Spatial variation in selection on corolla shape in a generalist plant is promoted by the preference patterns of its local pollinators

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An adaptive role of corolla shape has been often asserted without an empirical demonstration of how natural selection acts on this trait. In generalist plants, in which flowers are visited by diverse pollinator fauna that commonly vary spatially, detecting pollinator-mediated selection on corolla shape is even more difficult. In this study, we explore the mechanisms promoting selection on corolla shape in the generalist crucifer *Erysimum mediohispanicum* Polatschek (Brassicaceae). We found that the main pollinators of *E. mediohispanicum* (large bees, small bees and bee flies) discriminate between different corolla shapes when offered artificial flowers without reward. Importantly, different pollinators prefer different shapes: bees prefer flowers with narrow petals, whereas bee flies prefer flowers with rounded overlapping petals. We also found that flowers with narrow petals (those preferred by bees) produce both more pollen and nectar than those with rounded petals. Finally, different plant populations were visited by different faunas. As a result, we found spatial variation in the selection acting on corolla shape. Selection favoured flowers with narrow petals in the populations where large or small bees are the most abundant pollinator groups. Our study suggests that pollinators, by preferring flowers with high reward, exert strong selection on the *E. mediohispanicum* corolla shape. The geographical variation in the pollinator-mediated selection on *E. mediohispanicum* corolla shape suggests that phenotypic evolution and diversification can occur in this complex floral trait even without specialization.

Keywords: corolla shape evolution; pollinator preference; spatial variation; geometric morphometrics; nectar; pollen

1. INTRODUCTION

Angiosperms display an astonishing variety of flower shapes. Understanding the evolution of this morphological diversity has been a focus of evolutionary biology for many years (Coen *et al.* 1995; Donoghue *et al.* 1998; Endress 1999; Ree & Donoghue 1999; Kalisz *et al.* 2006), producing copious information gathered on macroevolution and developmental genetics of floral shape (Luo *et al.* 1995; Reeves & Olmstead 1998; Cubas *et al.* 1999; Ree & Donoghue 1999; Dilcher 2000; Cronk *et al.* 2005). By contrast, the selective mechanisms driving corolla shape evolution are far less understood (Herrera 1993; Campbell *et al.* 1996; Schemske & Bradshaw 1999; Galen & Cuba 2001). Consequently, despite many years of research, the adaptive significance of corolla shape remains largely elusive (Lloyd & Barrett 1996; Harder & Barrett 2006). According to the current models of floral evolution, corolla shape evolves in response to strong selection exerted by pollinators through an increase in flower attractiveness, which affects pollination quantity, and/or through enhanced pollen-transfer efficiency, which

affects pollination quality (Möller 1995; Möller & Sorci 1998; Neal *et al.* 1998; Endress 1999, 2001).

Generalist plants are visited by diverse pollinators that differ in preference patterns, visitation rate and per-visit effectiveness (Johnson & Steiner 2000; Gómez & Zamora 2006). Although both quantity and quality components are essential, abundant empirical evidence suggests that the importance of a given pollinator species for the fitness of generalist plants depends strongly on its visitation rates rather than its per-visit efficiency (Galen & Newport 1987; Olsen 1997; Gómez & Zamora 1999; Vazquez *et al.* 2005). Pollinator visitation rate is influenced in many plants by reward quantity and quality (Smithson & Macnair 1997; Cunningham *et al.* 1998; Scheiner *et al.* 1999; Waddington 2001; Blarer *et al.* 2002; Plepys *et al.* 2002; Schaefer *et al.* 2004; Biernaskie & Gegear 2007; Internicola *et al.* 2007). Under these circumstances, pollinators may indirectly promote the selection on corolla shape if this trait covaries with reward (Stanton & Preston 1988; Young & Stanton 1990; Ashman & Stanton 1991; Campbell *et al.* 1991; Cohen & Shmida 1993; Stanton & Young 1994; Blarer *et al.* 2002; Armbruster *et al.* 2005; Fenster *et al.* 2006).

For most species with generalized pollination, the pollinator assemblage often varies geographically as a consequence of changes in abundance and identity of the flower visitors (Moeller 2005, 2006 and references therein). When pollinators differ in morphology, foraging

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Table 1. Location and characteristics of the *E. mediohispanicum* populations studied in the Sierra Nevada (southeast Spain).

population	population characteristics				pollinator sampling effort			
	latitude north	longitude west	altitude	habitat	plants	minutes	flowers	pollinators
Em02	37°7.33′	3°25.86′	2099	shrubland	90	1720	2486	270
Em21	37°8.07′	3°25.71′	1723	forest	90	1955	1826	243
Em25	37°7.27′	3°26.05′	2064	shrubland	90	2195	1710	118

behaviour and flower trait preferences, spatial variation in the identity of the frequent pollinators may result in concomitant spatial variation in the selection affecting floral traits (Gómez & Zamora 2000; Herrera *et al.* 2006).

In this study we explore the mechanisms driving corolla shape evolution as a response to pollinator selection in *Erysimum mediohispanicum* Polatschek (Brassicaceae). This species is a good system to study pollinator-mediated corolla shape evolution for several reasons. First, corolla shape in *E. mediohispanicum* is heritable (J. M. Gómez, M. Abdelaziz, J. Muñoz & F. Perfectti 2005–2007, unpublished data). Second, phenotypic selection on corolla shape has already been established (Gómez *et al.* 2006). Third, the species exhibits extensive quantitative variation in corolla shape in the wild (see fig. 1 in Gómez *et al.* 2006). Fourth, although self-compatible, *E. mediohispanicum* needs pollen vectors for full seed set (Gómez 2005). Fifth, *E. mediohispanicum* is highly generalist, being visited by more than 130 species of insects (Gómez *et al.* 2008). Finally, pollinator fauna, and potential selective pressures, vary significantly among localities (Gómez *et al.* 2006, 2008).

Flower shape has usually been studied as a qualitative trait (e.g. radial versus bilateral symmetry) or as a variable composed of several linear measurements (e.g. Neal *et al.* 1998; Endress 1999, 2001; Ree & Donoghue 1999; Galen & Cuba 2001). This approach generates an over-simplified description of this complex trait. To estimate corolla shape variation accurately in *E. mediohispanicum*, we use geometric morphometrics that treat corolla shape as a single multidimensional trait (Zelditch *et al.* 2004). This approach allows the application of standard quantitative genetics and evolutionary ecology tools in the study of corolla shape microevolution. Our specific objectives are to (i) estimate phenotypic selection for corolla shape, (ii) test experimentally whether different *E. mediohispanicum* pollinators favour different corolla shapes, (iii) ascertain whether corolla shape covaries with floral reward, (iv) explore whether variation in local pollinator fauna leads to spatial variation in selection on corolla shape and (v) assess a link between pollinator preference patterns and phenotypic selection on floral shape.

2. MATERIAL AND METHODS

(a) *Study system*

Erysimum mediohispanicum is a biennial to perennial monocarpic herb, found in forests and subalpine scrublands of southeast Spain from 1000 to 2300 m elevation. Plants usually grow for 2–3 years as vegetative rosettes, and then die after producing one to eight reproductive stalks that can display a few to several hundred hermaphroditic, slightly protandrous bright yellow flowers (Gómez 2003). The most

important pollinator groups of *E. mediohispanicum* are large bees, small bees, bee flies and beetles (Gómez *et al.* 2008), including species that collect pollen, nectar and both (Gómez 2003, 2005).

The study was carried out during 2005 and 2006 in three *E. mediohispanicum* populations located in the Sierra Nevada high mountains (Granada province, southeast Spain; table 1).

(b) *Pollinator assemblage*

Pollinator assemblage was determined in each of the three populations using a standardized methodology (Gómez *et al.* 2008). Throughout peak bloom (10–15 days per population), we conducted five to seven pollinator samples per population, during which the number of open flowers on each labelled plant and the number of pollinators that landed on their flowers during 5 min were noted. Thus, each survey lasted 450 min and we conducted more than 3000 min of observation per population. The number of surveys per population was fitted to the local abundance of pollinators by means of accumulation curves (Gómez *et al.* 2008).

We grouped the insects visiting *E. mediohispanicum* flowers into functional groups. We define 'functional group' as flower visitors that interact with flowers similarly (Fenster *et al.* 2004). Basically, we used criteria of similarity in size, proboscis length, foraging behaviour and feeding habits. Thus, taxonomically related species were sometimes placed in different functional groups. We established eight functional groups: (i) large bees, mostly pollen- and nectar-collecting females measuring 10 mm in body length or larger; (ii) small bees, mostly pollen- and nectar-collecting females smaller than 10 mm; (iii) wasps, including aculeate wasps, large parasitic wasps and cleptoparasitic bees collecting only nectar; (iv) bee flies, long-tongued nectar-collecting Bombyliidae; (v) hoverflies, nectar- and pollen-collecting Syrphidae and short-tongued Bombyliidae; (vi) beetles, including species collecting nectar and/or pollen; (vii) butterflies, mostly Rhopalocera, all nectar collectors; and (viii) others, including nectar-collecting ants, small flies, small parasitic wasps, bugs and other occasional flower visitors.

(c) *Estimation of phenotypic selection on corolla shape*

In 2005, we analysed selection occurring on *E. mediohispanicum* corolla shape in each population. We marked 90 plants per population at the onset of the flowering period, using aluminium tags attached to the base of the flowering stalks. Corolla shape and lifetime female fitness were estimated for each labelled plant.

Corolla shape was quantified by means of geometric morphometrics (Gómez *et al.* 2006). Because the four petals of *E. mediohispanicum* flowers are presented in a single plane (Gómez *et al.* 2006), we used a two-dimensional analysis to describe their shape (Zelditch *et al.* 2004). We took a digital

photograph of one flower per labelled plant using a standardized procedure (front view and planar position). Flowers were photographed at anthesis to avoid ontogenetic effects on corolla shape. In addition, the flowers were located in the same position along the flowering stalk (central) to avoid any position effect on floral traits. For each flower, we defined 32 coplanar landmarks along the outline of the petals and the aperture of the corolla tube. The number of landmarks was chosen to provide comprehensive coverage of corolla shape (Zelditch et al. 2004; see Gómez et al. 2006 for further details). The two-dimensional coordinates of these landmarks were determined for each flower using the software TPSDIG v. 1.4 (http://life.bio.sunysb.edu/morph/morphmet.html). Then, the generalized orthogonal least-squares Procrustes average configuration of landmarks was computed using the Generalized Procrustes Analysis (GPA) superimposition method, using the software TPSRELW v. 1.11 (http://life.bio.sunysb.edu/morph/morphmet.html). Following GPA, the relative warps (RWs, principal components of the covariance matrix of the partial warp scores) were computed (Walker 2000; Adams et al. 2004). Each RW is characterized by a 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 used as a data matrix to perform standard statistical analyses (Zelditch et al. 2004).

Lifetime female fitness was estimated as the number of seeds produced per plant (Gómez et al. 2006). At the end of the reproductive season, we counted the number of fruits per labelled plant (90 per population, 270 plants in total) and collected between 50 and 75% of the fruits to determine the number of seeds produced per fruit in the laboratory.

Following Klingenberg & Leamy (2001) and Klingenberg & Monteiro (2005), selection for shape can be calculated using (multivariate) selection differentials (s) and gradients (β). The former is a descriptor of the total effect of selection on shape without distinguishing between direct and indirect selection. The latter provides information on how selection directly acts on each shape variable accounting statistically for the other measured variables (Klingenberg & Monteiro 2005). The selection differential was quantified as the vector of covariances between the fitness and the shape variables (the complete set of RWs in this study). We derived the vector of coefficients from a two-block partial least-squares (PLS) analysis between shape and fitness (Rohlf & Corti 2000). By means of cross-validation, we found the number of latent vectors displayed by the model with the lowest r.m.s.e. (root mean squared error) (Abdi 2007). We then determined the covariance between fitness and shape predicted by this parsimonious model. Selection gradients β for each population were computed from the standardized partial regression coefficients of a linear regression of relative fitness on all 60 RWs generated by the geometric morphometrics analyses (Lande & Arnold 1983; Klingenberg & Leamy 2001). Because we considered corolla shape as a single multidimensional trait, we included the complete set of RWs in these analyses (Adams et al. 2004). However, to improve the interpretation of the outcomes, we reduced the dimensionality of the covariance matrix (Klingenberg & Leamy 2001). For this reason, we evaluated only the first four RWs, a subset that explained most (more than 70%) of the variability in *E. mediohispanicum* corolla shape and that already has been shown to be subject to important selective effects (Gómez et al. 2006). In this model, we also included flower size and number as well as plant size to control for

indirect selection on flower shape (Gómez et al. 2006). Flower size was estimated by means of two variables, the corolla diameter (the distance in mm between the edges of two opposite petals, using a digital calipers with ± 0.1 mm error) and the corolla tube length (the distance between the corolla tube aperture and the base of the sepals). Plant size was estimated by means of three variables, the number of stalks growing from each rosette, the height of the tallest reproductive stalk (measured to the nearest 0.5 cm, the distance from the ground to the top of the highest opened flower) and the basal diameter of the tallest stalk (mm).

Selection gradients should not be visualized as shape changes (Adams & Rosenberg 1998; Klingenberg & Monteiro 2005). To visualize the expected shapes of individuals with different fitness, we followed Klingenberg & Monteiro (2005) and computed a predicted fitness score for each individual by multiplying its shape variables by the selection gradient. The regression of shape on this fitness score yields a vector of regression coefficients that can be visualized directly as changes in landmark positions (Rohlf et al. 1996).

(d) Experimental determination of pollinator preference for corolla shape

In 2005, we experimentally assessed flower shape preferences of *E. mediohispanicum*'s pollinators in the three populations by means of choice experiments. We built artificial flowers using yellow construction paper to match (from a human perspective) the colour of *E. mediohispanicum* flowers. We built artificial flowers of nine different shapes. Eight of these shapes corresponded to the two extremes (positive and negative) of the four RWs that define *E. mediohispanicum*'s corolla shape according to previous geometric morphometrics analyses (Gómez et al. 2006; see also figure 1). The ninth flower shape corresponded to the consensus shape obtained in the same analyses (Gómez et al. 2006). Artificial flowers were of the same size as natural flowers and were individually arranged in 20 cm tall wire stalks. The different shape models did not differ significantly in size, as measured by corolla diameter ($F_{15,88} = 1.21$, $p = 0.278$, one-way ANOVA). In addition, to avoid any side effect of reward on pollinator behaviour, we did not add any reward to the artificial flowers.

In each population, we set up experimental arenas (80×120 cm) with 12 flowers of each of the eight extreme shapes and 48 flowers of the consensus shape. Thus, each experimental arena had 144 artificial flowers. The flowers were randomly distributed within the arenas and the experiment was conducted during peak flowering of *E. mediohispanicum*. During the experiments, two to three observers (located 1 m away from the arenas) noted all insects approaching the artificial flowers. We considered an approach to be successful if the insect landed on a flower or contacted it. Each pollinator was visually identified based on our 2-year experience surveying *E. mediohispanicum* pollinators in the area. Our analyses included only those pollinator species seen visiting natural *E. mediohispanicum* flowers in the three populations.

Differences among pollinator functional groups in floral choice were explored by a nominal logistic model using a likelihood ratio test. Departures from random visitation (no pollinator choice as null hypothesis) were tested for each pollinator functional group separately by chi-squared tests for goodness of fit, with expected frequencies based on the frequency of each flower morph in the experimental arenas.
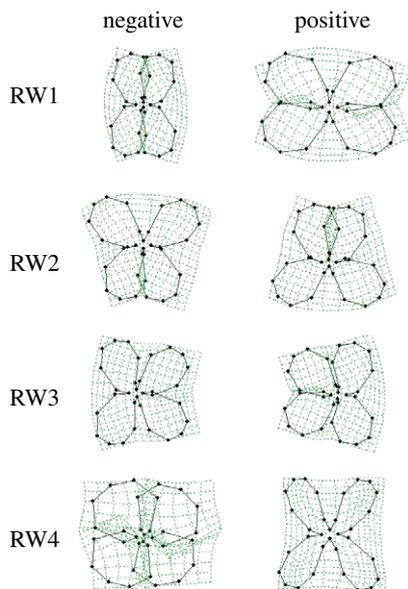
negative   positive

Figure 1. Corolla shape variation in *Erysimum mediohispani-cum*. Summary of the geometric morphometric analysis (2005 and 2006 data, $N = 420$ plants) showing the variation in corolla morphology produced by RWs explaining more than 5% of the overall variation in shape (RW1: 35.2%, RW2: 19.2%, RW3: 9.7%, RW4: 6.2%). The corolla shapes corresponding to the two extreme values (negative and positive) of the distribution along each shape component (RW1–RW4) are shown.

(e) *Determination of the relationship between corolla shape and flower reward*

Pollen and nectar were quantified during 2006 for 50 plants per population. We measured pollen production, as the total volume of pollen produced per flower. One flower bud per plant was taken and preserved in 70% ethanol. Three of the six anthers of each flower (two long and one short, as *E. mediohispanicum* has a typical Brassicaceae tetradynamous androecium) were placed in a vial with ethanol and sonicated for 3 min to dislodge pollen grains (Kearns & Inouye 1993). A known volume of saline solution was then added to the vial and the number of pollen grains per volume was counted in a Multisizer particle counter. Pollen grain diameter was measured on pollen slides at $400 \times$. From these two measures we obtained total pollen volume per flower. We measured nectar production as the volume produced during 24 hours by newly opened flowers. On each labelled plant we covered three to five flower buds with cellophane bags to prevent pollinator visitation. After 24 hours, we measured nectar volume in three flowers per plant using calibrated micropip-ettes (Kearns & Inouye 1993). Nectar volume was averaged per plant to avoid pseudoreplication. We also quantified corolla shape in these plants using the above methodology.

The relationship between flower shape and reward was analysed as the vector of covariances between reward production and flower shape variables (the complete set of RWs generated by the morphometric analyses). To accom-plish this, we derived the latent vector of coefficients from a two-block PLS analysis between the complete set of 60 RWs and reward (Rohlf & Corti 2000). Each reward trait (pollen and nectar) was used separately in the reward block. By means of cross-validation, we found the number of latent vectors displayed by the model with the lowest r.m.s.e. (Abdi 2007). Then, we determined the covariance between reward and shape predicted by this parsimonious

model. Prior to performing the PLS, we checked whether the corolla shape–reward relation was similar among populations by performing a general linear model (GLM) including corolla shape, population and the interaction between shape and population.

3. RESULTS
(a) *Differences between populations in pollinator fauna*

The assemblage of insects visiting *E. mediohispanicum* flowers differed among the three populations ($\chi^2_{16} = 108.63$, $p < 0.0001$, Monte Carlo contingency test with 100 000 iterations). The most abundant flower visitors in Em02 were beetles (42.2% of the visits), followed by large bees (18.1%), small bees (15.2%), hoverflies (8.8%) and bee flies (7.0%). In Em21, the most abundant floral visitors were small bees (44.4%), followed by bee flies (19.3%), beetles (16.1%) and large bees (5.6%). Finally, in Em25, the most abundant floral visitors were large bees (37.1%), followed by small bees (21.2%), beetles (28.8%) and bee flies (9.3%). The remaining functional groups were very scarce in all three studied populations (less than 5% of all visits).

(b) *Phenotypic selection for flower shape*

The geometric morphometric analysis generated 60 shape components (RWs, hereafter) of which the first four (RW1–RW4) explained more than 70% of the variation in corolla shape (figure 1, see appendix 1 in the electronic supplementary material for the complete set of RWs). In both years, RW1–RW4 were associated with the same pattern of corolla shape variation (figure 1). In addition, the outcome of the geometric morphometrics did not change when each population was analysed separately (data not shown).

The shape components did not correlate strongly with the phenotypic traits describing plant and flower size. RW4 was the only shape component correlating with any trait: it exhibited contrasting correlations with corolla diameter in two populations (Em02: $r = 0.371$, $n = 50$, $p = 0.0001$; Em21: $r = -0.317$, $n = 50$, $p = 0.002$, product–moment correlation) and varied positively with corolla tube length in one population (Em02: $r = 0.294$, $n = 50$, $p = 0.005$, significant after sequential Bonferroni correction; see appendix 2 in the electronic supplementary material for the complete correlation matrix). In addition, corolla shape did not vary significantly with the number of ovules produced per flower ($p > 0.43$ for all first four RWs, linear regression).

Corolla shape experienced significant total selection in the three populations (selection differential: $s = 0.77$, $n = 90$, $p < 0.0001$ (Em02); $s = 0.78$, $n = 90$, $p < 0.0001$ (Em21); $s = 0.76$, $n = 90$, $p < 0.0001$ (Em25); PLS analysis). However, different corolla shapes were selected in different populations (table 2). Thus, in populations Em21 and Em25, flowers with the highest female fitness had positive RW4 (Em21: $\beta \pm 1$ s.e. $= 0.15 \pm 0.08$, $n = 90$, $t = 2.06$, $p = 0.048$; Em25: $\beta \pm 1$ s.e. $= 0.28 \pm 0.11$, $n = 90$, $t = 2.64$, $p = 0.01$). In Em21, there was also a significant selection for flowers with positive RW1 ($\beta \pm 1$ s.e. $= 0.16 \pm 0.08$, $n = 90$, $t = 2.06$, $p = 0.048$). Finally, although we found selection on the overall shape in population Em02, it did not occur through any of the first four RWs (table 2).

Table 2. Standardized multivariate selection analysis on corolla shape for three *E. mediohispanicum* populations ($n = 90$ plants per population). Results of only the first four RWs (RW1–RW4) are shown, although the analysis included all RWs and traits describing flower size, plant size and flower number. The corolla shapes associated with the highest female fitness in each population are also illustrated. The selected flower shapes were visualized as changes in landmark positions after computing predicted fitness scores for each individual by multiplying its shape variables by the selection gradient (Klingenberg & Monteiro 2005).

shape component	$\beta \pm$ s.e.	t-ratio	p-value	selected flower shape
Em02				
Std RW1	-0.132 ± 0.185	2.32	0.479	
Std RW2	0.059 ± 0.119	0.50	0.622	
Std RW3	-0.058 ± 0.113	-0.51	0.609	
Std RW4	0.029 ± 0.109	0.26	0.793	
Em21				
Std RW1	0.159 ± 0.077	-2.06	0.048	
Std RW2	-0.059 ± 0.077	-0.77	0.447	
Std RW3	-0.051 ± 0.078	-0.65	0.515	
Std RW4	0.158 ± 0.077	2.06	0.048	
Em25				
Std RW1	-0.065 ± 0.108	-0.60	0.555	
Std RW2	-0.111 ± 0.101	-1.09	0.283	
Std RW3	0.073 ± 0.095	0.78	0.444	
Std RW4	0.285 ± 0.108	-2.64	0.013	

With respect to other phenotypic traits, we found significant selection only for flower number in one population, Em21 ($\beta \pm 1$ s.e. $= 0.64 \pm 0.17$, $t = 3.80$, $p = 0.0003$; see appendix 3 in the electronic supplementary material for the overall selection models).

(c) Experimental determination of pollinator preference for corolla shape

A total of 1300 pollinators belonging to 66 species visited the artificial flowers. These insects belonged to the main pollinator functional groups of *E. mediohispanicum*: large bees (144 visits); small bees (156 visits); bee flies (405 visits); hoverflies (427 visits); and beetles (168 visits). We frequently observed pollinator flights between natural and artificial flowers.

The major pollinators of *E. mediohispanicum* discriminated between flowers varying exclusively in corolla shape (figure 2). The results also show that different pollinator functional groups exhibited different preference patterns (likelihood ratio test $= 87.08$, d.f. $= 48$, $p < 0.0001$, nominal logistic model). Beetles did not show any clear preference for any flower type ($\chi^2_8 = 5.80$, $n = 168$, n.s., goodness of fit), suggesting that these pollinators visited the artificial flowers at random. The remaining functional groups departed significantly from random choice (figure 2a). Large bees visited mostly flowers with positive RW4. Small bees preferentially visited flowers with positive RW4, but also flowers with positive RW1. Bee flies visited mostly flowers with positive RW1 and then flowers with negative RW4. Finally, hoverflies favoured flowers with positive RW1 and positive RW4.

(d) Relationship between corolla shape and reward

Corolla shape varied significantly with both pollen and nectar production (figure 3). The latent vector for nectar production was significant (r.m.s.e. $= 1.142$, $n = 150$, $p < 0.0001$), explaining 30.1% of the variance. This vector was correlated with eight RWs, including positive
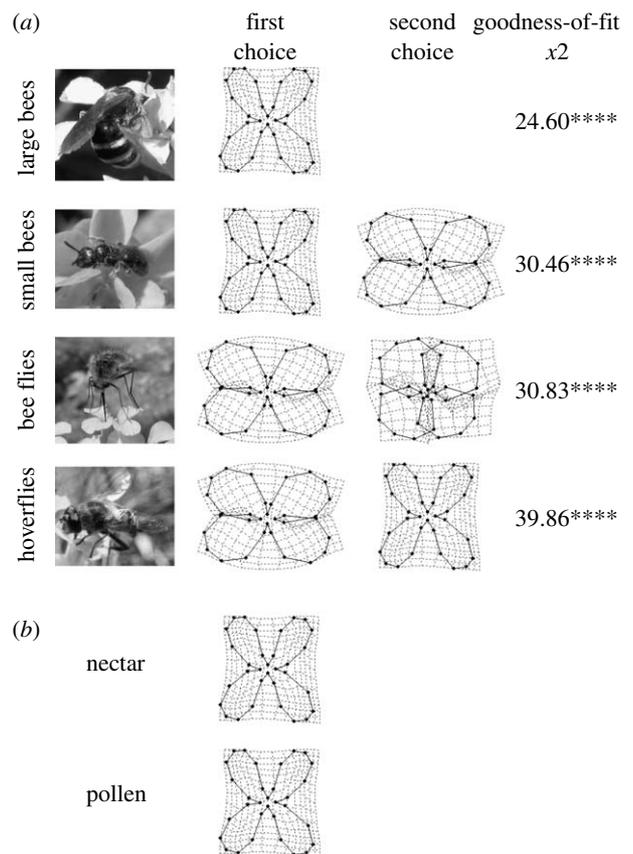

Figure 2. Corolla shapes associated with pollinator preference and floral rewards in *Erysimum mediohispanicum*. (a) Outcome of the pollinator choice experiment showing the flower shapes preferred by each *E. mediohispanicum* pollinator functional group. Goodness-of-fit tests assessed the departure from random choice based on the proportion of each corolla shape in the experimental arenas (see §2). ****$p < 0.0001$. Beetles showed no significant preferences. (b) Shape of the flowers producing the most nectar and pollen (PLS analysis; see the electronic supplementary material for details).
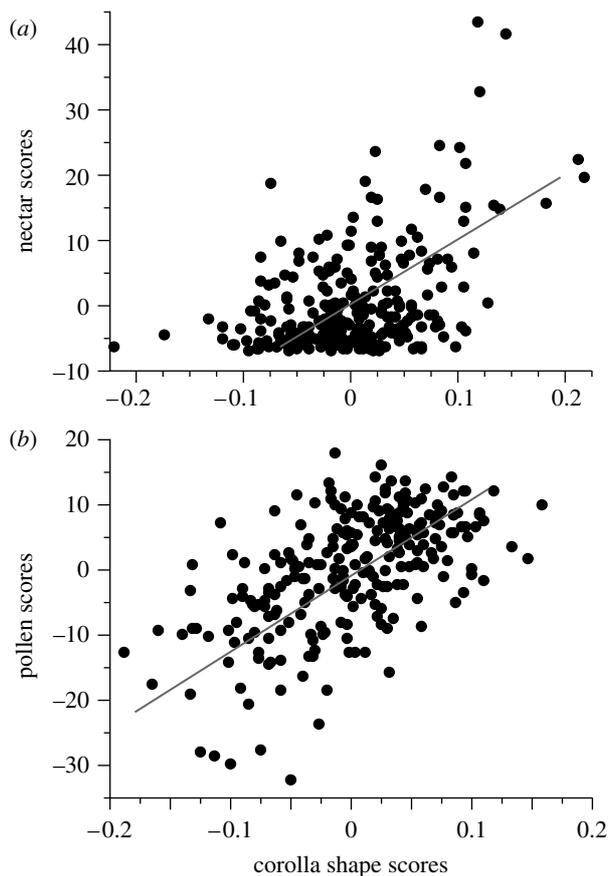
Figure 3. Relationship between flower reward and corolla shape. Outcomes of the PLS analysis showing the correlation between the scores of flower reward and corolla shape generated by the only significant latent vector. (*a*) Nectar scores ($R^2 = 0.30$, $p < 0.0001$), (*b*) pollen scores ($R^2 = 0.52$, $p < 0.0001$).

correlation with RW4 ($r = 0.194$, $p < 0.0001$) and RW2 ($r = 0.412$, $p < 0.0001$; figure 2*b*; see appendix 1 in the electronic supplementary material for the complete set of correlation coefficients). The latent vector for pollen production was also significant (r.m.s.e. $= 0.953$, $n = 150$, $p < 0.0001$), explaining 51.7% of the variance in pollen. Pollen production correlated with five RWs, again positively with RW4 ($r = 0.354$, $p < 0.0001$, figure 2*b*; see appendix 1 in the electronic supplementary material for the complete set of correlation coefficients). The GLM indicated similar relationships between corolla shape and reward among populations (no significant interaction).

4. DISCUSSION

Pollinators discriminated between different *E. mediohispanicum* corolla shapes and exhibited contrasting preference patterns. Whereas beetles visited indiscriminately, large and small bees preferred flowers with narrow petals and bee flies selected flowers with wide overlapping petals. These findings suggest that the shape of *E. mediohispanicum* corollas acts as a visual cue to flower visitors, as has been demonstrated for other species (Dafni & Kevan 1997; Neal *et al.* 1998; Giurfa & Lehrer 2001; Yoshioka *et al.* 2007). Several previous studies have established the ability of pollinators to discriminate among different models using artificial shapes (Gould 1985; Dafni & Kevan 1997; Johnson & Dafni 1998; Möller & Sorci 1998; Giurfa & Lehrer 2001), but few studies have been

conducted under natural conditions and using models mimicking natural flowers (Yoshioka *et al.* 2007).

Our experiment could confound corolla shape and size because, as illustrated in figure 1, flowers with different values of RWs have different areas, even though they have equal corolla diameter. In particular, flowers with positive RW1 or negative RW4 had larger areas than flowers with negative RW1 or positive RW4. However, differences in areas of our artificial flowers probably influenced pollinator behaviour less than shape differences because, as observed in figure 2, the corolla shape preferred by most pollinators in the choice experiments had a small area (positive RW4), whereas studies of the effects of flower size on pollinator preference, including some previous studies with *E. mediohispanicum*, have shown that bees choose large flowers (Gómez 2003; Gómez *et al.* 2006 and references therein). Because flower shape varied largely independently of flower size and ovule production, influences of shape on pollinator behaviour and seed production probably reflect direct effects, rather than correlated responses to flower size.

It is important to uncover the factors underlying the observed pollinator preference patterns, since they will provide the mechanisms promoting corolla shape evolution. Pollinator–flower shape associations often result from a functional link between floral traits and reward production (Ashman & Stanton 1991; Campbell *et al.* 1991; Cohen & Shmida 1993; Möller 1995; Blarer *et al.* 2002; Armbruster *et al.* 2005; Fenster *et al.* 2006). We found a link between corolla shape and both pollen and nectar production in *E. mediohispanicum*, and the most rewarding flowers matched the corolla shape of artificial flowers preferentially visited by large and small bees. These results suggest that bees can use corolla shape as a signal for reward production in *E. mediohispanicum*. Since we used non-rewarding artificial flowers visited by wild experienced pollinators, these presumably learned the association between shape and reward on natural flowers (Smithson & Macnair 1997; Neal *et al.* 1998; Boisvert *et al.* 2007; Makino & Sakai 2007).

The preference of bee flies for rounded flowers cannot be explained by a functional link between corolla shape and reward. We propose that bee flies prefer rounded flowers because the corolla serves as a landing platform (Neal *et al.* 1998). Bee flies always fly between consecutively visited flowers, even within the same plant. Sometimes bee flies hover while collecting nectar, but other times they land on the flowers and collect nectar while standing on their second and third pairs of legs (J. M. Gómez, J. Bosch & F. Perfectti 2005–2007, unpublished data). Because hovering is energetically costly (Heinrich 1993), bee flies may choose to feed while standing on flowers that offer an appropriate landing platform (flowers with large rounded petals). Interestingly, large bee-like hoverflies visiting *E. mediohispanicum* behave similarly to bees, frequently walking from flower to flower, while smaller wasp-like hoverflies behave like bee flies.

Corolla shape experienced phenotypic selection in all studied populations, with two specific shapes, those associated with RW1 and RW4, selected in two populations. Previous studies of the same species in other locations also found significant selection on flower shape (Gómez *et al.* 2006). Our findings demonstrate that selection on corolla shape matched the preference

patterns displayed by the most abundant local pollinators. Plants with the highest female fitness in population Em21 had flowers with positive RW4 and RW1, coinciding with the preference patterns of small bees, the most abundant pollinator group in this population, and of bee flies, the second most abundant groups. In population Em25, selection favoured flowers with positive RW4, in agreement with the preference pattern of large bees, the most abundant pollinator group in this population. Finally, no corolla shape was selected in population Em02, where the most abundant pollinators were beetles, the only functional group not showing preference for any corolla shape. In addition, the observed match between pollinator preference, corolla shape and reward production suggests that the evolution of corolla shape in this species with a generalized pollination system is mediated by the link between corolla shape and rewards. Corolla shape is heritable in *E. mediohispanicum*, due mostly to the significant heritability of two of the main shape components, RW1 and RW4 (J. M. Gómez, M. Abdelaziz, J. Muñoz & F. Perfectti 2005–2007, unpublished data), that are also associated with pollinator preferences and high fitness. Taken together, our results strongly indicate that corolla shape of *E. mediohispanicum* is evolving through pollinator-mediated natural selection.

The observed between-pollinator differences in preference patterns for corolla shape may have contrasting consequences for corolla shape evolution, depending on the pattern of spatial variation in pollinators. When plants in a single population are visited by many disparate pollinator species belonging to different functional groups, as occurs in some *E. mediohispanicum* populations (Gómez *et al.* 2008), between-pollinator differences in preference may lead to pollinator-mediated trade-offs. For example, any modification in corolla shape resulting in increased attraction to bees may be accompanied by a decrease in bee fly attraction, and vice versa (see also Wilson & Thomson 1996; Castellanos *et al.* 2004; Aigner 2006; Muchhala 2007). As a consequence, within-population variation in pollinator composition may cause disruptive selection maintaining intrapopulational phenotypic variability in corolla shape (Dilley *et al.* 2000; Gómez & Zamora 2006). By contrast, when the pollinator assemblage varies mostly among, rather than within populations, spatial variation in pollinators can promote variation in local selection across populations (Gómez & Zamora 2006; Herrera *et al.* 2006; Sargent & Otto 2006). Among-population differences in pollinator-mediated selection have been shown for some specialized systems, where an overall turnover of the main pollinator groups occurs at a geographical scale (Herrera *et al.* 2006). Our findings demonstrate that these differences also occur in generalized plants as a result of spatial changes in the relative abundance of different pollinators. In these kind of systems, complex floral traits, such as corolla shape, can also evolve and diversify without pollination specialization.

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