

# Natural Selection on *Erysimum mediohispanicum* Flower Shape: Insights into the Evolution of Zygomorphy

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**ABSTRACT:** Paleontological and phylogenetic studies have shown that floral zygomorphy (bilateral symmetry) has evolved independently in several plant groups from actinomorphic (radially symmetric) ancestors as a consequence of strong selection exerted by specialized pollinators. Most studies focused on unraveling the developmental genetics of flower symmetry, but little is known about the adaptive significance of intraspecific flower shape variation under natural conditions. We provide the first evidence for natural selection favoring zygomorphy in a wild population of *Erysimum mediohispanicum* (Brassicaceae), a plant showing extensive continuous variation in flower shape, ranging from actinomorphic to zygomorphic flowers. By using geometric morphometric tools to describe flower shape, we demonstrate that plants bearing zygomorphic flowers received more pollinator visits and had the highest fitness, measured not only by the number of seeds produced per plant but also by the number of seeds surviving to the juvenile stage. This study provides strong evidence for the existence of significant fitness differences associated with floral shape variation in *E. mediohispanicum*, thus illuminating a pathway for the evolution of zygomorphy in natural populations.

**Keywords:** *Erysimum mediohispanicum*, floral shape evolution, geometric morphometrics, natural selection, pollination, structural equation modeling.

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The evolution of flower shape is attracting attention from biologists working in disparate disciplines, such as developmental genetics and evolutionary development, evolutionary ecology, pollination biology, paleobiology, and

phylogeny (Coen et al. 1995; Donoghue et al. 1998; Cubas et al. 1999; Endress 1999; Ree and Donoghue 1999; Galen and Cuba 2001). Paleontological and phylogenetic studies have shown that the ancestral angiosperm flowers were actinomorphic (radially symmetric). Zygomorphy, or bilateral symmetry, in flowers arose independently several times from actinomorphic ancestors (Reeves and Olmstead 1998; Ree and Donoghue 1999; Dilcher 2000; Olson 2003; Rudall and Bateman 2003). Floral zygomorphy is considered a key innovation promoting speciation and diversification in angiosperms because it is associated with the largest plant families (Sargent 2004). Zygomorphy is thought to have evolved as a consequence of strong selection exerted by specialized pollinators (Neal et al. 1998; Endress 2001) because it increases both flower attractiveness to pollinators (Møller 1995; Rodríguez et al. 2004) and pollen transfer efficiency (Endress 1999).

Much empirical information on the developmental genetics of floral shape has accumulated in recent years (e.g., Cronk et al. 2002). While the genetic basis of zygomorphy is known for some model species, such as *Antirrhinum majus* or *Linaria vulgaris* (Luo et al. 1995; Cubas et al. 1999), it remains essential, in order to understand the role of natural selection in the evolution of flower symmetry, to link the genetic basis of flower shape to ecological and evolutionary contexts (Theißen 2000; Hileman et al. 2003). Few empirical studies have assessed the adaptive significance of flower shape (but see Herrera 1993; Bradshaw et al. 1998; Schemske and Bradshaw 1999; Galen and Cuba 2001), even though the quantitative study of natural selection has been greatly improved during the last two decades as a result of the development of robust statistical techniques. An important reason for this dearth of studies is the difficulty in quantifying intraspecific variation in flower shape and symmetry in plants. Selection on flower shape has been studied both by considering it qualitatively (i.e., radial vs. bilateral symmetry) and by describing it as a variable composed of linear measurements. To overcome this difficulty, we apply geometric morphometric tools (Bookstein 1991; Zelditch et al. 2004) to measure *Erysimum mediohispanicum* (Brassicaceae), a species showing

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high variation in flower shape in the wild (fig. 1). Our objectives are to determine the variation in flower shape occurring in *E. mediohispanicum*, to quantify the relationship between flower shape and pollinators, and to determine the effect of flower shape on plant fitness and thereby infer the effect of natural selection on flower shape.

## Material and Methods

### *Study System*

*Erysimum mediohispanicum* is a biennial to perennial monocarpic herb found in many montane regions of southeast Spain from 1,100 to 2,000 m elevation, inhabiting forests and subalpine scrublands. Plants usually grow for 2–3 years as vegetative rosettes and then die after producing one to eight reproductive stalks that can display between a few and several hundred hermaphroditic, slightly protandrous, bright yellow flowers (Gómez 2003). During anthesis, most flowers are oriented in a vertical or quasi-vertical plane with respect to the flowering stalk. Flowers are visited in the study site by several species of insects, particularly the pollen beetle *Meligethes maurus* (Nitidulidae), and several species of beetles, bumblebees, solitary bees, and syrphids (Gómez 2005). Although this crucifer is self-compatible, it requires pollen vectors to produce a full seed set. Selective exclusion experiments have demonstrated that *M. maurus* is a major pollinator for *E. mediohispanicum* at the study site (Gómez 2005).

We labeled 200 reproductive plants during 2003 and 100 plants during 2004 in a population located in the Sierra Nevada (southeast Spain; 37°4.8'N, 3°27.9'W; 1,830 m elevation; see Gómez 2003, 2005 for a detailed description of the study site).

### *Morphometric Analyses to Study Flower Shape*

Flower shape was studied by means of geometric morphometric tools, using a landmark-based methodology that eliminates the effect of variation in the location, orientation, and scale of the specimens (Bookstein 1991; Rohlf 2003; Zelditch et al. 2004). We took a digital photograph of one flower per plant, using a standardized procedure (front view and planar position). Flowers were photographed at anthesis to avoid ontogenetic effects. We defined 32 coplanar landmarks located along the outline of the flowers and the aperture of the corolla tube (fig. 1), the number of landmarks being chosen to provide comprehensive coverage of the flower shape (Roth 1993; Zelditch et al. 2004). Landmarks were defined by reference to the midrib (landmarks 1, 9, 17, 25), primary veins (landmarks 2, 8, 10, 16, 18, 24, 26, 32), and secondary veins (landmarks 3, 4, 6, 7, 11, 12, 14, 15, 19, 20, 22, 23,

27, 28, 30, 31) of each petal, as well as the connection between petals (landmarks 5, 13, 21, 29; see fig. 1). We consider all of these to be Type I landmarks; however, the last four are supported as much by geometric evidence as by histological evidence and may be considered Type II landmarks (see Zelditch et al. 2004 for landmark definitions). We captured the landmarks using the computer program tpsDig, version 1.4 (available from the State University of New York [SUNY] Stony Brook morphometrics Web site at <http://life.bio.sunysb.edu/morph/index.html>). Afterward, the two-dimensional coordinates of these landmarks were determined for each plant, and the generalized orthogonal least squares Procrustes average configuration of landmarks was computed using the generalized Procrustes analysis (GPA) superimposition method (Rohlf and Slice 1990; Slice 2001). We used this method because of its low bias (Rohlf 2000, 2003). This procedure eliminates nonshape variation in configurations of landmarks by superimposing landmark configurations using least squares estimates for translation and rotation parameters. GPA was performed using the software tpsRelw, version 1.11 (available from the SUNY Stony Brook morphometrics Web site at <http://life.bio.sunysb.edu/morph/index.html>). In these analyses, we have considered the flower as a nonarticulated structure because the relative positions of the petals do not change during their functional life (see Adams 1999 for a discussion on articulated structures). After GPA, the relative warps (RWs, which are principal components of the covariance matrix of the partial warp scores) were computed (Walker 2000; Adams et al. 2004). Unit centroid size was used as the alignment-scaling method and orthogonal as the alignment-projection method. This procedure generates a consensus configuration, the central trend of an observed sample of landmarks, which is similar to a multidimensional average. In addition, it also generates  $2p - 4$  orthogonal RWs ( $p$  = number of landmarks). Each RW is characterized by its singular value and explains a given variation in shape among specimens. Thus, RWs summarize shape differences among specimens (Adams et al. 2004), and their scores can be saved to be used as a data matrix to perform standard statistical analyses (Zelditch et al. 2004).

### *Flower and Plant Size*

Plant size was estimated by means of three raw variables, the number of reproductive stalks growing from each rosette, the height of the tallest stalk (distance from the ground to the top of the highest opened flower, measured to the nearest 0.5 cm) and the basal diameter of the tallest stalk (mm; measured by digital calipers with  $\pm 0.1$  mm error). These three variables are significantly correlated ( $P < .01$  in all cases). We performed a principal compo-



**Figure 1:** Diverse floral forms of *Erysimum mediohispanicum* occurring in the population studied. The uppermost left panel shows the location of the 32 landmarks used in the geometric morphometric analysis.

nents analysis (PCA) on the covariance matrix of the three original variables. This analysis yielded two factors (eigenvalues = 0.31 and 0.07), the first one explaining 75% of the variation. We extracted the scores from the first factor, which is considered to be a plant-size factor (Pugesek 2003a).

Flower size in the same flowers used to study shape was estimated by three variables: length of the showy part of one petal, diameter of the corolla, and corolla tube length. These variables were measured by using digital calipers with  $\pm 0.1$  mm of error. These three variables were strongly correlated ( $P < .0001$  in all cases), and therefore we also performed a PCA to obtain a linear combination of them. We obtained one factor (eigenvalue = 4.32) explaining almost 90% of the variation in the three original variables and, consequently, showing very high correlation with them ( $t > 200.0$ ,  $P < .0001$ ,  $R^2 > 0.99$  for all cases). As above, we obtained the value of this new variable, which we called flower size, for each labeled plant. We did not use centroid size as a measure of flower size for two reasons. First, we included the corolla tube depth as a variable contributing to flower size, this being a key variable to understanding the interaction between plants and pollinators but one that is not represented by the landmark configurations because it is located on a different plane. Second, to get analogous data, we decided to use an approach similar to the one used in all of the other studies on flower size evolution.

#### *Pollinator Preference*

In 2003, we determined the identity and abundance of pollinators for each labeled plant by counting all insects visiting flowers and making contact with the anthers and stigmas in 1-min censuses ( $N = 2,000$  censuses, corresponding to 34 observation hours evenly distributed among experimental plants). The censuses were made about 1 m from the flowering plants to monitor all the floral visitors without alarming them or disturbing their foraging behavior (see Gómez 2003, 2005 for a detailed description). As an estimate of the pollinator preference pattern, we performed a forward stepwise multiple regression of the pollinator visitation rate on morphological traits of the plants. In this analysis, we included the three flower size traits, the three plant size traits, and the RWs. We always provided standardized regression coefficients by using JMP 5.1 (SAS Institute 2003).

#### *Estimates of Plant Fitness*

We used two estimates of plant fitness: the number of seeds ( $w_s$ ) and the number of juveniles ( $w_j$ ) produced per plant during its entire life. The first one, the most widely

used estimate in selection studies, considers only individual fitness, whereas the second estimate includes progeny performance in early development (Wolf and Wade 2001). To calculate  $w_s$ , we counted the number of fruits per plant at the end of the reproductive season and collected a sample to determine in the lab their numbers of seeds (this species is monocarpic, reproducing only once). To calculate  $w_j$  during the fall of 2003, we sowed eight seeds from each individual plant (1,600 seeds in total) in four blocks located in the population studied. Seeds from each family were randomly assigned to a position in each block. We registered seed germination and seedling emergence every 2 weeks from March to May of 2004 and then survival monthly until September of 2004.

#### *Estimating Phenotypic Selection for Flower Shape*

No formal approach has been developed to examine selection for shape. In this study, we followed the methodology proposed by Klingenberg and Leamy (2001) and Klingenberg and Monteiro (2005). According to these authors, selection for shape can be calculated by selection differential  $s$  and selection gradient  $\beta$ . The former is a descriptor of the total effect of selection on shape without distinguishing between direct and indirect selection, whereas the latter allows inquiring into the causal basis of selection and represents the direct effect of each shape variable separately (Klingenberg and Monteiro 2005).

The selection differential was quantified as the vector of covariances between fitness and the shape variables (the complete set of RWs in this study). For this, we derived the vector of coefficients from a two-block partial least squares (PLS) analysis between shape and fitness (Rohlf and Corti 2000). Each fitness variable was used separately in the fitness block. By means of cross-validation, we found the number of latent vectors displayed by the model with the lowest root mean square error (Abdi 2003). After this, we determined the covariance between fitness and shape predicted by this parsimonious model. The PLS analysis was performed by the PLS platform in JMP 5.1 (SAS Institute 2003).

The multivariate selection gradients were estimated by multiple regressions of fitness on shape variables (Klingenberg and Monteiro 2005). We estimated the linear, nonlinear, and correlational Lande and Arnold standardized selection gradients (Lande and Arnold 1983). The linear selection gradient  $\beta$  was computed from the standardized partial regression coefficients of a linear regression of relative fitness on all traits, whereas the nonlinear selection gradient  $\gamma$  was calculated from the second-order standardized coefficient in a quadratic regression. In this last quadratic model, we partitioned selection into direct selection gradients for the character  $i$  ( $\gamma_{ii}$ ) and gradients

**Table 1:** Summary of the goodness-of-fit tests ( $\chi^2$ ) and the Akaike Information Criterion (AIC) of the saturated and nested models for each fitness estimate

|                        |           | $\chi^2$ | df | <i>P</i> | AIC   | Paths constrained to zero         |
|------------------------|-----------|----------|----|----------|-------|-----------------------------------|
| <i>w<sub>s</sub></i> : |           |          |    |          |       |                                   |
| Model 1                | Saturated | 80.093   | 63 | .004     | .920  |                                   |
| Model 2                | Nested    | 80.927   | 51 | .005     | .912  | Plant size → <i>w<sub>s</sub></i> |
| Model 3                | Nested    | 52.401   | 41 | .109     | .692  | <i>N</i> stalks                   |
| Model 4                | Nested    | 45.062   | 32 | .063     | .615  | RW3                               |
| Model 5                | Nested    | 36.412   | 25 | .066     | .516  | RW2                               |
| Model 6                | Nested    | 26.876   | 18 | .081     | .425  | RW1                               |
| <i>w<sub>j</sub></i> : |           |          |    |          |       |                                   |
| Model 1                | Saturated | 127.039  | 53 | .0001    | 1.165 | Plant size → <i>w<sub>j</sub></i> |
| Model 2                | Nested    | 104.271  | 43 | .0001    | .989  | <i>N</i> stalks                   |
| Model 3                | Nested    | 64.719   | 43 | .022     | .715  | Plant size → flower size          |
| Model 4                | Nested    | 123.909  | 45 | .022     | 1.092 | Plant size → flower number        |
| Model 5                | Nested    | 27.266   | 27 | .499     | .416  | Stalk diameter + stalk height     |
| Model 6                | Nested    | 23.322   | 20 | .273     | .364  | RW3                               |
| Model 7                | Nested    | 17.025   | 14 | .255     | .296  | RW1                               |
| Model 8                | Nested    | 11.176   | 9  | .264     | .231  | RW4                               |

Note: We constrained to zero those paths reaching the highest *P* values in the previous models. We used the rescaled AIC, which remains more stable across differing sample sizes. This criterion is calculated as  $F_{ML,k} + (f_k/N - 1)$ , where  $F_{ML,k}$  is the maximum likelihood discrepancy function,  $f_k$  is the number of free parameters for the model  $M_k$ , and *N* is the sample size. RW = relative warp.

describing selection on the correlation between characters *i* and *j* ( $\gamma_{ij}$ ). These multivariate models were built by introducing flower number, the two PCA-generated traits (flower size and plant size), and the GPA-generated RWs as independent variables. Unfortunately, finding the non-linear and correlational gradients was not possible when introducing the complete set of RWs because the matrix proved to be singular. To decrease the dimensionality of the model, we analyzed only the RWs explaining more than 5% of the variation in shape (see Klingenberg and Leamy 2001 for a similar procedure). To visualize the expected shape of individuals with different fitness, we followed the recommendations of Klingenberg and Monteiro (2005) and visualized the shape directly as changes in landmark positions (Rohlf et al. 1996; Adams and Rosenberg 1998). To determine the effect of each phenotypic trait on fitness independent of the other correlated traits, we used the partial-regression leverage plots of each trait on fitness residuals (Rawling et al. 1998). This method calculates a confidence function with respect to each variable, from which it can give the sign and percentage of the variation in fitness explained by each variable. In addition, we also determine the standardized selection differential (*i*) for each of the variables included in the multivariate selection gradients as the covariance of each trait and the fitness components divided by the trait standard deviation.

Finally, we also used structural equation modeling with latent constructs (SEM; Shipley 2000; Pugsek 2003b) to estimate the relationship between the phenotypic traits and

fitness. This method allowed us to consider flower shape as a single, inclusive, and multidimensional character (Adams and Rosenberg 1998). We created three latent constructs: flower size, flower shape, and plant size, each defined by a set of indicator variables (note that flower size and plant size in the structural equation modeling are not the same as the two PC factors obtained above and used for the selection gradient analysis). We built an a priori overidentified saturated model in which the latent variables directly affected plant fitness and were connected to the phenotypic traits measured, which acted as indicator variables. Flower shape was connected to the four RWs explaining more than 5% of variation (analyses incorporating all RWs yielded identical results but were omitted for clarity). In addition, we built five alternative nested models where we constrained some of the causal paths to zero (see table 1). All models were solved minimizing yield-parameter estimates through an iterative process that used generalized least squares shifting to maximum likelihood as discrepancy functions. We used maximum-likelihood estimation (MLE) on the variance-covariance matrix to test the goodness of fit of the model and to calculate the Akaike Information Criterion (AIC). To select the best-fitting model, we chose the one with the highest *P* value and the lowest  $\chi^2$  and AIC. Structural equation modeling was performed with the SEPATH procedure in Statistica 7.0 (StatSoft 2002). Finally, we introduced pollinator abundance connected to each indicator variable remaining in the best-fitted models.

2003

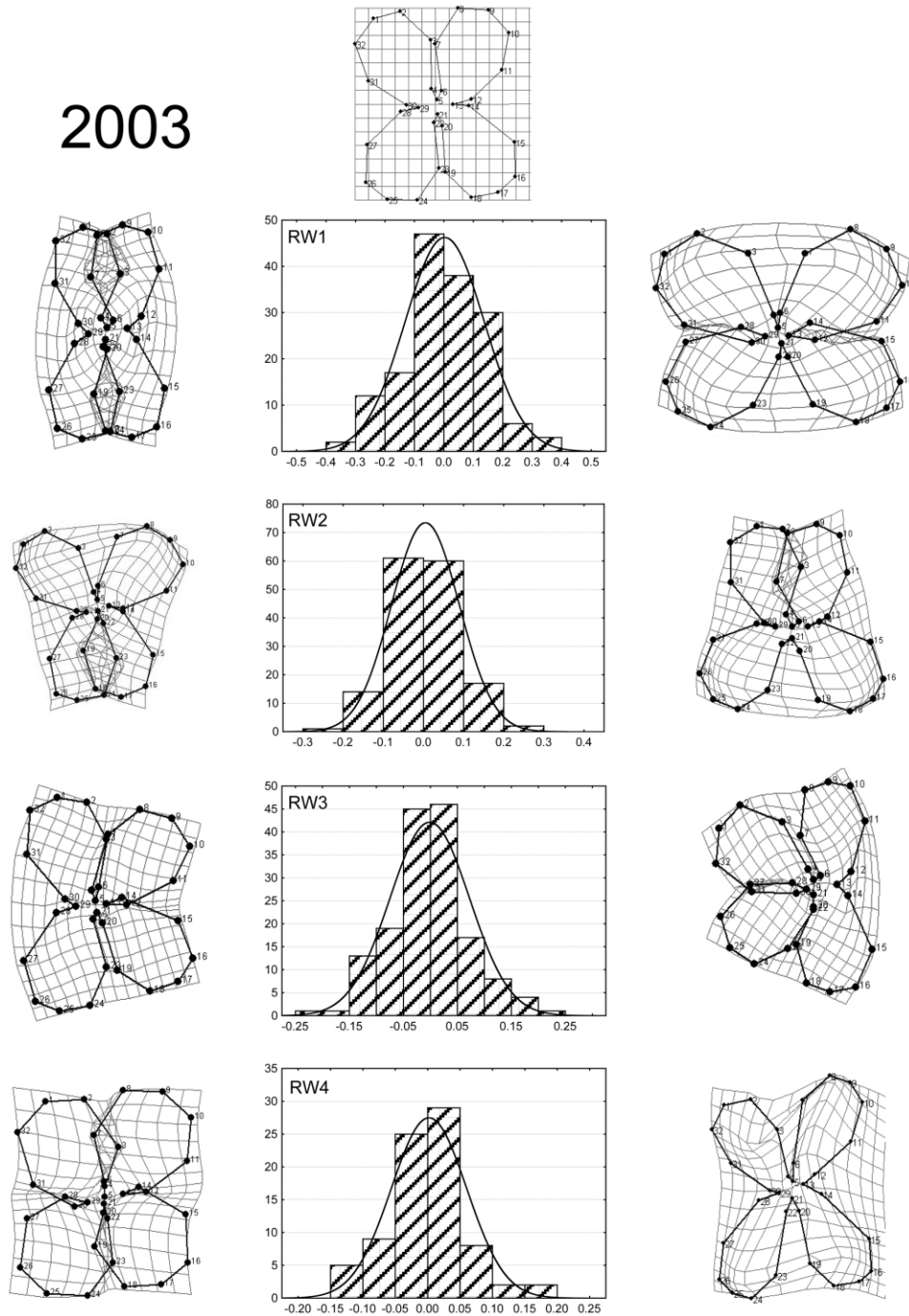


Figure 2: Summary of the geometric morphometric analysis ( $N = 155$  plants in 2003 and 80 plants in 2004) showing the consensus morphology (*uppermost panels*) and the variation in flower morphology produced by the relative warps (RW) explaining more than 5% of the overall variation in shape (2003: RW1 = 35.97%, RW2 = 14.90%, RW3 = 11.91%, RW4 = 5.19%; 2004: RW1 = 37.90%, RW2 = 11.52%, RW3 = 10.32%, RW4 = 6.80%, RW5 = 5.50%). The distribution of each RW statistically fitted a normal distribution with mean = 0 ( $\chi^2 < 0.993$ ,  $P > .34$  in all cases; Shapiro-Wilks  $W$  test).

2004

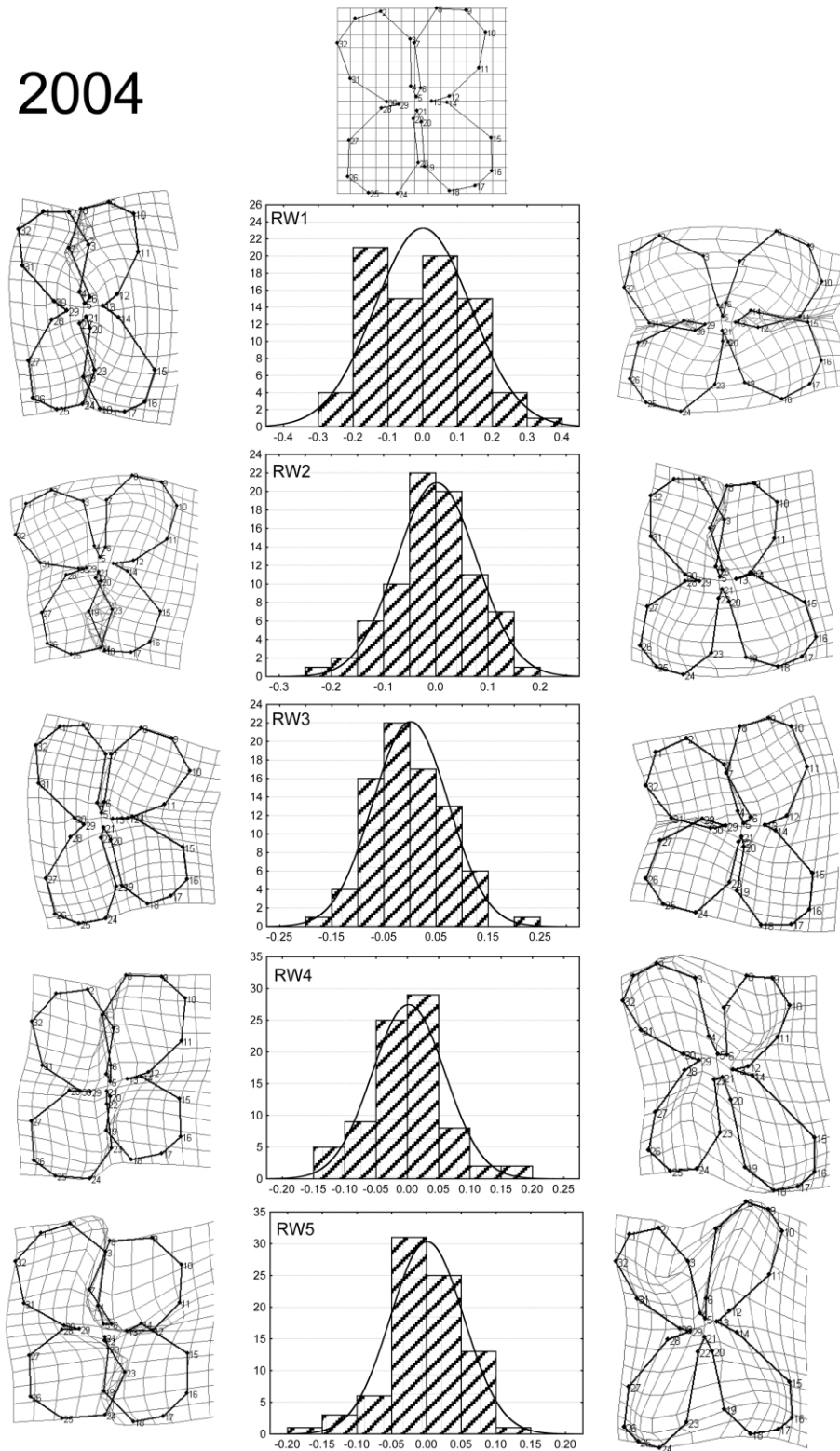
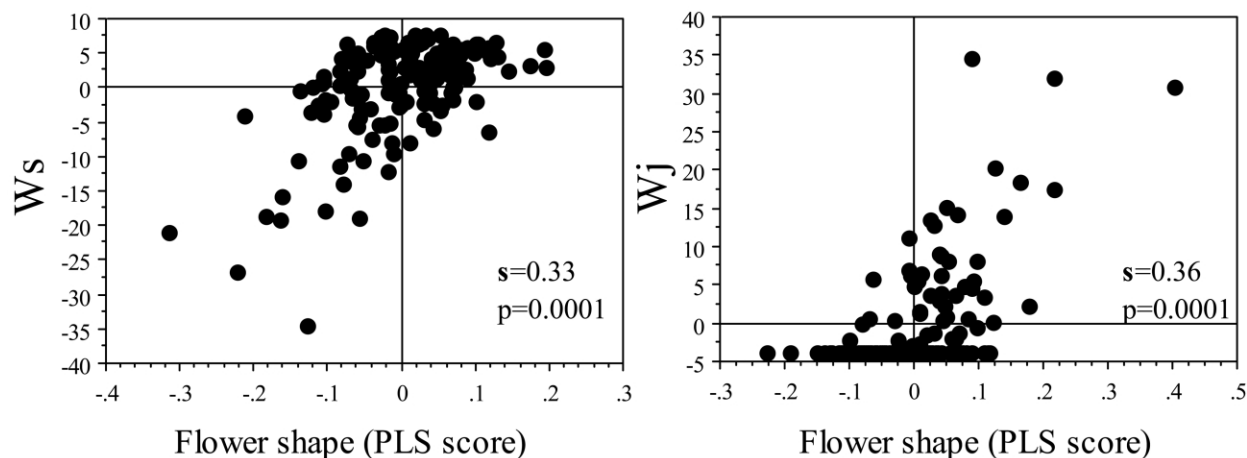


Figure 2 (Continued)



**Figure 3:** Results of the two-block partial least squares analysis to estimate the vector of selection differential ( $s$ ) estimate as the covariance between fitness and shape.

## Results

### *Description of Flower Shape*

The geometric morphometric analysis showed that the consensus flower of *Erysimum mediohispanicum* for the population studied was disymmetric, with two adaxial and two abaxial petals and only two reflexional planes (fig. 2). The morphometric analysis yielded 60 orthogonal shape variables (RWs) each study year. Four RWs in 2003 and five in 2004 explained more than 5% of the variance in each shape (app. A in the online edition of the *American Naturalist*). The first, RW1, was associated with a change in petal parallelism, RW2 with the development of true zygomorphic flowers with dorsoventral asymmetry (and therefore only one reflexional plane), RW3 with a change in lateral development of petals, and RW4 and RW5 with changes in flower roundness (fig. 2).

We detected no correlation between the flower shape variables and any other phenotypic trait ( $P > .05$ ; PLS analysis). By contrast, we found significant positive phenotypic correlations of number of flowers with stalk diameter (2003:  $r = 0.573$ ,  $n = 155$ ,  $P = .0001$ ; 2004:  $r = 0.643$ ,  $n = 81$ ,  $P = .0001$ ; pairwise Pearson correlations), stalk height (2003:  $r = 0.238$ ,  $n = 155$ ,  $P = .003$ ; 2004:  $r = 0.411$ ,  $n = 81$ ,  $P = .0001$ ), petal length (2003:  $r = 0.173$ ,  $n = 155$ ,  $P = .033$ ; 2004:  $r = 0.323$ ,  $n = 81$ ,  $P = .003$ ), corolla diameter (2003:  $r = 0.165$ ,  $n = 155$ ,  $P = .042$ ; 2004:  $r = 0.577$ ,  $n = 81$ ,  $P = .0002$ ), and corolla tube depth (2004:  $r = 0.338$ ,  $n = 81$ ,  $P = .002$ ). Stalk height also correlated with petal length (2003:  $r = 0.235$ ,  $n = 155$ ,  $P = .003$ ; 2004:  $r = 0.420$ ,  $n = 81$ ,  $P = .0001$ ), corolla diameter (2003:  $r = 0.277$ ,  $n = 155$ ,  $P = .0005$ ; 2004:  $r = 0.489$ ,  $n = 81$ ,  $P = .0001$ ), and corolla tube

depth (2003:  $r = 0.277$ ,  $n = 155$ ,  $P = .0005$ ; 2004:  $r = 0.338$ ,  $n = 81$ ,  $P = .002$ ).

### *Pollinator Preferences*

Flowers were visited by more than 20 species of insects ( $1.90 \pm 0.13$  insects plant<sup>-1</sup> min<sup>-1</sup>;  $N = 2,000$  censuses; net 34 h of observation) belonging to Coleoptera, Hymenoptera, and Diptera. The beetle *Meligethes maurus* (Nitidulidae) was the most abundant flower visitor (>80% of relative abundance). Pollinators were more abundant in taller plants (stepwise multiple regression:  $\beta = 0.30 \pm 0.14$ ,  $t = 2.1$ ,  $df = 1,153$ ,  $P = .03$ ) with wider stalks ( $\beta = 0.25 \pm 0.07$ ,  $t = 3.5$ ,  $df = 1,153$ ,  $P = .0006$ ). More importantly, pollinators were also more abundant in plants with parallel petals, that is, negative values of RW1 ( $\beta = -0.34 \pm 0.14$ ,  $t = 2.4$ ,  $df = 1,153$ ,  $P = .02$ ), and zygomorphic flowers with larger abaxial petals, that is, positive values of RW2 ( $\beta = 0.56 \pm 0.23$ ,  $t = 2.5$ ,  $df = 1,153$ ,  $P = .01$ ).

### *Phenotypic Selection for Flower Shape*

Significant selection for flower shape was consistently found by all three analytical procedures used in this study. First, according to the PLS analysis, we found a significant selection differential on flower shape for both fitness estimates  $w_s$  and  $w_j$  (fig. 3). The regression of the fitness latent vector on the flower shape latent vector suggested that this flower trait explains more than 86% of the variation in fitness. The multivariate selection gradient analysis also found selection for shape since at least one RW was significant for each fitness estimate (app. B in the



**Table 2:** Linear selection intensity ( $i$ ) and linear ( $\beta$ ), nonlinear ( $\gamma_{ii}$ ), and correlational ( $\gamma_{ij}$ ) phenotypic standardized selection on plant size, flower size, and flower shape in *Erysimum mediohispanicum* through both fitness estimates

| Plant trait | $i$      | $\beta \pm 1 \text{ SE}$ | $\gamma_{ii} \pm 1 \text{ SE}$ | $\gamma_{ij} \pm 1 \text{ SE}$ |                   |                  |                  |                  |                    |
|-------------|----------|--------------------------|--------------------------------|--------------------------------|-------------------|------------------|------------------|------------------|--------------------|
|             |          |                          |                                | $N$ flowers                    | Flower size       | RW1              | RW2              | RW3              | RW4                |
| $w_s$ :     |          |                          |                                |                                |                   |                  |                  |                  |                    |
| Plant size  | .265***  | .033 $\pm$ .007          | -.012 $\pm$ .049               | -.065 $\pm$ .062               | .024 $\pm$ .068   | -.017 $\pm$ .073 | -.080 $\pm$ .074 | -.051 $\pm$ .073 | -.017 $\pm$ .076   |
| $N$ flowers | .670**** | .631 $\pm$ .061****      | .130 $\pm$ .045**              |                                | .276 $\pm$ .088** | .064 $\pm$ .074  | -.117 $\pm$ .070 | -.016 $\pm$ .069 | -.195 $\pm$ .074** |
| Flower size | .325**** | .205 $\pm$ .066***       | .075 $\pm$ .042                |                                |                   | .083 $\pm$ .067  | -.013 $\pm$ .078 | .092 $\pm$ .057  | .077 $\pm$ .070    |
| RW1         | -.075    | -.003 $\pm$ .060         | -.023 $\pm$ .044               |                                |                   |                  | -.012 $\pm$ .062 | .074 $\pm$ .064  | -.032 $\pm$ .070   |
| RW2         | -.066    | -.032 $\pm$ .060         | -.010 $\pm$ .043               |                                |                   |                  |                  | -.001 $\pm$ .061 | -.003 $\pm$ .067   |
| RW3         | .091     | .031 $\pm$ .060          | -.057 $\pm$ .038               |                                |                   |                  |                  |                  | .083 $\pm$ .072    |
| RW4         | -.070    | -.139 $\pm$ .061**       | -.032 $\pm$ .046               |                                |                   |                  |                  |                  |                    |
| $w_l$ :     |          |                          |                                |                                |                   |                  |                  |                  |                    |
| Plant size  | .156*    | .027 $\pm$ .088          | -.013 $\pm$ .068               | -.035 $\pm$ .084               | .020 $\pm$ .093   | .096 $\pm$ .104  | -.141 $\pm$ .104 | -.035 $\pm$ .099 | .187 $\pm$ .109    |
| $N$ flowers | .221**   | .194 $\pm$ .082*         | -.017 $\pm$ .063               |                                | .148 $\pm$ .120   | -.046 $\pm$ .100 | .211 $\pm$ .095* | -.070 $\pm$ .094 | -.078 $\pm$ .101   |
| Flower size | .213**   | .179 $\pm$ .089*         | .112 $\pm$ .059                |                                |                   | .042 $\pm$ .092  | .039 $\pm$ .108  | .110 $\pm$ .082  | .195 $\pm$ .097*   |
| RW1         | -.025    | .023 $\pm$ .081          | .007 $\pm$ .061                |                                |                   |                  | -.024 $\pm$ .085 | .153 $\pm$ .091  | .102 $\pm$ .096    |
| RW2         | .211**   | .243 $\pm$ .080***       | .073 $\pm$ .059                |                                |                   |                  |                  | .063 $\pm$ .085  | -.187 $\pm$ .091*  |
| RW3         | .037     | .046 $\pm$ .083          | -.028 $\pm$ .053               |                                |                   |                  |                  |                  | .121 $\pm$ .105    |
| RW4         | .047     | .052 $\pm$ .084          | -.014 $\pm$ .064               |                                |                   |                  |                  |                  |                    |

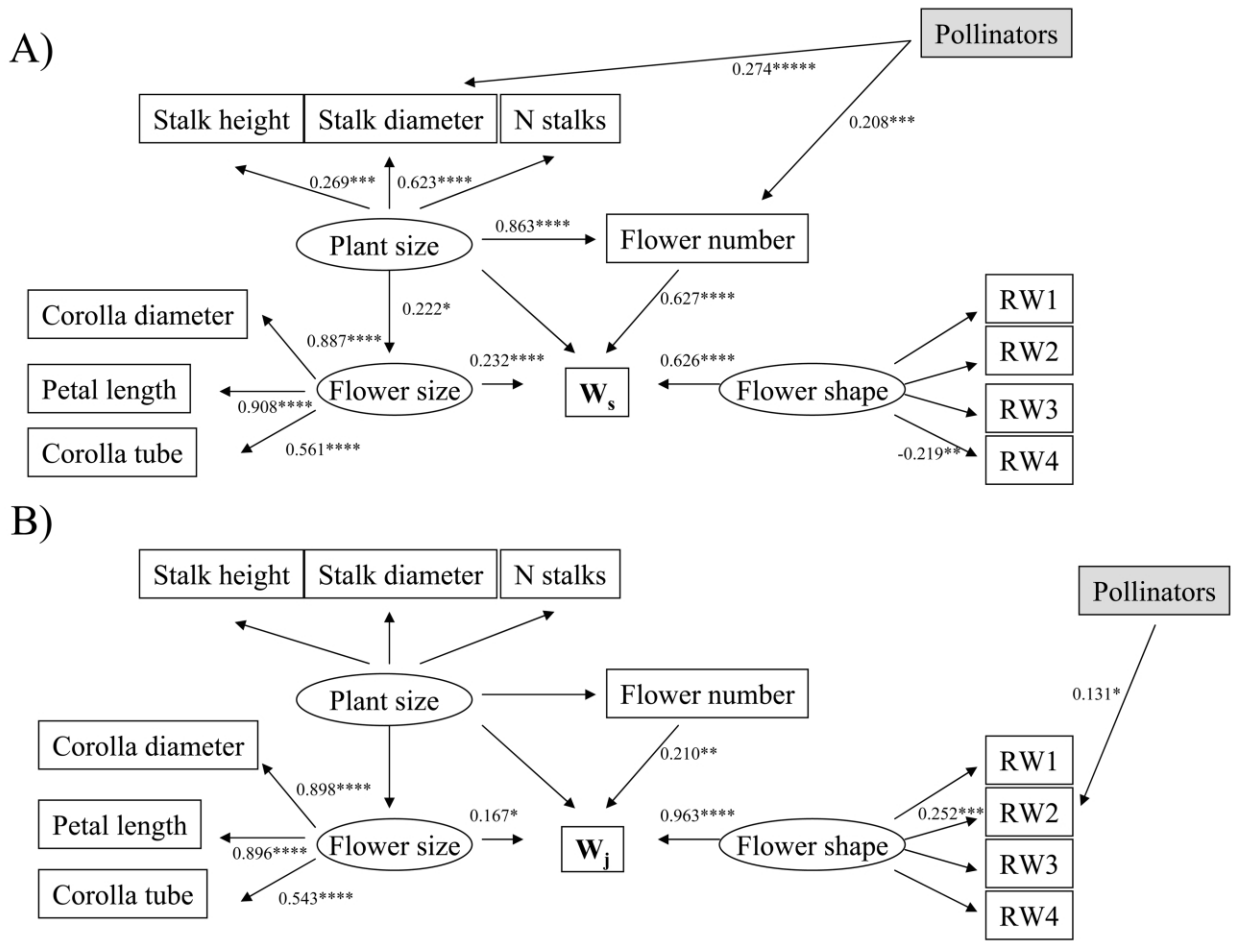
Note: Plant size and flower size are the principal component scores obtained from the principal component analyses from the original variables (see “Material and Methods”). The Lande-Arnold linear and quadratic multivariate models on  $w_s$  were significant ( $F = 21.1$ ,  $df = 7, 140$ ,  $P = .0001$ ,  $R^2 = 0.52$  and  $F = 12.1$ ,  $df = 14, 133$ ,  $P = .0001$ ,  $R^2 = 0.51$ , respectively). The Lande-Arnold linear model on  $w_l$  was also significant ( $F = 21.1$ ,  $df = 7, 140$ ,  $P = .0001$ ,  $R^2 = 0.15$ ). Only plants from 2003 were included in this analysis.  $P$  values are Bonferroni corrected. Only four RWs are shown; see appendix B in the online edition of the *American Naturalist* for the multiple linear regression with the complete set of 60 RWs.

\*  $P < .05$ .

\*\*  $P < .01$ .

\*\*\*  $P < .001$ .

\*\*\*\*  $P < .0001$ .



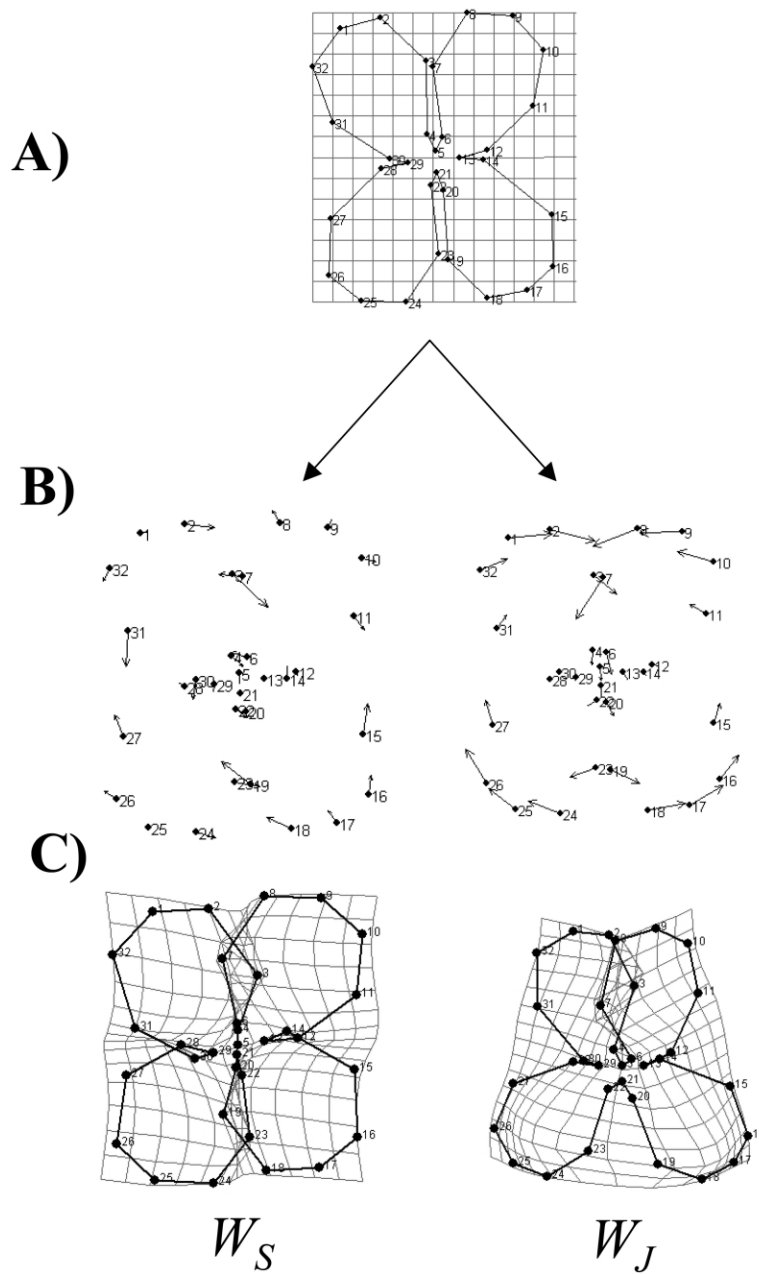
**Figure 4:** Saturated structural models showing the causal relationships between the set of indicator variables (*squares*), the latent variables (*circles*), and the two fitness estimates during 2003 ( $N = 155$  plants). In the path diagrams, we show only the values of the path coefficients from the most parsimonious models (model 6 for  $w_s$ , model 8 for  $w_j$ ); one asterisk =  $P < .05$ , two asterisks =  $P < .01$ , three asterisks =  $P < .001$ , four asterisks =  $P < .0001$ . The saturated structural models in both cases were inadequate ( $\chi^2 = 80.09$ ,  $df = 50$ ,  $P = .004$  for  $w_s$ ;  $\chi^2 = 127.04$ ,  $df = 53$ ,  $P = .0001$  for  $w_j$ ). Pollinators were included in the most parsimonious models, connected to every remaining indicator variable, although we show only the significant path coefficients.

online edition of the *American Naturalist*; table 2). Finally, the most parsimonious SEM models were model 6 for seed production ( $w_s$ ) and model 8 for seedling production ( $w_j$ ; table 1; fig. 4). In both models, there was a significant selection for flower shape (fig. 4). Selection for flower shape was stronger through seedling production than through seed production (figs. 3, 4; table 2). The selected shape variable depended on which fitness estimate was considered. Thus, plants showing the highest  $w_s$  had rounded flowers (fig. 5). This shape variable explained 3.5% of  $w_s$  variation (leverage plot analyses). However, the shape change associated with the highest  $w_j$  involved a spreading out of the landmarks located in the bottom half of the flower and a coming together of the landmarks

located in the upper half. Consequently, plants with the highest  $w_j$  displayed zygomorphic flowers with large abaxial petals (fig. 5). This latter shape variable explained 6% of  $w_j$  variation.

Finally, the selection gradient analysis detected positive correlational selection between flower number and floral zygomorphy (RW2) and negative correlational selection between flower number and corolla roundness (RW4; table 2). More importantly, there was negative correlational selection between shape variables RW2 and RW4 (table 2).

Flower number was more strongly related to seed production than to seedling production (43% and 4% of explained variation in fitness, respectively; table 2; fig. 4). There was also significant quadratic selection for flower



**Figure 5:** Patterns of flower shape transformation predicted by the multivariate selection models on the fitness components considered ( $w_s$  and  $w_j$ ). A, Consensus flower shape; B, visualization of the landmark displacements with respect to the consensus morphology in flowers scoring the highest fitness; C, resulting flower shape of selected individuals for each fitness component.

number through seed production (table 2). Flower size was also more strongly related to seed production than to seedling production (6% and 3% of explained variation; table 2; fig. 4). Flower size was significantly connected to its three indicators, corolla diameter, petal length, and corolla tube length, in the two most parsimonious structural models (fig. 4).

Our study also detected selection for plant size when the fitness estimate was  $w_s$ , although according to both SEM and the selection differential/gradient, it was indirect, mediated by the effect that plant size has on both flower number and size (fig. 4; total path coefficient for  $w_s$ :  $0.592 \pm 0.078$ ,  $t = 2.60$ ,  $P = .009$ ). As observed in figure 4, plant size was significantly connected to only two in-

dicators, stalk height and stalk diameter. The SEM suggests that the selection for plant size, both direct and indirect, fully vanished for  $w_1$  (fig. 4B).

The structural equation modeling suggests that pollinators were significantly related to flower number and stalk diameter through seed production ( $w_s$ ) and to RW2 through seedling production ( $w_j$ ; fig. 4).

## Discussion

### *Intrapopulational Variation in Flower Shape*

It was remarkable that the average floral shape of *Erysimum mediohispanicum* in the population studied was disymmetrical in both study years rather than actinomorphic (radially symmetric), as expected from an ideal crucifer (Sargent 2004; Mitchell-Olds et al. 2005). Disymmetry is thought to be one of the most highly derived types of symmetry, together with zygomorphy (Neal et al. 1998; Endress 1999). It is widely accepted that the evolution of bilateral symmetry from radial symmetry is a crucial step in the evolution and diversification of flowering plants (Coen et al. 1995; Sargent 2004). In fact, it has independently evolved repeatedly as a specialized adaptation to animal pollinators (Donoghue et al. 1998; Rudall and Bateman 2004). While flowers of the Brassicaceae are thought to be radially symmetrical (Hall et al. 2002; Sargent 2004), some *Arabidopsis* genes are expressed asymmetrically in the floral meristem without affecting the radial symmetry of flowers (Cubas et al. 2001), suggesting the existence of incipient molecular potential for floral asymmetry in a radially symmetrical species.

The geometric morphometric analysis also revealed that individuals of the same population vary widely in flower shape. Our morphometric geometric analysis suggests that flower shape in *E. mediohispanicum* is continuously variable, in a way similar to the variation in other flower traits such as nectar production, size, or spur length. Although the genetic and epigenetic mechanisms for flower shape variation are still unknown, this variation pattern allows the use of quantitative genetic tools to ascertain the evolution of flower shape.

### *Natural Selection for Flower Shape*

All of the analytical techniques used in this study suggest the existence of phenotypic selection for flower shape. According to the PLS analysis, flower shape, considered as a multidimensional inclusive trait, affects plant fitness, with the effect increasing rather than decreasing from  $w_s$  to  $w_j$ . This outcome is very interesting because it means that the effect of flower shape can manifest itself and even amplify beyond seed production in this plant. Both the selection

gradient analysis and the structural equation modeling suggest that selection occurs mostly through two shape variables, RW4 and RW2. Plants with rounded flowers (RW4) produced more seeds (higher  $w_s$ ) whereas plants with bilaterally symmetric flowers (RW2) produced more juveniles (higher  $w_j$ ; fig. 5). Furthermore, the significant correlational selection gradients occurring between these two shape variables and with flower number (table 2) also suggest that plants with many zygomorphic and rounded flowers seem to be favored by natural selection.

The selection found for *E. mediohispanicum* flower shape is noteworthy because zygomorphy has been traditionally associated with efficient pollinators rather than beetles, as seen in this article (Neal et al. 1998; Endress 2001). Selection for flower shape can be mediated by pollinator preference. In this study, we found that plants with parallel petals and, most importantly, with overdeveloped abaxial petals, were preferred by pollinating insects. This finding implies that these insects use flower shape to discriminate among plants. Many studies have already reported pollinator ability to differentiate between specific flower shapes (Schemske and Bradshaw 1999; Galen and Cuba 2001). Moreover, it also has been found that pollinators prefer bilaterally symmetric flowers (Møller 1995; Neal et al. 1998; Giurfa et al. 1999; Rodríguez et al. 2004). However, these studies have focused on testing whether effective flower visitors, such as honeybees, bumblebees, or other large bees, prefer symmetrical flowers to asymmetrical ones in species that are either bilaterally or radially symmetric. In this respect, our study is unusual because we found that generalist pollinators such as *Meligethes maurus*, the most abundant flower visitor at the study site (>90% of relative abundance; Gómez 2005), can display a clear preference (but see Møller and Sorci 1998). This preference can be mediated by a correlation between shape and rewards; pollinators prefer those flower shapes associated with higher amounts of reward (J. M. Gómez, F. Perfectti, and J. P. M. Camacho, unpublished data). As an additional mechanism, overdeveloped abaxial petals in the selected flower shapes could act as landing platforms, facilitating pollinator stops.

Selection for flower shape can also be explained by differential efficiency of pollinators visiting differently shaped flowers (Endress 1999; Galen and Cuba 2001). For example, the selected flower shapes could cause an increase in the amount of pollen deposited per pollinator visit, perhaps due to a differential arrangement of the flower's sexual organs (see, e.g., Mayfield et al. 2001; Castellanos et al. 2003; Ivey et al. 2003). If this caused reduced inbreeding and increased seed vigor, then we have a potential explanation for the observed relationship between bilateral symmetry (RW2) and juvenile production ( $w_j$ ). Corolla morphology plays a critical mechanical role in both out-

crossing and self-fertilization in *Mimulus guttatus* (Arathi and Kelly 2004). In the absence of pollinators, the lower portion of the *M. guttatus* corolla facilitates autogamy by retaining pollen released from the anthers. When pollinators are present, the corolla facilitates outcrossing before, during, and after insect visitation (Arathi and Kelly 2004). Unfortunately, we lack sufficient data to infer the actual mechanism provoking the fitness benefit of a given flower shape. Further experimental studies are mandatory to discern the underlying mechanisms causing the observed selection on flower shape and to discover whether this selection regime varies depending on the local pollinator fauna. Pollinator abundance was associated with petal parallelism (RW1) that was not related to seed or juvenile production. This finding suggests that flower shape could be related to male fitness in addition to female fitness. However, no information on male fitness is available for this species.

We also found positive directional selection on flower size and number, consistent with previous studies (Gómez 2003). This selection can be mediated by pollinators, as suggested by the significant relationship between pollinator abundance and flower number in the structural equation models (Gross et al. 1998; Gómez 2000; Totland 2001). However, this selection can be also a consequence of the relationship between number of flowers per plant and potential fecundity, without any effect of pollinator behavior or visitation rate (Galen 1989, 2000; Herrera 1993; Conner and Rush 1996; Gómez 2000; Maad 2000; Thompson 2001). In fact, the selection strength on these two traits, although still significant, decreased when it was quantified through seedling production ( $w_j$ ), where the number-size trade-off is already expressed (seed number-size phenotypic correlation:  $r = -0.244$ ,  $P = .003$ ,  $n = 141$  plants).

Finally, there was also significant selection for plant size, although, according to the selection gradient and the SEM, this selection is indirect, mediated by the effect that plant size has on both flower number and size. The plant traits providing this selection for plant size were stalk diameter and, mostly, stalk height, two traits significantly correlated with plant size. Plant size was also under selection in the same population during 2001 and 2002 (Gómez 2003), indicating that selection for this trait is temporally consistent. However, in 2003, selection for plant size was indirect rather than direct, as observed in previous years, even though the main *E. mediohispanicum* pollinators at the study site also preferred taller plants and wider stalks in that year. In summary, although estimates of flower shape heritability are not available (experiments are ongoing), our results provide strong evidence of significant fitness differences associated with floral shape variation in *E. mediohispanicum*.

#### Use of Geometric Morphometrics to Study Floral Evolution

Geometric morphometrics has proved to be very successful as a tool to analyze the evolution of complex morphological structures (e.g., Klingenberg and Leamy 2001; Adams et al. 2004; Lockwood et al. 2004; Klingenberg and Monteiro 2005 and references therein). Here, we suggest extending the use of this technique to the study of phenotypic variation and selection for flower shape in natural populations. Nevertheless, we need to be cautious in the use of geometric morphometric methods to study natural selection. Geometric morphometrics considers the flower as a single multidimensional character, whereas RWs cannot be considered independent variables with specific biological meanings. According to geometric morphometrics, shape variables are not biological characters (Adams et al. 2004). In most selection models, it is difficult to predict evolutionary rates from these kinds of complex traits. In this way, the structural equation modeling with latent variables may successfully (operationally) address this problem because it allows the inclusion of latent variables, which are nonmeasured multidimensional variables (e.g., floral shape) that are defined by a set of manifest variables. It would be informative to explore the ability of structural equation modeling to cope with multidimensional characters such as flower shape. Notwithstanding the need for caution, this study shows that this approach can help unravel and accurately explore the adaptive significance of this multidimensional floral trait. Further interdisciplinary studies bridging developmental genetics and evolutionary ecology are essential for moving beyond descriptions of flower development to further our knowledge concerning flower shape evolution, and the application of geometric morphometrics can help in this advance.

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