $F=70.51$ ,  $p < 0.0001$ ;  $DF=1, 1153$  in all cases, see Fig. 2).

Flower traits taken together did not show any significant differences between morphs in isoplethic populations (MANOVA; population 1: Wilks' lambda=0.128,  $F_{14,1}=0.487$ ,  $p=0.8263$ ; population 2: Wilk's lambda=0.118,  $F_{14,1}=0.53$ ,  $p=0.807$ ; population 3: Wilks' lambda=0.012,  $F_{13,1}=6.97$ ,  $p=0.289$ ; population 16: Wilks' lambda=0.021,  $F_{12,1}=4$ ,  $p=0.382$ ). Results from one-way ANOVAs on each flower trait were similar, except for flower-tube width in population 1, where a significant difference between morphs was found ( $F_{1,98}=19.032$ ,  $p=0.000032$ ).

### Patterns of variation and covariation of flower and vegetative traits

#### Patterns of correlation

A two-way ANOVA failed to show a significant effect of isoplethic vs monomorphic populations on the mean correlation coefficient ( $r$ ). However, the type of correlation (inter-vegetative, inter-floral, or floral-vegetative) did show a significant effect, and the interaction term was moderately significant (Table 3). Consistent with our expectations, correlations between flower traits were stronger than between vegetative traits in moth-pollinated (isoplethic) populations ( $p=0.008$ , after bootstrapping estimation), whereas these correlations were not significantly different in fly-pollinated (monomorphic) populations ( $p=0.20$ ; Fig. 3a; Table 3). Correlations between flower and vegetative traits were lower than other correlations in all comparisons. They were significantly lower than the floral correlations in isoplethic populations ( $p=0.0078$ ), and significantly lower than both flower ( $p=0.0005$ ) and vegetative correlations ( $p=0.0005$ ) in monomorphic populations. The heterogeneity in flower, vegetative, and flower-vegetative correlations was greater in isoplethic populations than in monomorphic ones (Fig. 3a), as indicated by the marginally significant interaction term in the ANOVA (Table 3).

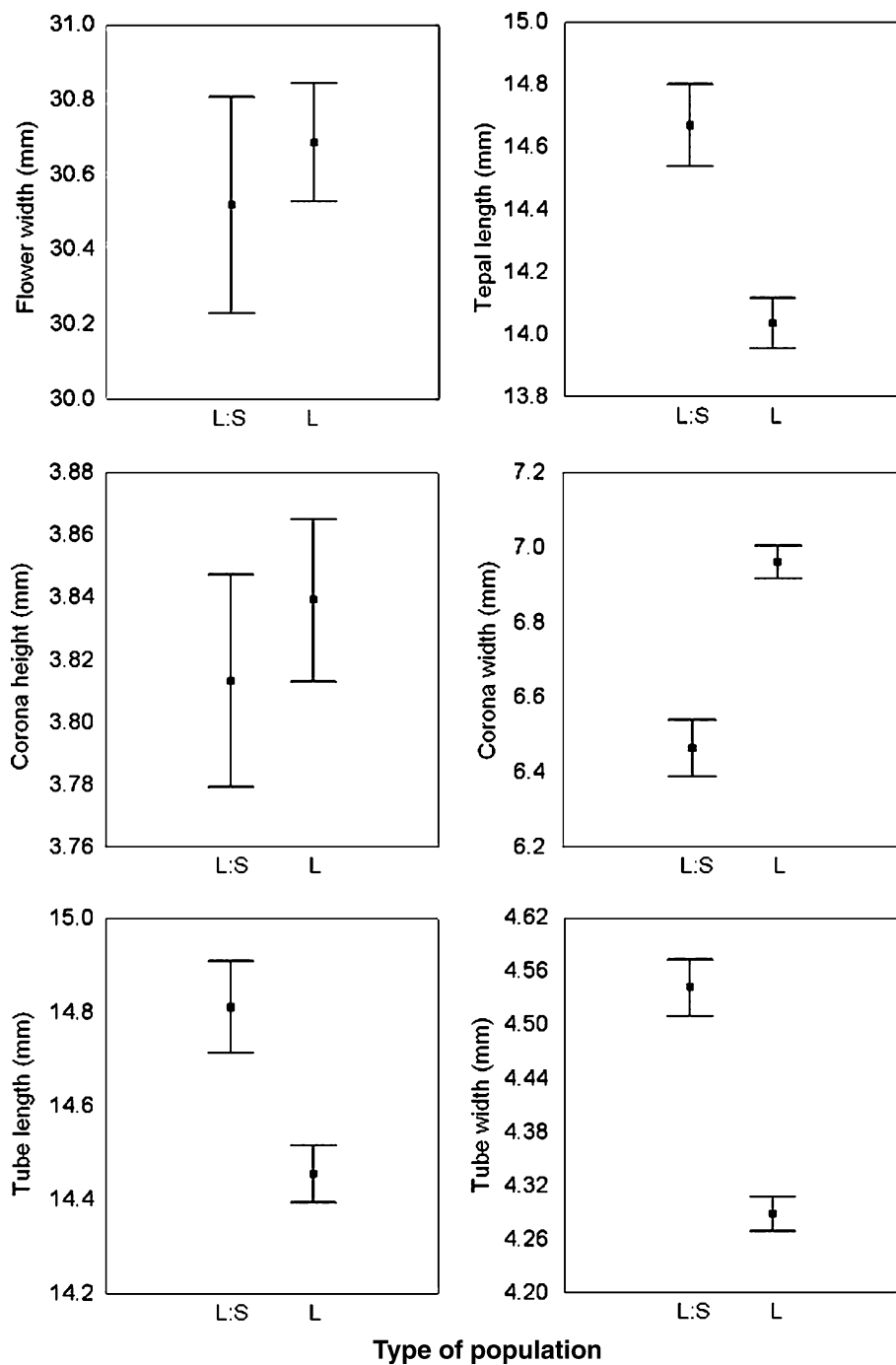


Fig. 2. Mean values ( $\pm$ SE) of flower traits in four isoplethic (L:S) and ten monomorphic (L) populations of *Narcissus papyraceus*.

**Regression coefficients**

Two-way ANOVA on the regression coefficients (slopes of trait relationships) failed to detect an effect of population type, but did show significant effect of regression type (inter-floral, inter-vegetative, or

floral-vegetative). The interaction term was not significant (Table 3). Indeed, the slopes of the relationships between flower traits and between vegetative traits were much steeper than those between flower and vegetative traits in both types of populations ( $p=0.0078$  for

Table 3. Summarized two-way ANOVA results of the comparison of the mean values of coefficients of correlation, regression, variation and phenotypic integration index in isoplethic and monomorphic populations ("population type", fixed factor) and on flower, vegetative and, if applicable, flower-vegetative traits (see text) ("trait type", fixed factor) of *Narcissus papyraceus*.

	SS	DF	MS	F	p
Dependent variable					
Correlation coefficient					
Population type	0.0033	1	0.0033	0.7060	0.4090
Trait type	0.2933	2	0.1467	31.0700	0.0000
Population × Trait	0.0312	2	0.0156	3.3050	0.0540
Error	0.1133	24	0.0047		
Regression coefficient					
Population type	0.0043	1	0.0043	0.2121	0.6493
Trait type	0.9157	2	0.4578	22.4908	0.0000
Population × Trait	0.0581	2	0.029	1.4270	0.2597
Error	0.4886	24	0.0204		
Coefficient of variation					
Population type	0.0066	1	0.0066	3.1950	0.0928
Trait type	0.1162	1	0.1162	55.9552	0.0000
Population × Trait	0.0028	1	0.0028	1.3669	0.2595
Error	0.0332	16	0.0021		
Integration index					
Population type	8.5020	1	8.5020	0.2706	0.6100
Trait type	326.5440	1	326.5440	10.3952	0.0053
Population × Trait	116.8870	1	116.8870	3.7210	0.0717
Error	502.6100	16	31.4130		

isoplethic and  $p=0.0005$  for monomorphic populations, bootstrapping estimation). This difference in slopes indicates a degree of decoupling of floral from vegetative traits in both populations types (Table 3, Fig. 3b), as predicted by Armbruster et al. (1999).

#### **Coefficient of variation**

Floral traits were less variable than vegetative traits in both types of populations (Fig. 3c, Table 3). Although this trend appeared stronger in the isoplethic populations, the non-significant population-type and interaction terms (Table 3) indicated that the pattern in across population type did not differ detectably.

#### **Patterns of standardized phenotypic integration**

Considering the phenotype correlations as a whole, using an index of integration, we found a clear difference in the integration of flower vs vegetative traits overall ( $p=0.005$ ; Table 3). This pattern did not differ significantly between population types, but the interaction term (population type × trait type) was marginally significant ( $p=0.07$ ). Hence, although flower integration was always higher than vegetative integration, this difference was stronger in isoplethic populations ( $p=0.008$ , bootstrapping estimation) than in monomorphic populations ( $p=0.166$ ; Fig. 3d).

## **Discussion**

Pollinators commonly vary across the geographic distribution of wide-ranging species. This offers an opportunity to test their possible selective effect on flower traits at the population level. In this paper we have shown that *Narcissus papyraceus* is pollinated largely by different pollinators in different parts of its range, and that this difference may have generated differences in flower morphologies, both in trait means and variances and in the patterns of traits covariation. Although population analyses have many advantages over species-level comparisons (Armbruster et al. 1999), they depend on a critical assumption: that the study populations are independent, similarly to phylogenetic independency (Ebbel 2004). Although the close proximity of the only known isoplethic, moth-pollinated populations to one another raises concern, we think the assumption of our analysis is justified. A study of population-genetic relatedness by means of AFLPs markers, including all our study populations (Rodríguez-Sánchez et al. 2007), revealed a well supported group including both Portuguese L-monomorphic populations and dimorphic populations close to the Strait of Gibraltar. The remaining L-monomorphic populations form a heterogeneous set. Although a full phylogeographic analysis of these populations is lacking, these genetic analyses show that the difference in patterns of trait correlation, variation, and integration of isoplethic and monomorphic

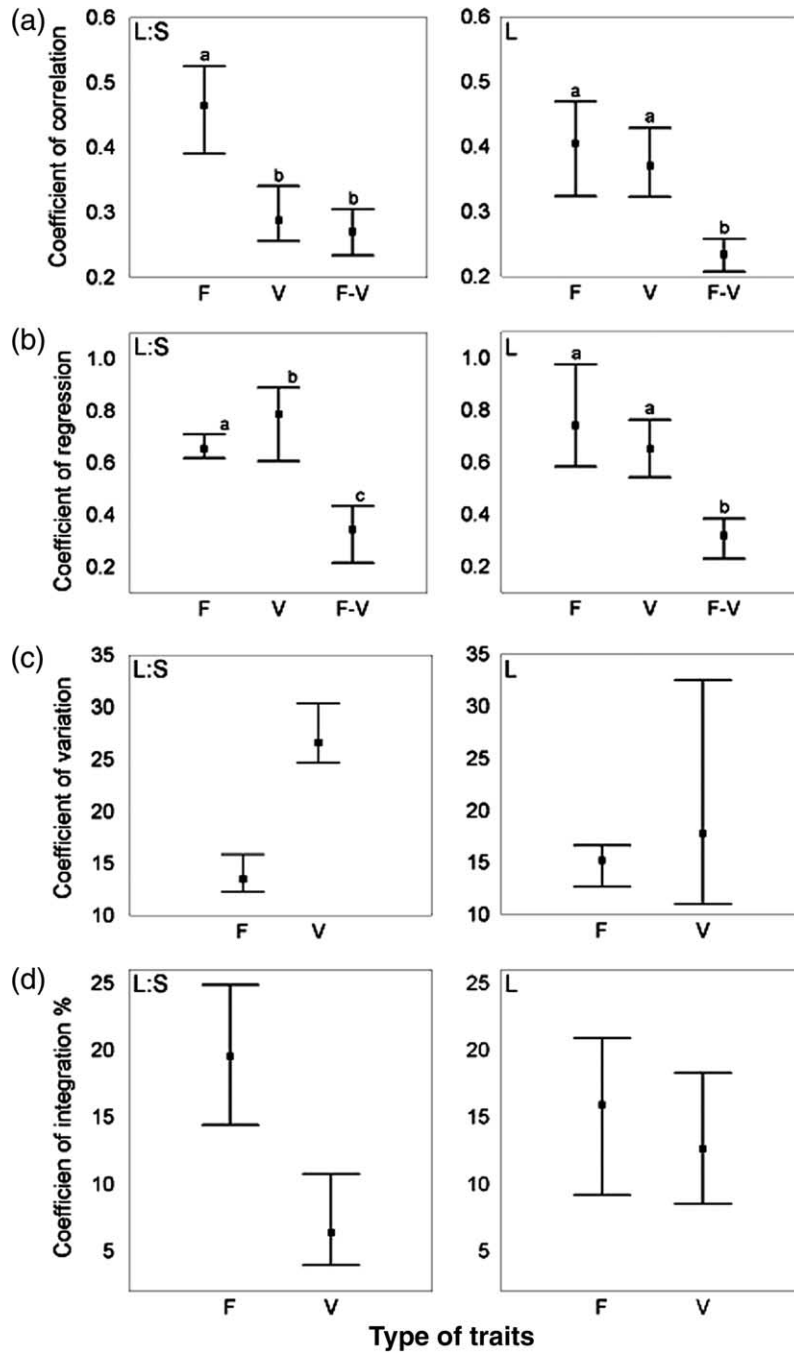


Fig. 3. Population mean values and confidence intervals ( $p < 0.05$ ) of coefficients of correlation, regression, variation, and phenotypic integration index (see Methods for details) of flower (F), vegetative (V) traits and, when applicable, between them (F-V) of isoplethic (L:S) and monomorphic (L) populations of *Narcissus papyraecus*. Different letters over the bars indicate significant differences ( $p < 0.05$ ). The mean values of coefficient of variation and index of integration showed significant differences between types of traits (F, V see Table 3). Confidence intervals and differences between mean values of correlation coefficients and integration index were estimated by bootstrapping (20 000 runs).

populations is not likely to have been generated by recent coancestry of the populations of each type.

### Differences in pollinator faunas

Different populations of *N. papyraceus* had different pollinator faunas and visitation rates. The two long-styled (L) monomorphic populations, located in the interior, far from the Strait of Gibraltar, were pollinated primarily by syrphid flies, particularly *Eristalis* spp. These flies were also recorded as the most frequent pollinator in observations made on several other monomorphic populations (Arroyo et al. 2002). Although these insects usually feed on both nectar and pollen (Wacht et al. 1996, Dinkel and Lunau 2001), their short proboscides preclude them from reaching nectar in flower tubes deeper than 10 mm; indeed these flies seldom forage nectar from flowers whose tubes are deeper than 3 mm (Gilbert 1981). Their short tongues make it virtually impossible for syrphids to use *N. papyraceus* as a nectar source, because the floral tube is nearly 15 mm long. Moreover, it has been reported that the hoverfly *Eristalis tenax* shows predilection for white flat flowers with a central yellow spot (Dinkel and Lunau 2001). This pattern is matched by the yellow stamens on white flat tepals of *N. papyraceus*, and by the yellow corona and white tepals of *N. tazetta*, which has *E. tenax* as the main flower visitor in populations of the eastern Mediterranean (Arroyo and Dafni 1995). Thus, these flies are treating *Narcissus* flowers effectively as a flat, open flower offering only pollen.

The relative abundance of butterfly visitors to monomorphic populations in 1998 was due to a population explosion of *Pieris brassicae* in that year. Unlike with the syrphids, their presence was not consistent across years. During the two consecutive years of nocturnal observations and captures, we failed to observe even a single moth on or near the flowers of monomorphic populations. This may be a result of the fact that the blooming period of *N. papyraceus* is January–February, when freezing temperatures at night are very frequent (as it has been observed in a parallel study of the pollinator effectiveness; R. Pérez-Barrales and J. Arroyo, unpubl.). It seems likely that moth activity is strongly limited by such weather conditions (Boggs 1987). The relatively frequent presence of long-tongued bees as flower visitors in monomorphic populations may have an effect on the pollination success. Nonetheless, a low floral constancy to *N. papyraceus* has been observed in these insects; this is probably related to their difficulties in reaching nectar. Female bees use the species primarily as a source of pollen, which they can reach, for their offspring.

In contrast, the two isoplethic populations close to the Strait of Gibraltar (with a strong maritime influence), showed markedly greater visitation by nocturnal pollinators. Most of the moths we captured bore pollen of *N. papyraceus* attached to their bodies. The proximity of these populations to the coast, where winter temperatures are mild and frost virtually absent when *N. papyraceus* is in bloom, allows moth activity (Boggs 1987). Because of their long tongues and feeding behaviour, probing into the floral tube, moths have a close fit into the flowers (tube and corona), and they probably respond to larger, more nectar rewarding and scented flowers, as well as variation in tube length. Due to strong nectar dependence of Lepidoptera, this fit could be critical for moths, which have no alternative nectar sources (Rusterholz and Erhardt 1998) at the time of year *N. papyraceus* is in full bloom. During the day, flower visitors included a wide array of insects, both long- and short-tongued insects; thus emphasizing the higher incidence of long-tongued pollinators (Lepidoptera), as a whole, in isoplethic populations. The selective effects of Lepidoptera on nectar tube length and correlated traits in flowers with concealed nectar have been shown several times (Robertson and Wyatt 1990, Johnson 1997, Nilsson 1998).

The difference in pollinator faunas between isoplethic and monomorphic populations of *N. papyraceus* is similar to that found across populations of *N. tazetta* (Arroyo and Dafni 1995). Pérez-Barrales et al. (2006) have also suggested similar pollinator shifts across species of *Narcissus*. There is solid evidence of multiple secondary reversals to long-styled monomorphism, apparently associated with changes in pollinator faunas, both within and between other species in the genus (Arroyo and Dafni 1995, Arroyo et al. 2002, Baker et al. 2000b, Pérez et al. 2004, Pérez-Barrales et al. 2006), although the exact number and directions of shifts remains to be determined. This type of shift in pollinators is particularly important in the present context because it means a change from nectar-feeding pollinators that potentially interact with most of the morphological components of the flower (i.e. pollinators with a “good” fit with their deep-tubed flowers, thus being “specialized pollinators” sensu Armbruster et al. 1999) to pollination by pollen-feeding insects that interact only with the flat top part of flowers (i.e. pollinators with “poor” fit between flower and pollinator, thus being “unspecialized pollinators” sensu Armbruster et al. 1999; see Berg 1959, 1960, Fenster et al. 2004). These two interactions very likely generate different selective pressures both on sex-organ position (Arroyo et al. 2002) and perianth morphology in *Narcissus*. In fact, Pérez-Barrales (2005) found that components of female fitness in experimental *N. papyraceus* populations are consistent with this expectation: L flowers are pollinated by nearly all visiting

insects, whereas S flowers are pollinated only by long-tongued insects. Although an estimate of pollination efficiency of each pollinator type in each perianth phenotype is lacking, the key issue is that short-tongued insects have virtually no interaction with the internal parts of long-tubed flowers, and thus selection on these floral parts is probably relaxed.

### Mean flower traits across populations

Several studies of the effects of pollinators on the evolution of flower traits have found evidence for directional selection by the analysis of means across divergent pollination ecotypes (Armbruster 1985, Robertson and Wyatt 1990, Johnson 1997). In this study, we found that four out of the six flower traits considered had significant differences between population types. Tepals were larger, and tubes longer and wider in isoplethic populations close to the Strait of Gibraltar, where moth-pollination was important. These differences could facilitate attraction and improve the flower-pollinator fit of these insects (Nilsson 1998, Robertson and Wyatt 1990, Johnson 1997, Kelber 1997; but see Herrera 1996). In contrast, the corona was significantly wider in monomorphic, syrphid-pollinated populations. This may be related to syrphid choice of more attractive or more suitable flowers when collecting pollen.

Some studies have found significant differences in perianth traits between floral morphs (Faivre 2000). Thus variation in morph ratios among populations could generate among-population differences in trait means. However, we do not think this generated the differences we observed, because the only difference detected was that in population no. 1 where perianth tube width was slightly greater in S flowers than in L flowers. This difference might account for the wider flower tube reported for isoplethic populations, but is unlikely to account for strong inter-population differences involving the floral tube. We do not have evidence for an adaptive significance of this inter-morph difference in morphology, but it may be due to developmental differences between morphs (Richards and Barrett 1992) or an allometric relationship related to floral-tube elongation in isoplethic populations.

### Patterns of variation, correlation, and integration of flower and vegetative traits

When studying the effect of selective forces on plant traits, it is necessary to take into account the correlation and degree of dependence between them (reviewed by Murren 2002), especially when traits belong to functional units, such as flowers. Berg (1959, 1960) established explicit hypotheses about flower-trait

covariation based on the prevalent pollination system – generalized vs specialized in terms of flower-pollinator fit – as a selective agent, following the correlation-pleiades concept of Terentjev (1931). In plant species with elaborate flower structures pollinated by close-fitting pollinators, selection would favour the position of all flower organs to optimize pollen deposition and receipt (Castellanos et al. 2003, Armbruster et al. 2004). As an extension of this argument, Berg predicted the degree of coupling or decoupling floral and vegetative traits should exhibit. Obviously, vegetative traits are not directly subjected to selection by pollinators, but serve as a “null” expectation against which floral covariation can be compared.

Few studies testing Berg’s hypothesis use population-level comparisons and the results provide mixed support (Waite and Levin 1993, Herrera 2001, Herrera et al. 2002). That our data show inconsistent support of Berg’s hypothesis, that floral traits should be largely decoupled from vegetative traits and correlated, *inter alia*, when flowers and pollinators fit closely, may be the result of 1) real genetic/developmental constraints, 2) limited population sampling per species, 3) lack of robust data on pollinators, or 4) possible effects of phenotypic plasticity in traits measured in field-collected samples. In general, however, our results do support the major components of Berg’s hypotheses.

The patterns in our data may have been influenced by plastic responses of traits to the different environments in which populations were sampled. However, we think this source of error is minor because *N. papyraceus* grows almost exclusively in homogeneous, rich, clay soils in a limited geographic range under Mediterranean climate (Arroyo et al. 2002). Secondly, a meta-analysis revealed that phenotypic correlations in plants generally reflect genetic correlations (Waite and Levin 1998). Thus, it seems reasonable to argue for a minor role of plasticity in this study. In any case, ongoing studies of plants in common-garden cultivation will allow estimation of plasticity vs genetic effects.

Another issue to consider is that, because monomorphic populations are generally smaller than isoplethic populations, random processes (drift, founder events) could have influenced the results through a reduction of genetic and phenotypic variability (reduced CV) and hence less phenotypic integration of monomorphic populations. This may be the case of vegetative traits which showed smaller mean CV in the populations, but high CV variability across populations, probably due to spatially variable climate conditions among them. The CVs for floral traits were, however, higher in monomorphic populations, which cannot be expected from small-populations effects.

Many of Berg’s predictions require analyses that involve comparisons of variables that are not independent by nature. We have therefore employed two

statistical approaches in our study: parametric ANOVAs and non-parametric analyses through bootstrapping estimation. Both procedures produced similar results, which may indicate that required assumptions in parametric methods are not dramatically violated (Manly 1998). We found that flower traits were more intercorrelated than vegetative traits, and that this difference was more pronounced in moth-pollinated populations than in hoverfly-pollinated ones, as predicted by the integration hypothesis. However, this pattern is partly due to higher floral correlations in isoplethic populations, as predicted, and partly due to higher vegetative correlations in monomorphic populations. The latter is an unexpected result. Although a decoupling of flower and vegetative traits was observed, as detected by low coefficients of regression between these two types of traits (Berg 1960, Conner and Sterling 1996, Armbruster et al. 1999), there was no detectable difference in degree of decoupling between moth- and hoverfly-pollinated populations. Thus, the integration hypothesis was supported by the differences in floral and vegetative correlations, and the modularity hypothesis was supported by the generally low floral-vegetative correlation and regression coefficients. However, the specialisation component of the Berg hypothesis (that populations with tight-fitting pollinators should have greater modularity) was not supported; we saw only small differences in regression and correlation coefficients between populations with different pollinators.

Berg's arguments addressed primarily those flower traits related to the fit of pollinators in flowers, and hence those traits affecting flower handling by insects rather than traits affecting attractiveness. Thus accessibility traits should be more tightly intercorrelated when the main pollinators access the hidden nectar reward (e.g. moths), than when they do not (e.g. syrphid flies). Indeed the intercorrelation of accessibility (tube length and width) traits was higher in isoplethic populations (mean  $r \pm SE = 0.357 \pm 0.240$ ) than in monomorphic ones ( $0.283 \pm 0.279$ ), as predicted (see also Conner and Sterling 1995). This occurred despite the reverse being true for attractiveness traits (flower and corona diameter) (monomorphic populations:  $0.364 \pm 0.071$ ; isoplethic populations:  $0.299 \pm 0.198$ ).

The patterns of the coefficients of variation (CV) fully agree with Berg's hypothesis. In both population types the CV was higher for vegetative than flower traits, these differences being strongly significant. The difference between population types was marginally significant in the expected direction (isoplethic populations had smaller flower CVs and larger for vegetative CVs than in the monomorphic populations). This result supports the hypothesis of moth pollinators exerting stabilizing selection on flower traits (Fenster 1991).

The pairwise covariation patterns give a limited picture of flower covariation, because all variables are potentially interrelated. Although Berg did not use multivariate methods, we have taken advantage of them as a further test of her hypothesis. We used the approach of eigenvalue distribution (Herrera et al. 2002, Armbruster et al. 2004) for this purpose. This standardized method (Wagner 1984, Cheverud et al. 1989) for measuring phenotypic integration allows comparisons among traits, populations, taxa, and studies. Using this method, we obtained a mean floral integration index of 19.6% in moth-pollinated, isoplethic populations and 16% in hoverfly-pollinated, monomorphic populations. These values fit within the range recorded by Herrera et al. (2002) for a heterogeneous set of species. Mean values of integration for vegetative traits were 6.4% and 12.6% for isoplethic and monomorphic populations, respectively. The differences between integration of floral and vegetative traits again fit the floral-integration hypothesis. Similarly, the greater floral integration of moth-pollinated than hoverfly-pollinated populations fits our prediction that measured traits covary more tightly when they all interact with pollinators (moth pollination) than when only some do (hoverfly pollination; Conner and Via 1993, Armbruster et al. 1999, 2004).

In conclusion, we have shown that most of the tests we performed at the univariate, bivariate, and multivariate levels support the hypothesis of pollinator-mediated selection on perianth traits. In this context, it seems advisable that future research on heterostyly and other floral polymorphisms include consideration of population differentiation in response to variation in pollinator faunas, and on the effects of faunal variation on the variation in, and integration of, perianth parts, as well as sexual organs.

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