



## Research

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# Phenotypic integration in style dimorphic daffodils (*Narcissus*, Amaryllidaceae) with different pollinators

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Different pollinators can exert different selective pressures on floral traits, depending on how they fit with flowers, which should be reflected in the patterns of variation and covariation of traits. Surprisingly, empirical evidence in support of this view is scarce. Here, we have studied whether the variation observed in floral phenotypic integration and covariation of traits in *Narcissus* species is associated with different groups of pollinators. Phenotypic integration was studied in two style dimorphic species, both with dimorphic populations mostly visited by long-tongued pollinators (close fit with flowers), and monomorphic populations visited by short-tongued insects (loose fit). For *N. papyraceus*, the patterns of variation and correlation among traits involved in different functions (attraction and fit with pollinators, transfer of pollen) were compared within and between population types. The genetic diversity of populations was also studied to control for possible effects on phenotypic variation. In both species, populations with long-tongued pollinators displayed greater phenotypic integration than those with short-tongued pollinators. Also, the correlations among traits involved in the same function were stronger than across functions. Furthermore, traits involved in the transfer of pollen were consistently more correlated and less variable than traits involved in the attraction of insects, and these differences were larger in dimorphic than monomorphic populations. In addition, population genetic parameters did not correlate with phenotypic integration or variation. Altogether, our results support current views of the role of pollinators in the evolution of floral integration.

## 1. Introduction

Most organisms display complex and integrated phenotypes with multiple traits involved in different and coordinated functions. This morphological complexity has long intrigued evolutionary biologists and it has stimulated discussions from both theoretical and empirical perspectives to understand how integrated phenotypes evolve [1–8]. Perhaps, one of the most influential works was that by Olson & Miller [1], which represented a turning point and inspired current views on phenotypic integration. These authors viewed integration as resulting mostly from the genetic and developmental programmes of organisms. As interpreted by Cheverud [3, p. 499], 'the degree of interdependence in development and function among morphological characters is directly related to the degree of phenotypic morphological integration among these characters'. This perspective has stimulated research agendas, with most of the empirical case studies coming from animal biology [1,3,9–16] and less frequently from plant biology [2,17–20].

Adaptive evolution can also influence the strength and the patterns of phenotypic integration of traits. Following the ideas of correlation pleiades developed by Terentjev [21], Berg [2,22] developed the concept of integration as a result of natural selection. In these papers, plant–pollinator relationships were used as a theoretical framework to illustrate the mechanisms by which natural selection could shape the structure and intensity of correlations between



**Figure 1.** Floral traits measured in (a) *Narcissus papyraceus*: flower diameter (1), corona diameter (2) and height (3), flower tube length (4) and width (5), style length (6), upper anther height (7) and lower anther (8) height in long- (L) and short-style (S) flowers, and (b) *Narcissus tazetta*: flower diameter (1), outer tepal length (2) and width (3), corona diameter (4) and height (5) and flower tube length (6). (Online version in colour.)

traits. Specifically, flowers with tight relationships with their pollinators should undergo stronger selection and display less variation than those without ‘precise’ fit. These contrasting selection scenarios should be reflected in the strength of floral correlations, and by the magnitude of modularity and decoupling between groups of traits involved in different functions, such as floral and vegetative traits, the latter being unaffected by pollinators [18,23–29].

Berg’s ideas have been expanded further in relation to the magnitude of intra-floral correlations with different functions and modularity [18,30–32]. The flower as a unit can be divided into semi-autonomous modules involved in the attraction of pollinators (e.g. flower size and display) and the transfer of pollen (e.g. pollen pick-up and delivery), and variation within these modules is usually restricted by genetic control and architectural constraints [17,19,20,32]. Despite these constraints, it could be expected that the adaptive peak of traits involved in the pollen transfer function may be narrower than those traits involved in the pollinator attraction function. Attraction traits are fundamental to receive visits and move pollen from anthers to stigmas [33,34], but more pollen should be transferred if pollinators pick up the pollen and touch the stigmas with the same body parts, which requires a precise position of these organs [35–37]. Furthermore, differences in the fitness surface between attraction traits and those involved in the pollen transfer should be larger in species and populations with close fit between flowers and pollinators (e.g. narrower adaptive peak for traits involved in pollen transfer than in attraction) than those with loose fit [2,18,29,38]. In addition, floral modularity will be favoured when traits involved in different functions experience different selective pressures [30].

Historical processes at lineage and population levels can influence trait correlation and covariation, but these have not been discussed much in the context of phenotypic integration [3,4,38–41]. Following the argument that genetic variation is a precondition for adaptive evolution [17,42], part of the variation in the strength of trait correlations could be explained by the variation in the gene pool of populations. This is supported by the fact that phenotypic correlation matrices and genetic correlation matrices do not differ much [17,19,39]. When studying the distribution of genetic diversity across species’ geographical ranges, genetic diversity is usually larger in central than peripheral populations [43]. Thus, population genetic processes might influence phenotypic variation strongly [44–47], which could in turn be reflected in the patterns of correlation and covariation of traits (but see [48]).

In this paper, we wished to test Berg’s hypothesis of different patterns of flower integration when plants are under selection by different functional groups of pollinators, using *Narcissus* species and populations as a case study. The research on *Narcissus* has provided important evidence to understand the mechanisms by which shifts in pollinators can drive floral phenotypic variation and evolution. Many *Narcissus* species present style dimorphism, a sex polymorphism where populations present two floral morphs with either long- or short-style flowers (hereafter L and S flowers), and two anther levels (upper and lower) attached to the flower tube (the position of the upper and lower anther level does not differ between morphs; figure 1a). In a macroevolutionary context, changes in the polymorphism correlate with the evolution of long and narrow floral tubes, which seem to be the result of selection mediated by long-tongued nectarivorous insects [49]. Many style dimorphic *Narcissus* display great variation in the

morph ratio, from dimorphic populations (L : S and L-biased) to L-monomorphic populations (although uncommon, S-biased populations can occur, [50–53]), and this variation is frequently associated with shifts in pollinators. For example, populations of *N. papyraceus* in the west of the Mediterranean Basin and *N. tazetta* in Israel can be either dimorphic (L : S,  $L > S$  and  $S > L$  in *N. tazetta*) and visited mostly by long-tongued nectarivorous pollinators, or L-monomorphic and highly L-biased ( $L > 95\%$ ) with short-tongued pollinivorous insects as main pollinators [29,50,53–55]. These variations in morph ratio can occur because, although the species are self-incompatible, crosses between different plants of the same morph render viable seeds [53,55,56]. Experimental manipulations have revealed that the maintenance of S flowers depends upon the presence of long-tongued insects, which transfer pollen (mostly from the lower anther level of L-flowers) to S-stigmas (short-tongued insects, such as syrphid flies, do not reach S-stigmas; [55,57–60]). Stigmas of long-styled flowers can receive pollen from either L- or S- anthers and both long- and short-tongued insects are able to deliver pollen. In *Narcissus* and other polymorphic species, the absence of one morph seems to be a derived condition [41,61–65]. Hence, it is reasonable to argue that L-monomorphic populations of *N. papyraceus* and *N. tazetta* are derived from dimorphic populations, although it is unclear how many times the polymorphism has been lost at the population level (but see [62]).

Most investigations on polymorphic species have focused on how pollinators select for and maintain discrete floral phenotypes [58,59,66–68], ignoring possible effects on the continuous variation (but see [69]). For example, species of *Lithodora* with closer reciprocal placement of anthers and stigmas display greater phenotypic integration values [70], and these patterns correspond to the efficiency of different pollinators [71]. Here, we wished to assess whether populations of *N. papyraceus* and *N. tazetta* with contrasting functional groups of pollinators differed in their levels of floral integration and trait correlation. In dimorphic populations, long-tongued insects should exert strong selection, particularly on the flower tube length and the position of the anthers and the stigma, because these insects closely fit with the flower tube to reach the nectar (specialized pollinators *sensu* [2,22,18,72]). By contrast, selection exerted by short-tongued insects in L-monomorphic populations should be weaker on these traits. Short-tongued pollinators feed on the pollen from the upper anther level (they do not reach the nectar hidden at the bottom of the narrow flower tube) and their interaction with the flower is loose in terms of morphological fit (unspecialized pollinators *sensu* [2,22,18,72]). If the previous scenario holds, these different selective pressures should be reflected in the strength of phenotypic correlation and integration. In fact, in *N. papyraceus*, decoupling between floral and vegetative traits was greater in dimorphic populations than in L-monomorphic population [29], fitting Berg's predictions [2,22].

The first aim of this study was to assess whether phenotypic integration in dimorphic populations with long-tongued pollinators (hereafter LT pollinators) was greater than in L-monomorphic populations with short-tongued pollinators (hereafter ST pollinators) in *N. papyraceus* and *N. tazetta*. Secondly, modularity of *N. papyraceus* flowers was assessed by analysing the strength of correlations of sets of traits considered to play the same function with the correlations of traits involved in different functions. To test whether LT and ST pollinators could exert different selective pressures on floral traits, within and between population types, the phenotypic variation and

phenotypic correlations of traits involved in the attraction of pollinators and access to the flower (i.e. flower diameter, corona diameter and height, flower tube length and width) was compared to that from traits involved in the transfer of pollen (i.e. style length, upper anther height and lower anther height). Finally, to control for possible population genetic constraints and marginal range effects on phenotypic integration (monomorphic populations are smaller and tend to occur more peripherally than dimorphic populations; [50,53]), the genetic diversity of dimorphic and L-monomorphic populations was studied using microsatellite markers. Population genetic parameters were used to explore possible associations with phenotypic integration, variation and correlation of floral traits. The comparisons across species and populations allowed validation of current views of selection on floral trait covariation and modularity caused by different pollinators [2,20,30,73].

## 2. Material and methods

### (a) Population sampling for floral measurements

Flowers were collected from 17 populations of *N. papyraceus* (seven dimorphic and 10 L-monomorphic and highly L-biased,  $L > 95\%$ , see [29,54]) and nine populations of *N. tazetta* (three dimorphic and six L-monomorphic and highly L-biased,  $L > 95\%$ , see [50] for sampling details and table 1). For simplicity, we will call the group represented by L-monomorphic and highly L-biased populations as L-monomorphic. Flower measurements in *N. papyraceus* were taken by R.P.-B. (figure 1a) and included flower diameter (1), corona diameter (2) and height (3), flower tube length (4) and width (5), style length (6), upper (7) and lower (8) anther height in L and S flowers. Flower measurements in *N. tazetta* were taken by J.A. (figure 1b) and they included flower diameter (1), outer tepal length (2) and width (3), corona diameter (4) and height (5) and flower tube length (6). Details on pollinators, their ability to pick up and deliver pollen and select for L and S flowers can be found elsewhere [29,50,55,56].

### (b) Phenotypic integration in dimorphic and L-monomorphic populations of *Narcissus papyraceus* and *N. tazetta*

We used the method developed by Wagner [74] and Cheverud *et al.* [75] to calculate the phenotypic integration index for each species and population. The phenotypic integration index was estimated as the variance of the eigenvalues of the correlation matrix. Sample size varied among populations (table 1); hence, the integration index was corrected by subtracting the expected phenotypic integration under the assumption of random covariation of traits (see [26,29,74] for details). The integration index was expressed as percentage of the maximum value, which is the number of traits included [26]. In dimorphic populations, the phenotypic integration index was estimated by pooling the data from L and S flowers (style length and upper and lower anther height were not included in this analysis as these data were only available for *N. papyraceus*; see description of traits measured above, figure 1a,b). The average phenotypic integration between the two types of populations was analysed with an unpaired *t*-test. To control by the lack of independence (phenotypic integration index is based on a correlation matrix), we implemented a bootstrap procedure ( $n = 20,000$  permutations with replacement; see [31,76] for details) in R [77] to detect significant differences.

**Table 1.** *Narcissus papyraceus* and *N. tazetta* populations surveyed for flower measurements and analyses of phenotypic integration, and patterns of floral variation and phenotypic correlation (see the electronic supplementary material, appendices S1, S2 and S3). Estimates of population morph ratio were done on a larger sample size (see [29,49–51,53,54] for detailed information on population sampling). Confidence intervals (CIs) for the raw integration index were obtained by bootstrapping.

species and populations	sample size for flower measurements (L : S)	L morph (%)	phenotypic integration index (%)	raw phenotypic integration index	95% CI
<i>Narcissus papyraceus</i>					
Morocco: Tánger-Tetuán, Oued Lediane	100 (57 : 42)	57	10.28	0.55	0.01–0.95
Morocco: Tetuán-Larache, Souk el Arba Ayacha	100 (95 : 100)	96.3	3.76	0.23	0.01–0.38
Morocco: Tánger-Tetuán, Ragaia	100 (41 : 59)	50	5.08	0.29	0.01–0.51
Spain: Málaga, Casares-Manilva	100 (87 : 13)	87.4	14.99	0.79	0.04–1.37
Spain: Málaga, San Pedro de Alcántara	67 (66 : 1)	98.53	13.39	0.73	0.01–1.23
Spain: Cádiz, Tarifa-Bolonia	100 (52 : 48)	50	23.64	1.22	0.01–2.05
Spain: Cádiz, Los Barrios	100 (50 : 50)	50	19.11	1.00	0.00–1.62
Spain: Cádiz, El Bosque	48 (32 : 16)	66	19.36	1.05	0.01–1.74
Spain: Huelva, Villanueva de los Castillejos	100 (99 : 1)	99	19.46	1.01	0.02–1.69
Spain: Huelva, Hinojos, El Caoso	24	100	8.15	0.57	0.13–0.94
Spain: Huelva, Hinojos, Coto del Rey	100	100	4.45	0.26	0.02–0.39
Spain: Huelva, Almonte, El Rocío	98 (95 : 3)	98.5	5.44	0.31	0.01–0.55
Spain: Sevilla, Aznalcázar	98	100	8.33	0.46	0.02–0.78
Spain: Córdoba, Carcabuey, Valdecañas	100	100	5.59	0.32	0.01–0.57
Portugal: Algarve, Barranco São Miguel	60 (55 : 6)	90.6	7.73	0.45	0.02–0.78
Portugal: Algarve, Mesines-Alte	100	100	10.49	0.57	0.01–0.98
Portugal: Algarve, Tavira	100	100	10.50	0.57	0.02–0.98
<i>Narcissus tazetta</i>					
Israel: Yuvalim	30 (15 : 15)	96	9.88	0.63	0.05–1.15
Israel: Stella Maris	28 (25 : 3)	95	13.4	0.89	0.12–1.5
Israel: Megadim	32 (30 : 2)	100	15.7	0.94	0.08–1.7
Israel: Nahal Mearot West	16	100	10.1	0.92	0.08–1.53
Israel: Nahal Mearot North	11	100	20.43	1.68	0.08–0.04

(Continued.)

**Table 1.** (Continued.)

species and populations	sample size for flower measurements (L : S)	L morph (%)	phenotypic integration index (%)	raw phenotypic integration index	95% CI
Israel: Nahal Ma'sad	19 (17 : 2)	89.5	9.41	0.83	0.06–1.41
Israel: Yagur	20 (10 : 10)	54	21.66	1.55	0.03–2.42
Israel: Kfar Yeoshua	13 (3 : 10)	20	19.55	1.56	0.12–2.77
Israel: Kishon River	34 (10 : 24)	10	23.57	1.33	0.06–2.35

**Table 2.** Genetic diversity parameters ( $\pm$  s.d.) for each of the selected *Narcissus papyraceus* populations. Percentage of polymorphic loci (PL), mean number of alleles ( $n_a$ ), genetic diversity ( $H_S$ ), allelic richness ( $R_S$ ) and the fixation index ( $F_{IS}$ ) per locus.

population	sample size	PL	$n_a$	$H_S$	$R_S$	$F_{IS}$
Morocco: Tánger-Tetuán, Ragaia	20	100	11.4 (1.2)	0.81 (0.06)	3.2 (0.2)	0.40 (0.07)
Spain: Málaga, Casares-Manilva	15	87.5	7.6 (1.5)	0.68 (0.10)	2.8 (0.3)	0.45 (0.08)
Spain: Cádiz, Tarifa-Bolonia	18	100	9.0 (1.1)	0.75 (0.06)	3.0 (0.2)	0.38 (0.10)
Spain: Cádiz, Los Barrios	15	100	8.6 (0.9)	0.74 (0.06)	3.0 (0.2)	0.37 (0.09)
Spain: Cádiz, El Bosque	15	100	7.6 (1.0)	0.69 (0.10)	2.8 (0.3)	0.42 (0.10)
Spain: Huelva, Villanueva de los Castillejos	16	100	5.8 (0.7)	0.71 (0.06)	2.8 (0.2)	0.47 (0.12)
Spain: Huelva, Hinojos, El Caoso	19	100	8.4 (1.0)	0.77 (0.05)	3.0 (0.2)	0.55 (0.08)
Spain: Sevilla, Aznalcázar	15	100	8.4 (1.3)	0.69 (0.09)	2.9 (0.3)	0.46 (0.08)
Spain: Córdoba, Carcabuey, Valdecañas	15	87.5	6.9 (1.5)	0.65 (0.10)	2.7 (0.3)	0.27 (0.10)
Portugal: Algarve, Barranco São Miguel	16	87.5	5.0 (1.0)	0.52 (0.10)	2.3 (0.3)	0.46 (0.13)

### (c) Patterns of phenotypic variation and phenotypic correlations in *Narcissus papyraceus*

To evaluate if *N. papyraceus* flowers could be divided into different functional modules, we tested whether the average of the correlation coefficients of the set of traits included within the same function (attraction: diameter and corona diameter; access: corona height and flower tube length and width; pollen transfer: style length, upper and lower anther position) was larger than the average of the correlation coefficients between traits belonging to different functions. These comparisons were conducted within population type.

The phenotypic variation of traits involved in pollinator attraction and access, and pollen pick-up and deposition was analysed within and between population types. Within population type, pairwise comparisons were used to test for differences in the average coefficient of variation (hereafter CV) between groups of traits (attraction versus pollinator access and fit, attraction versus pollen pick-up and deposition, and pollinator access and fit versus pollen pick-up and deposition). Between populations, pairwise comparisons were implemented to test for differences on the average CV of the same type of trait (e.g. differences in the CV of attraction traits in dimorphic versus L-monomorphic populations).

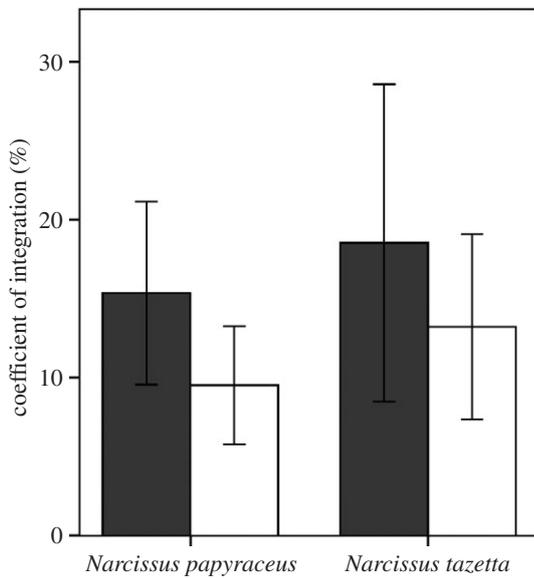
The strength of the correlation coefficient of sets of traits included in the same function was also studied. Within population type, the correlations of traits involved in pollen transfer (the style length–flower tube length correlation, the upper anther height–flower tube correlation and the lower anther height–flower tube correlation) were compared against the average correlations among traits involved in attraction or access (diameter, corona diameter and height, and flower width).

In addition, comparisons were established to detect possible differences between population types in the style length–flower tube length correlation, the upper and lower anther height–flower tube length correlations and the average correlations among traits involved in attraction or access.

We used the resampling procedure described above to detect significant differences in all the comparisons.

### (d) Genetic diversity in *Narcissus papyraceus* populations

Leaf tissue was collected from 15 to 20 individuals chosen randomly, totalling 164 *N. papyraceus* plants in six dimorphic populations and four L-monomorphic populations (tables 1 and 2). Sampled plants were separated from each other by at least 1 m. Leaf tissue was dried out in silica gel and later frozen at  $-80^{\circ}\text{C}$  until DNA extraction. DNA was isolated following Bernartzky & Tanksley's [78] protocol without mercaptoethanol. All samples were genotyped according to eight nuclear microsatellite markers previously tested for polymorphism (A5, A109, A116, A121, B7, B104, B109 and B112; [79]). We performed polymerase chain reactions (PCR) in 25  $\mu\text{l}$  of reaction mixture containing 50 ng of template DNA, 1 $\times$  PCR buffer, 1.5 mM  $\text{MgCl}_2$ , 0.1  $\mu\text{M}$  fluorescently labelled (6-FAMTM, VIC, NEDTM and PET dyes) forward primer, 0.1  $\mu\text{M}$  reverse primer, 0.05 mM each dNTP and 1.25 U Taq polymerase. PCRs were performed in a Biometra Gradient Thermal Cycler (Biometra, Göttingen, Germany), with an initial 5 min of denaturation at  $94^{\circ}\text{C}$ , 45 cycles at  $94^{\circ}\text{C}$  for 30 s, annealing at different temperatures depending on the marker ( $57^{\circ}\text{C}$  for A109 and B7;  $58^{\circ}\text{C}$  for



**Figure 2.** Means and 95% CI of the phenotypic integration index in dimorphic (black bars) and L-monomorphic (white bars) populations with mainly long- and short-tongued pollinators, respectively, in *N. papyraceus* and *N. tazetta*.

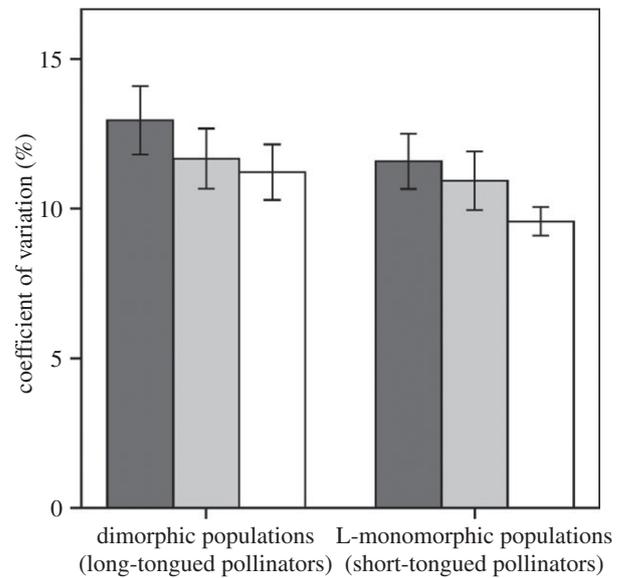
A116, A121 and B109; 59°C for B104 and B112) for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. Polymerase chain reaction products were analysed on an ABI 3130 × 1 Genetic Analyser and sized using GENEMAPPER v. 4.0 (Applied Biosystems, Foster City, CA, USA) and GeneScanTM 500 LIZ size standard.

For each *N. papyraceus* population, the mean number of alleles per locus ( $n_a$ ), the mean genetic diversity ( $H_s$ ), the fixation index ( $F_{IS}$ ) and the proportion of polymorphic loci (PL) was calculated using GENALEX v. 6 [80]. Allelic richness ( $R_s$ ) was estimated with HP-RARE v. 1 [81]. Non-parametric Kruskal–Wallis one-way ANOVA was used to detect possible differences in the population genetic parameters between dimorphic and L-monomorphic populations. The relationship between population genetic parameters and the phenotypic integration index, the average CV of floral traits and the average coefficient of correlation of floral traits was analysed with Spearman's rank correlation.

### 3. Results

#### (a) Phenotypic integration in *Narcissus papyraceus* and *N. tazetta* populations

Our results supported the hypothesis that dimorphic populations visited by LT pollinators should display higher integration values than L-monomorphic mostly visited by ST pollinators. Table 1 includes the phenotypic integration index and 95% CI estimates for *N. papyraceus* and *N. tazetta* populations. The magnitude of the phenotypic integration index in *N. papyraceus* ranged from 3.7 to 23.6%, and dimorphic populations showed greater integration than L-monomorphic populations (dimorphic populations mean (95% CI): 15.6% (9.1, 20.7); L-monomorphic populations: 10.2% (7.1, 13.5),  $p = 0.02$ , figure 2). Phenotypic integration values for *N. tazetta* ranged from 9.9 to 23.6% (table 1), and they also showed that dimorphic populations displayed larger integration values (18.6% (9.4, 23.6)) than L-monomorphic populations (13.2% (9.9, 20.4)), but the significance of the differences were marginal ( $p = 0.07$ , figure 2).



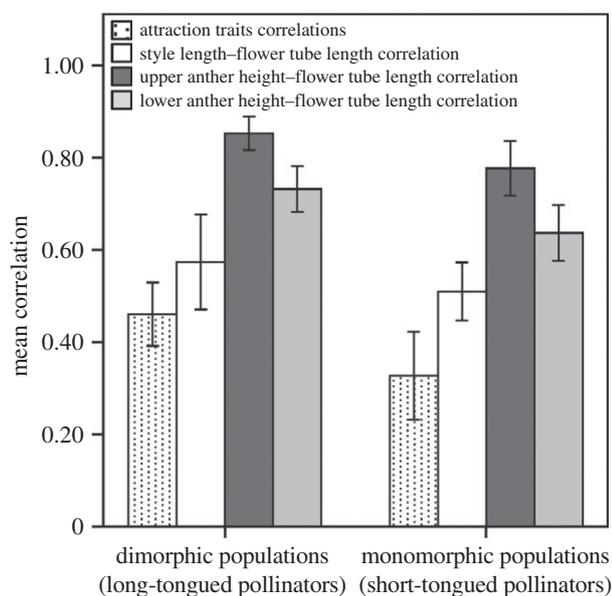
**Figure 3.** Mean and 95% CI of the coefficient of variation of floral traits involved in the pollinator attraction (black bars), pollinator access and fit (grey bars) and in pollen pick-up and deposition (white bars) in dimorphic and L-monomorphic populations of *N. papyraceus*.

#### (b) Patterns of variation and phenotypic correlation in groups of traits with shared function in *Narcissus papyraceus*

Electronic supplementary material, appendices S1, S2 and S3, include the coefficients of variation and correlation for *N. papyraceus* populations (estimates for L and S morph were calculated separately, and differences in the coefficients among floral traits for L and S flowers were not significant, results not shown).

The comparisons of the correlation coefficients of sets of traits included in the same function and in different functions supported the hypothesis of floral modularity in *N. papyraceus*. In dimorphic populations, the average correlation coefficient of sets of traits involved in the same function was larger than the average correlation coefficient of traits belonging to different functions (correlation coefficient of sets of traits within function: 0.64 (0.60, 0.67); correlation coefficient of sets of traits between functions: 0.33 (0.31, 0.36),  $p < 0.0001$ ). The same results were found in L-monomorphic populations (correlation coefficient within function: 0.55 (0.51, 0.59); correlation coefficient between functions: 0.21 (0.18, 0.23),  $p < 0.0001$ ).

The comparisons of the CV aimed at testing whether patterns of floral phenotypic variability in dimorphic populations with LT pollinators differed from those found in L-monomorphic populations with ST pollinators. In dimorphic populations, the CV of floral traits involved in the attraction of pollinators (12.9 (10.5, 11.9)) was significantly larger than the CV of floral traits involved in the access and fit with pollinators (11.7 (10.9, 12.6),  $p = 0.02$ ) and than the CV of floral traits involved in pollen pick-up and deposition (11.2 (10.5, 12.1),  $p = 0.0036$ , figure 3). By contrast, the CV of traits involved in pollinator access and fit did not differ from the CV of traits involved in pollen pick-up and deposition ( $p = 0.23$ , figure 3). In L-monomorphic populations, the CV of floral traits to attract pollinators (11.6 (11.0, 12.8)) did not differ from the CV of floral traits involved in the access and fit with



**Figure 4.** Mean and 95% CI of the correlation coefficients among traits involved in the attraction of pollinators and traits involved in the pollination function (style length–flower tube length correlation, upper anther height–flower tube correlation and lower anther height–flower tube correlation) in dimorphic and L-monomorphic populations of *Narcissus papyraceus*.

pollinators (10.9 (10.2, 11.8),  $p = 0.11$ , figure 3), whereas differences between the CV of floral traits involved in pollen pick-up and deposition (9.6 (9.1, 9.9)) and traits involved in attraction and pollinator access and fit were significant ( $p < 0.0001$  and  $p = 0.0002$ , respectively, see figure 3). Comparisons between population types showed that, on average, the CV of attraction traits and traits involved in pollen pick-up and deposition were larger in dimorphic populations than in L-monomorphic populations ( $p = 0.05$  and  $p < 0.0001$ , respectively); in contrast, differences in the CV of traits involved in the fit with pollinators were not significant ( $p = 0.13$ , figure 3).

In dimorphic and L-monomorphic populations, the style length–flower tube length correlation, and the anther height–flower tube correlation, both upper and lower anther level, were larger than the average phenotypic floral correlations among attraction traits and these differences were significant (dimorphic populations: style versus attraction correlations,  $p = 0.02$ ; upper anther versus attraction correlations,  $p < 0.0001$ ; lower anther versus attraction correlations,  $p < 0.0001$ ; L-monomorphic populations: style versus attraction correlations,  $p < 0.0001$ , upper anther versus attraction correlations,  $p < 0.0001$ , lower anther versus attraction correlation,  $p < 0.0001$ , figure 4). These results supported the prediction that the fitness surface for traits involved in pollen pick-up and delivery should be steeper than in traits involved in the attraction of pollinators. Comparisons between population types showed that upper anther–flower tube correlations (dimorphic populations: 0.85 (0.82, 0.88); L-monomorphic populations: 0.78 (0.73, 0.82)) and lower anther height–flower tube length correlations (dimorphic populations: 0.73 (0.68, 0.76); L-monomorphic populations: 0.64 (0.58, 0.68)) were larger in dimorphic than L-monomorphic populations, and the differences were significant ( $p = 0.01$  for both comparisons, figure 4). By contrast, style length–flower tube length correlations did not differ between dimorphic and L-monomorphic populations

(dimorphic populations: 0.57 (0.48, 0.66); L-monomorphic populations: 0.51 (0.46, 0.57),  $p = 0.12$ ). The average floral correlations among attraction traits were significantly larger in dimorphic than L-monomorphic populations (dimorphic populations: 0.46 (0.40, 0.52); L-monomorphic populations: 0.33 (0.25, 0.41),  $p = 0.005$ , figure 4). These comparisons between population types agreed with the expectation that the adaptive peak of floral traits should be narrower when selection is mediated by specialized LT-pollinators than by generalized ST-pollinators.

### (c) Genetic diversity in *Narcissus papyraceus* populations

The percentage of PL among *N. papyraceus* populations varied between 87.5 and 100% (table 2). The mean number of alleles per locus ( $n_a$ ) ranged between 5.0 and 11.4, and genetic diversity ( $H_S$ ) between 0.52 and 0.51. The allelic richness ( $R_S$ ) varied between 2.3 and 3.2, and the fixation indices  $F_{IS}$  were all positive and ranged from 0.27 to 0.55. The non-parametric Kruskal–Wallis one-way ANOVA showed that dimorphic and L-monomorphic populations did not differ in the population genetic parameters estimated (PL:  $H = 0.071$ ,  $n_a$ :  $H = 0.736$ ,  $H_S$ :  $H = 0.011$ ,  $R_S$ :  $H = 0.191$  and  $F_{IS}$ :  $H = 1.183$ , in all cases d.f. = 1 and  $p > 0.2$ ). The Spearman's correlation coefficients between  $H_S$  and the integration index ( $\rho = -0.024$ ), the mean CV ( $\rho = 0.248$ ) and the average correlation coefficients ( $\rho = 0.041$ ) for all floral traits were not significant ( $N = 10$  and  $p > 0.5$  in all estimates).

## 4. Discussion

### (a) Patterns of phenotypic integration in *Narcissus* species

*Narcissus papyraceus* and *N. tazetta* both have dimorphic populations with mostly long-tongued diurnal and nocturnal pollinators, and highly L-biased and L-monomorphic populations pollinated mainly by short-tongued syrphid flies [29,51,53–55]. Shifts from long- to short-tongued pollinators seem to select against the S morph and favour the L morph [59,60]. Thus, a steeper fitness surface could be expected in populations where flowers have close fit with pollinators (long-tongued insects) than in populations in which pollinators fit loosely with flowers (short-tongued insects), and this should be reflected in the patterns of phenotypic integration [2,18]. Our results confirmed this expectation: the phenotypic integration index in dimorphic populations was larger than in L-monomorphic or highly L-biased populations, and these trends were consistent across the two species.

The phenotypic integration observed in the two species could reflect possible effects of common ancestry [38,40,41]. However, *N. papyraceus* and *N. tazetta* are not sister species [49,82], and other species with different styler condition (fixed monomorphism in *N. serotinus* or dimorphism in *N. broussonetii*) are in the same clade. Assuming that legitimate pollinators in dimorphic species and populations are long-tongued insects, as the floral syndrome suggests [49], and that L-monomorphism with pollination by short-tongued insects is a derived condition [49,59,83], similar levels of integration in dimorphic populations (15.6% for *N. papyraceus* and 18.5% for *N. tazetta*), which differ greatly from the 10-fold variation in other species of the clade

(3–30%; R Pérez-Barrales, R Santos-Gally and J Arroyo 2013, unpublished data), may reflect factors other than common ancestry. More evidence in support of pollinators as drivers of floral integration includes the similar patterns of variation in population morph ratio, and the similar shifts in pollinators and patterns of floral integration in two species at the edges of the Mediterranean Basin (ca 4000 km distance), which are unlikely to be caused by phylogenetic effects. Nevertheless, detailed evolutionary reconstruction of flower phenotypic integration would help to elucidate this question.

Colonization of rocky habitats with severe temperature fluctuations, which determine early blooming, has been proposed as a cause for the shift of pollinators in *N. tazetta* populations [50]. An expansion to inland from coastal ranges seems to have played a similar role in *N. papyraceus* populations [84]. Hence, the lower integration in L-monomorphic populations could also be explained by (i) historical effects, if all L-monomorphic populations represent a single evolutionary event, and they have inherited the patterns of trait correlation and covariation (but see discussion above); and (ii) a reduction in genetic variation associated with the colonization of marginal ranges [43]. At present, we do not have sufficient phylogeographic information for these species to trace the colonization history of populations, and therefore reconstruct variation of integration across the ranges. However, population neutral genetic variation based on microsatellite markers did not differ in dimorphic and L-monomorphic populations of *N. papyraceus*; neither was significant the non-parametric correlations with integration, average floral variation and average correlation among traits (see discussion below). This evidence suggests that population genetic processes other than selection may have played a minor role in the patterns observed [85,86] and that low integration values are not due to a reduction in population genetic variation. However, our interpretations must be taken cautiously due to the limited number of populations in which we could relate phenotypic and genetic variation.

### (b) Patterns of floral variation, modularity and correlation in *Narcissus papyraceus*

Traits involved in attraction of pollinators (flower diameter, corona diameter and height) were significantly more variable than traits putatively involved in pollen pick-up and delivery (style length, upper and lower anther height) in dimorphic and L-monomorphic populations. This is not surprising because, regardless of the level of pollination specialization, developmental canalization and selection for precision in the pollination function reduces phenotypic variation [35–37,87,88]. By contrast, access and fit traits (flower tube width and length) displayed different patterns: their CV was lower than attraction traits and similar to pollination traits in dimorphic populations; while in L-monomorphic populations, the CV of access and fit traits was within the same range as attraction traits and substantially larger than pollination traits (figure 3). Small values for the CV of traits involved in pollen pick-up and delivery (e.g. access and fit, position of sexual organs) might reflect stronger directional selection or steeper stabilizing selection caused by long-tongued pollinators compared with short-tongued pollinators [38,87,88].

Comparisons of correlation coefficients of traits involved in attraction and transfer of pollen, as well as correlations of traits across functions revealed interesting patterns. The hypothesis

of floral modularity in *N. papyraceus* was supported. In both dimorphic and L-monomorphic populations, correlations of sets of traits associated with attraction, access and pollen transfer were larger than correlations of traits involved in different functions. From a developmental and genetic perspective this is expected, as shown for both plants [25,26,32] and animals [2,3]. Floral modularity can be selected for, and this can cause low floral integration [30,32]. In this study, we lack fitness estimates to measure the adaptive value of traits involved in different functions, and hence cannot say that modularity is the cause of low integration. Interestingly, dimorphic and L-monomorphic populations displayed similar modularity but different phenotypic integration.

Despite the fact that selection for modularity may act against high integration, the comparisons of groups of traits related to attraction, access and transfer of pollen in dimorphic and L-monomorphic populations agreed with the hypothesis that selection by different pollinators can generate differences in levels of integration. Within population type, the correlations between organs involved in pollen pick-up and deposition (upper and lower anther length, style length and flower tube length) were consistently larger than the average correlations among floral traits involved in attraction. In addition, the correlation coefficient was substantially larger for the anther height–flower tube correlation than the style length–flower tube length correlation (figure 4). An important aspect of *Narcissus* flowers is that the filaments are fused to the flower tube. Hence, the high correlations observed for anther position and flower tube length both in dimorphic and L-monomorphic populations might reflect important developmental constraints, genetic correlation and pleiotropy (see discussion above, [89–91]). However, we detected differences in the strength of correlations as predicted by Berg [2,18], and the results fitted the expectations that LT-pollinators exert stronger selection on anther position than ST-pollinators. Furthermore, differences between population types in the average correlation between anther height and flower tube length were larger for the lower anther level than the upper anther level. This may reflect two processes that are not mutually exclusive: (i) selection generated by LT-pollinators for a precise position of the lower anther level to donate pollen to S-stigmas in dimorphic populations [58,92]; and/or (ii) relaxation of selection on the lower anther level in L-monomorphic populations because, unlike long-tongued insects, short-tongued pollinators interact only with the upper anther level (R Pérez-Barrales 2003, personal observation; [60]).

In contrast to anther height and flower tube length, the correlation between style length and flower tube length did not differ between population types (although the average correlation was smaller in L-monomorphic than in dimorphic populations, figure 4). In addition to pollinators, the position of the stigma may be constrained by additional factors. For example, avoidance of self-interference through stigma clogging can affect stigma position in self-incompatible species and increase the deviation from optimal position for pollen arrival [35–37,93,94].

## 5. Concluding remarks

In a previous paper, we documented a pattern of floral phenotypic integration in *N. papyraceus* consistent with the role of different pollinators in different populations. Here, we

expanded our results using *N. tazetta*, a different species from a distant geographical range, but displaying similar variation in pollinators. We also studied patterns of variation and correlation of traits involved in different functions, and incorporated a population genetic dataset to assess possible effects of demographic population processes on the phenotypic patterns described. Taken together, the results suggest that pollinator-mediated selection plays an important role in the phenotypic integration of *N. papyraceus* and *N. tazetta* flowers: selection probably maintains the correlation structure in dimorphic populations pollinated by long-tongued pollinators, whereas this structure is weakened when these pollinators are mostly substituted by short-tongued pollinators in other populations. Our findings agree with a number of studies supporting the idea that plant species with specialized pollinators present larger values of floral integration than species with generalized pollinators

[2,22,18,29,38]. Notwithstanding this, our study did not include female and male fitness estimates to quantify the adaptive value of integration, nor could we assess the adaptive value of the traits taking part in attraction, access and pollen transfer with different functional groups of pollinators. Future research will require combining phenotypic selection studies with developmental and quantitative genetics to better understand how pollinators can select for integrated phenotypes.

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