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Selection of trait combinations through bee and fly visitation to flowers of *Polemonium foliosissimum*

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Abstract

Pollinators are known to exert natural selection on floral traits, but the extent to which combinations of floral traits are subject to correlational selection (nonadditive effects of two traits on fitness) is not well understood. Over two years, we used phenotypic manipulations of plant traits to test for effects of flower colour, flower shape and their interaction on rates of pollinator visitation to *Polemonium foliosissimum*. We also tested for correlational selection based on weighting visitation by the amount of conspecific pollen delivered per visit by each category of insect visitor. Although bumblebees were the presumed pollinators, solitary bees and flies contributed substantially (42%) to pollination. In manipulations of one trait at a time, insects visited flowers presenting the natural colour and shape over flowers manipulated to present artificial mutants with either paler colour or a more open or more tubular flower. When both colour and shape were manipulated in combination, selection on both traits arose, with bumblebees responding mainly to colour and flies responding mainly to shape. Despite selection on both floral traits, in a year with many bumblebees, we saw no evidence for correlational selection of these traits. In a year when flies predominated, fly visitation showed a pattern of correlational selection, but not favouring the natural phenotype, and correlational selection was still not detected for expected pollen receipt. These results show that flower colour and shape are subject to pollinator-mediated selection and that correlational selection can be generated based on pollinator visitation alone, but provide no evidence for correlational selection specifically for the current phenotype.

Introduction

Natural selection of phenotypic traits can take multiple forms. A single trait can experience directional selection, in which fitness increases with one extreme of the trait, stabilizing selection in which an intermediate value is favoured, or disruptive selection in which both extremes of large and small values are favoured. When multiple traits are considered, there is the possibility of correlational selection in which there are synergistic (or antagonistic) effects of two traits on fitness that are not predicted by adding together selection on single traits (Phillips & Arnold, 1989). Correlational selection is thought to strengthen (or weaken) genetic correlations between traits and cause the functional integration necessary for operation of a complex organ (Jones *et al.*, 2003; Roff & Fairbairn, 2012). The process is therefore thought to be important in nature, and yet it is infrequently studied in comparison with directional selection (Kingsolver *et al.*, 2001). Flowers provide an excellent model for studying correlational selection; functional integration of floral traits has been hypothesized to result in part from correlational selection (Armbruster & Schwaegerle, 1996; Pérez *et al.*, 2007), and floral traits are particularly amenable to study with experimental manipulations that provide strong evidence for selection (Campbell, 2009). Correlational

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selection on floral traits has received, however, relatively little study, with the evidence for or against it still based largely on phenotypic selection analysis of unmanipulated plants (O'Connell & Johnston, 1998; Maad, 2000; Benitez-Vieyra *et al.*, 2006; Gómez *et al.*, 2006; Reynolds *et al.*, 2010). In contrast with these observational studies, experimental alterations of traits in factorial combination would allow correlational and other forms of selection to be distinguished from spurious effects on fitness caused by unmeasured characters or features of the environment (Rausher, 1992; Campbell, 2009).

Most experimental studies of natural selection of floral characters have focused on a single trait or type of trait (e.g. colour only or scent only or shape only; reviews in Raguso, 2008; Campbell, 2009), with relatively few studies manipulating multiple traits (Wilson, 1995; Dudash *et al.*, 2011). Only a handful of studies used experimental manipulations of flower traits to test for correlational selection mediated by pollinators. Three of these studies found no evidence for correlational selection, but also found no evidence that both traits affected fitness at all so that the traits tested turned out not to be ones where correlational selection would be expected. Manipulations of the upper and lower corolla lips of *Lavandula latifolia* had no detectable effects on male or female pollination success (Herrera, 2001), only spur length and not perianth size influenced pollen removal and fruit set in a *Platanthera* orchid (Boberg & Ågren, 2009), and reduction in neither the lateral sepals nor the labellar lobe appeared to influence pollinia removal or fruit production in another orchid (Cuartas-Dominguez & Medel, 2010). In contrast with these three cases, manipulation of number of flowers and spur length had nonadditive effects on fruit mass, indicating correlational selection, in the orchid *Dactylorhiza lapponica* (Sletvold & Ågren, 2011). The authors suggested that correlational selection of floral traits is more likely to arise when one trait influences the rate of pollinator visitation and the other trait influences pollinator effectiveness per visit, because plant reproductive success is a product of those two components of pollination, leading to nonadditive (or correlational) selection. This process in which incorporating two successive episodes of directional selection can itself lead to correlational selection was demonstrated theoretically in McGlothlin (2010). None of the empirical cases, however, examined the effects of the trait manipulations on visitation rate, on pollinator effectiveness per visit or both, making it hard to establish the potential mechanisms involved.

In the current study, we manipulated two floral traits, petal colour and corolla shape, both singly and in factorial combination and examined the effects on rate of pollinator visitation. Number of pollinator visits is a fitness component corresponding to a single selective episode, and we tested whether that single component could itself show correlational selection. Visitation rate could show nonadditive effects of traits through a variety of mechanisms. First, two floral traits might be required in combination for an insect to use a food source if one trait allows recognition of flower species identity and the other trait reveals location of the reward (Leonard *et al.*, 2011) or otherwise allows greater reward extraction. In one example, hawkmoths required both olfactory and visual cues to feed from flowers (Raguso & Willis, 2005). Although selection of plant traits was not measured in that study, such behaviour would be expected to generate correlational selection. Second, when multiple pollinator types are involved, overall visitation rate by all pollinators could show nonadditivity if one kind of pollinator strongly preferred, for example dark, tubular flowers and another kind preferred light, flat flowers, even if selection by each pollinator individually was directional.

We focused on the insect-pollinated *Polemonium foliosissimum* (Polemoniaceae). This species has purple, campanulate flowers and has been considered primarily bumblebee pollinated (Zimmerman, 1980; Brody, 1997), although it is also visited by solitary bees and by flies (see Results). In addition to examining effects of floral traits on pollinator visitation rate, we compared the pollinator effectiveness of bumblebees, solitary bees and flies to determine whether weighting visits by effectiveness would change the pattern of selection. We tested for correlational selection in 2 years. Pollinators can show considerable temporal variation (Price *et al.*, 2005) in response to changing environmental conditions (Alarcón *et al.*, 2008) and that variability may alter patterns of selection on floral traits (Benitez-Vieyra *et al.*, 2012). We asked the following questions:

Q1. Do the presumed primary pollinators (bumblebees) and secondary pollinators (other bees and flies) preferentially visit in response to flower colour or shape? If so, do they differ in the floral traits they overvisit?

Q2. Do the overall patterns of pollinator visitation generate correlational selection or other forms of selection for flower colour and shape, and do these patterns of selection vary between years?

Q3. Is the pattern of selection in either year altered by considering the relative effectiveness of pollen transfer in addition to visitation rate, and does this overall selection favour the naturally occurring flower traits while selecting against experimental mutants?

Materials and methods

Study system

We studied *Polemonium foliosissimum* in subalpine meadows at the Rocky Mountain Biological Laboratory (RMBL), Gunnison County, CO, USA. The campanulate flowers of this species contain five purple petals that

overlap basally to form a broad tube that flares outward. The species is almost completely self-incompatible (Zimmerman, 1980), and populations are either hermaphroditic or gynodioecious (both females and hermaphrodites). For consistency, we used only hermaphroditic flowers for our experiments.

Flower traits and manipulations

Flower colour and shape (from flat to strongly tubed) were measured for natural flowers for comparison with our experimental manipulations. Petal colour was measured for three flowers between the wavelengths of 400–700 nm using an Ocean Optics (Ocean Optics Inc., Dunedin, FL, USA) Red Tide USB650 spectrometer with a LS-1 light source, standardized by a white reflectance standard, and a fibre optic probe held at a 45° angle. Measurements of additional flowers in this species using an AvaSpec-2048 spectrometer with AvaLight-XE xenon light source (Avantes, Broomfield, CO, USA) have shown that they do not reflect in the ultraviolet (K. Whitney, personal communication). The reflectance peaked at 450–455 nm, which is near maximal sensitivity of 440 nm for the blue receptor in most insects (Briscoe & Chittka, 2001), and again at 700 nm or higher. As a proxy for our shape measure of the tube, we measured flower diameter along a bisecting axis from the tip of one petal across the flower face to the tips of the two petals on the far side. Diameter was measured with callipers for 97 unmanipulated flowers for comparison with manipulated flowers.

We generated experimental mutants by manipulating colour and shape. With this approach, we extended variation beyond that naturally present within populations of this species, allowing us to test for selection maintaining the current phenotype over potential mutants (Kessler *et al.*, 2008). Our experimentally generated treatments did fall within the range of colour and shapes seen in other North American species of *Polemonium* (Worley *et al.*, 2009). For colour manipulations, we painted flowers either titanium white or purple, the latter in a way that matched the average floral reflectance spectrum, using mixes of water-based acrylic paints as have been used successfully in other pollinator studies with insects (Waser & Price, 1985; Campbell *et al.*, 2010, 2012; Pohl *et al.*, 2011). Tube shape was manipulated for other sets of flowers to create three experimental phenotypic values: open, tubular and control. Open flowers had the largest diameter, whereas tubular flowers had the smallest diameter. We made open flowers by smoothing the petals onto five-pointed stars cut from mylar, using double-sided tape so that flowers presented a nearly flat display. Tubular flowers were created by inserting the flowers into tubes of mylar sheeting to constrict the corolla. Control flowers with the same average diameter and shape as natural flowers were created by forming shallow tubes from the five-pointed mylar stars and resting the natural flowers inside. In this way, all three flower shapes had constructions built from mylar for consistency. The diameters of experimental flowers were measured for comparison with natural flowers. Tubular, open, control and unmanipulated flowers differed significantly in diameter (means = 12.9, 22.3, 19.7 and 20.1 mm, respectively, ANOVA, $F_{3,200} = 250.91$, $P < 0.0001$), but with the control flowers indistinguishable from natural unmanipulated flowers (range = 16.5–28.9 mm, $P > 0.20$).

Experimental arrays

We set up four types of experimental arrays in meadows at the RMBL: (i) colour arrays, (ii) paint-control arrays, (iii) shape arrays and (iv) factorial arrays in which both colour and shape were manipulated. Each array was established in a 0.7 m × 0.7 m plot with the 36 flowers representing the set of treatments randomly distributed and evenly spaced at 10-cm intervals within the plot. Each flower was placed with 1.0 mL of plant food (Miracle-Gro® Quick Start Planting & Transplant Starting Solution) in a numbered 1.5-mL microcentrifuge tube attached to a wooden skewer staked into the ground.

For arrays of all four types, we followed each sequence of flower probes by an insect from the time it entered its first flower in the array until the time it exited the array. Such a sequence was considered a foraging bout. For each bout, we recorded the identity of the insect, the numbered sequence of flowers probed and the total time spent in the bout. For analyses, we categorized insects as bumblebees (*Bombus* spp.), solitary bees or flies. This coarse grouping may obscure variation across individual species in visitation preferences; however, the large number of species involved made analysis at species level impractical. Of 474 foraging bouts observed, only 18 were by other insects not in these categories (lepidopterans, beetles, wasps and ants). Flies were identified to family level, and bees were identified to family, genus or species (for bumblebees).

Responses of bees and flies to colour and shape

The first three types of arrays were used in answering Q1 about behavioural responses of insects to single floral traits. To assess responses of bees and flies to manipulation of petal colour alone, we used 'colour arrays' with 18 flowers painted the natural purple colour and 18 flowers painted white. Five arrays were set up, and each was observed for 4 h (20 h in total) between 10:00 and 15:00 when bee and fly visitors were most active.

To test whether insects respond to other features of the paint manipulation rather than to the colour itself,

we set up two 'paint-control arrays' (for a total of 8 h of observation) with 18 unmanipulated flowers and 18 flowers painted purple on the calyx (to expose pollinators to the paint odour). In principle, the paint could also influence insect responses by changing the texture of the petal surface or fine details such as venation patterns; however, in previous tests with a wide variety of insects, no effects of these acrylic paints on insect choice have been seen (Waser & Price, 1983; Campbell *et al.*, 2010; Pohl *et al.*, 2011).

To assess responses of bees and flies to manipulation of shape alone, we used 'shape arrays' with our three shape treatments. Each array had 12 open flowers, 12 tubular flowers and 12 control flowers. Five arrays were set up and observed for 4 h (20 h in total) between 10:00 and 15:00. These first three types of arrays were observed between 6 July 2010 and 27 July 2010.

Our aim with these single-trait manipulations was to test for behavioural responses of bumblebees, other bees and flies, rather than to estimate selection on plant traits. For this reason, we performed analyses using an insect foraging bout as a replicate. As individual insects were not marked, it is possible that some insects made multiple foraging bouts in the same observation session. However, all experiments were replicated with multiple arrays using different flowers on different days, reducing the extent of this nonindependence. For each category of insect, we analysed two variables: the kind of flower first visited upon entering the array, and the proportion of visits made to a given type of flower during the foraging bout. The first variable was compared with the null hypothesis that all types were visited equally, using a likelihood ratio test. The second variable was first arcsin-square-root-transformed to improve normality. For the colour arrays, we then used one-sample *t*-tests to compare the proportion of visits to purple with the null hypothesis of 0.5. To compare responses of the three types of insects, we used one-way ANOVA on the proportion of visits to purple. For the paint-control arrays, we similarly compared the proportion of visits to painted with the null hypothesis of 0.5. For the shape arrays, which included three treatments, we compared the proportion of visits to open flowers with the null hypothesis of 0.3333, and the proportion of visits to tubular flowers with 0.3333 using one-sample t tests. Again, ANOVA was used to compare these proportions among types of insects.

Tests for correlational selection of flower traits in two years

To test for correlational selection during visitation, we set up arrays in which both floral traits were manipulated in factorial combination. Each array contained six flowers each of six treatments: control/purple, control/white, open/purple, open/white, tubular/purple, and tubular/white. Arrays were observed between the hours of 10:00 and 15:00 from 30 July 2010 to 6 August 2010 and 15 July 2011 to 9 August 2011. We employed five arrays, each with a unique random assignment of treatments to positions. The first array was observed for a total of 13.5 h in 2010, substituting fresh manipulated flowers prior to each of three observation periods spread over 3 days. The second array was observed for a total of 7 h in 2010, substituting fresh flowers between two observation periods. Three more arrays were observed in 2011, each for 4 h, increasing our total hours of observation to 32. We analysed each year separately, as the 2 years differed considerably in visitor composition. This portion of the study was designed to measure selection of plant traits, so the unit of replication was a flower of a particular treatment type in a particular array position. We had 12 independent replicates per treatment in 2010 (2 arrays × 6 flowers) and 18 in 2011. Visits per hour to each such flower were analysed with a generalized linear model with two crossed factors of colour (purple vs. white) and shape (open vs. control vs. tube) and a blocking factor corresponding to the array. Correlational selection would be indicated by a colour x shape interaction. We also tested for a specific type of correlational selection in which the difference in visitation to the two extreme shapes (tube vs. open) depends on colour, employing a contrast statement in a model with treatment and array. We compared the fit of these models, using Proc GENMOD in SAS (version 9.2, SAS Institute, Inc., Cary, NC, USA), based on three assumptions about residuals (normal distribution, Poisson or zero-inflated Poisson), and chose the model with the lowest AIC value, in both years a model with a normal distribution. Likelihood ratio tests for type 3 analysis are reported. In addition to analysing overall visitation rate, we also analysed separately visit rate by type of insect (for all three types in 2010 and for flies only in 2011, as other insect visitors were scarce that year).

Incorporating pollinator effectiveness

Pollinator effectiveness was assessed by counting the conspecific pollen grains deposited in a single flower visit. Flowers were emasculated while in bud stage and then covered with fine mesh jewellery bags to exclude insects. Once stigmas had expanded so that the flowers were female receptive, bags were removed for observation. After a single visit by an insect, the flower was re-bagged. The stigmas from these visited flowers and from control flowers that were treated in the same manner, but with no observed insect visit, were excised with forceps and mounted on slides in fuchsin gel to stain the pollen grains. Pollen grains of *Polemonium foliosissimum* were easily distinguishable from pollen grains of other species in the area and were counted under a compound microscope. Only those flowers that were

the first experimental flower to be visited by an insect were included in analysis, as later flowers in the foraging bout would have been preceded by visits to emasculated flowers where no conspecific pollen was present. Experiments were carried out in 2010 in meadows at the RMBL and in 2011 at both that site and in meadows along Gunnison County Road 12 west of Kebler Pass because of low bee visitation at RMBL that year. In total, we obtained stigmatic pollen counts for 37 flowers visited by bumblebees, 25 by solitary bees, 35 by flies and 31 unvisited controls. Sample sizes for individual species were generally too small to divide the three types of insects up more finely for analysis, with the exception of a comparison of bumblebee species.

As many cases had zero pollen (1 for bumblebees, 2 for solitary bees, 7 for flies and 14 for unvisited controls), we employed a generalized linear model with a zero-inflated Poisson distribution to compare single-visit pollinator effectiveness among these four groups. This analysis, implemented in Proc GENMOD, modelled both the incidence of zeros and the nonzero values.

To predict overall pollen receipt in our factorial manipulation arrays in 2010 and 2011, we incorporated pollinator effectiveness into a model of expected pollen receipt for a replicate flower. For each flower treatment, we modelled visits per hour, after taking residuals from the mean for each array, with a factor of type of insect, which yields estimates comparable with a model with both array and type of insect. This procedure yielded a coefficient for each type of insect, allowing us to describe expected pollen receipt as the sum of the products of bumblebee pollinator effectiveness by bumblebee visits per hour, solitary bee effectiveness by solitary bee visits per hour and fly effectiveness by fly visits per hour. Approximate standard errors were found for each treatment, using the delta method to find the variance of a function of random variables, implemented with the 'msm' package in R ('deltamethod' function, R Core Team, 2012).

Results

In 80 h of observation at the four types of experimental arrays, 985 flower visits were observed. Of these, only 35.9% were made by bumblebees (*Bombus appositus, B. bifarius, B. californicus, B. flavifrons*), even though *Polemonium foliosissimum* is described as bumblebee-pollinated (Zimmerman, 1980). Bumblebee visits were common in 2010 but exceedingly rare in 2011 (42% vs. 3% of the total visits). Over both years combined, other bees (families Andrenidae, Colletidae, Halictidae, Megachilidae) made 13.8% of the visits, flies (families Anthomyiidae, Bombyliidae, Calliphoridae, Sarcophagidae, Syrphidae) made 48.7% and other insects accounted for the remaining 1.5%. Examining just the flowers in the paint-control arrays in 2010, all of which were purple with unmanipulated shape, a mere 10% of the flower visits were by bumblebees, 31% by solitary bees and 53% by flies. So, the observation that bumblebees made a minority of the flower visits overall even in 2010 when they were abundant was not a result of presenting manipulated phenotypes.

Responses of bees and flies to colour and shape

In the arrays in which petal colour was manipulated, the colour first visited by an insect differed across insect type (likelihood ratio $\chi^2 = 12.3525$, d.f. = 2, $P = 0.0021$). All eight bumblebees that entered an array first visited only the purple flowers, and 17 of 18 solitary bees did likewise ($\chi^2 = 14.2$, $P = 0.0002$). In contrast, flies did not choose to start at one colour significantly more often than the other, making 23 of 37 first visits to purple ($\chi^2 = 2.19$, $P = 0.1390$). Similar responses to purple vs. white were seen in terms of the proportion of visits in a foraging bout made to purple. Bumblebees and solitary bees averaged significantly higher than half their visit to purple (Fig. 1; one-sample t tests, $P < 0.0001$ for both types of insects). Flies showed no detectable discrimination, averaging 0.62 of visits in a foraging bout to purple ($t_{36} = 1.77$, $P = 0.0859$). Analysis of variance indicated a significant difference among these insect types in the proportion of purple flowers visited in a bout ($F_{3,61} = 25.44$, $P = 0.0022$). Applying paint to flowers without changing the colour did not influence visitation of these insects. Of the 31 foraging bouts by bees and flies at the paint-control arrays, 16 started with a painted flower ($\chi^2 = 0.0323$, $P = 0.8575$), and these insects averaged a proportion of 0.52 of their visits to painted flowers, which did not differ significantly from the null hypothesis of 0.5 ($t_{30} = 0.329$, $P = 0.7490$).

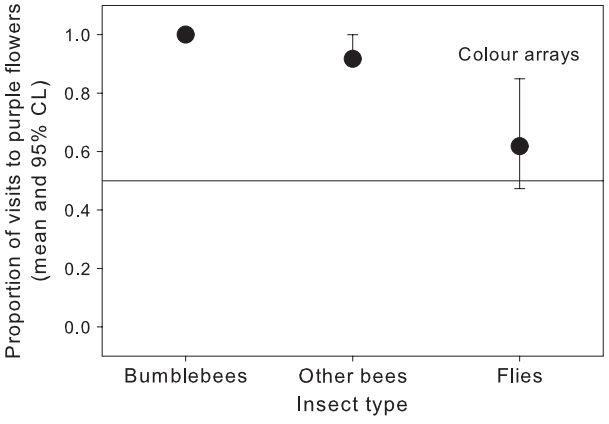

Fig. 1 Proportion of visits in a foraging bout made to purple rather than white flowers in the colour manipulation arrays. The horizontal line at 0.5 indicates the null expectation in the absence of discrimination.

When flower shape was manipulated, the three types of insects responded similarly with their first flower visits ($\chi^2 = 0.1895$, $P = 0.9096$), with all types more often choosing control or open flowers than tube flowers. All three types of insects made less than 1/3 of visits during a foraging bout to tube flowers (Fig. 2; one-sample t tests, $P < 0.01$ in all cases). The proportion of visits to open flowers did not differ significantly from 1/3 for any of the insect types (all $P > 0.25$). Analysis of variance detected no difference among insect types in the proportion of visits during a foraging bout made to tube flowers ($F_{3,78} = 1.04$, $P = 0.3801$). In sum, for the arrays in which a single floral trait was manipulated, all three types of insects discriminated against tube-shaped flowers to a similar extent, and bumblebees and solitary bees discriminated against white petal colour.

Tests for correlational selection based on visit rate in two years

When both floral traits were manipulated in factorial combination, both petal colour and flower shape influenced the overall rate of insect visitation to flowers in 2010 (Proc GENMOD, likelihood ratio χ^2_1 for colour = 4.72, $P = 0.0299$ and likelihood ratio χ^2_2 for shape = 9.55, $P = 0.0084$). Purple flowers received more visits than white, and control and open flowers more visits than tube flowers (Fig. 3a). The main effects of petal colour and flower shape were also significant in 2011, again with purple favoured over white and tube flowers discriminated against (χ^2_1 for colour = 4.45, $P = 0.035$, χ^2_2 for shape = 14.25, $P = 0.0008$; Fig. 3b). Examining each insect type separately in 2010, bumblebee visit rate was significantly affected only by colour ($P < 0.0001$), solitary bee visit rate was relatively constant across treatments ($P > 0.05$ for all effects), and fly visit rate was significantly affected only by shape ($P = 0.0010$). No measure of visit rate in 2010 showed a significant interaction between the effects of colour and shape (all $P > 0.05$, χ^2_2 for total visit rate = 2.34). In 2011, however, the difference between fly visit rate to open vs. tube flowers was greater if flowers were coloured purple (contrast estimate $\chi^2 = 5.20$, $P = 0.02$; overall interaction between colour and shape including all three types, $P = 0.05$). In sum, we detected differential visitation according to both colour and shape and found evidence for correlational selection arising from the rate of fly visitation to flowers in 2011 but not in 2010.

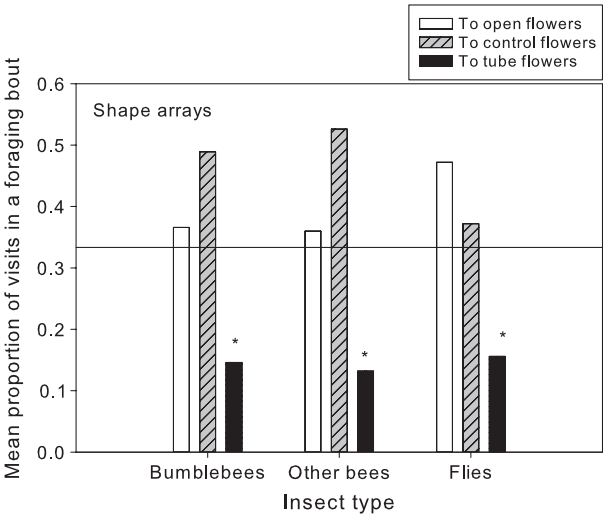

Fig. 2 Proportion of visits in a foraging bout made to each of three flower shapes in the shape manipulation arrays. * Mean proportion differs from the null expectation of 0.33 at $P < 0.05$ using a one-sample *t*-test on arcsin-square-root-transformed data.

Incorporating pollinator effectiveness

In our effectiveness study, we obtained single visits by five species of bumblebees (*Bombus appositus*, *B. bifarius*, *B. flavifrons*, *B. kirbyellis* and *B. sylvicola*), two families of solitary bees (mostly Andrenidae and 1 Halictidae) and at least three families of flies (Anthomyiidae, Syrphidae and others). Bumblebees were the most effective pollinators, solitary bees the second most effective pollinators, and flies the least effective based on amount of pollen transferred to a stigma in a single visit (Fig. 4). A generalized linear model analysing these three groups and the control unvisited flowers detected differences in both the probability that a flower received any pollen (likelihood ratio χ^2 for zero-inflation portion of model = 21.16, $P < 0.0001$) and the count of pollen grains in the nonzero cases ($P < 0.0001$). Each type of insect differed in pollinator effectiveness from the control flowers for both the count portion of the model (all $P < 0.0001$) and the zero-inflation portion ($P = 0.0016$, 0.0117 and 0.0320 for bumblebees, solitary bees and flies, respectively). Thus, all types of insects were effective pollinators. Bumblebees were significantly better at delivering pollen than solitary bees (contrast estimate $\chi^2 = 161.66$, $P < 0.0001$), and solitary bees were similar to flies ($\chi^2 = 3.20$, $P = 0.0735$). On average, bumblebees transferred 44 conspecific pollen grains, solitary bees transferred 21 and flies transferred 17 in a single visit. Within bumblebees, *B. appositus* and *B. bifarius* were similarly effective (mean pollen grains = 44 vs. 38, $n = 11$ vs. 13, one-way ANOVA, $P = 0.79$).

For each type of insect we multiplied its single-visit effectiveness by the proportion of flower visits it was responsible for (across all types of arrays) to estimate its overall contribution to pollination in terms of deposition of compatible pollen on the stigma (pollination success *sensu* Ne'eman *et al.*, 2010). In 2010, for bumblebees, solitary bees and flies, we obtained values of 18.35 (43.8 grains × 41.9% of visits), 3.45 and 6.81, respectively. From these values, we estimate that in

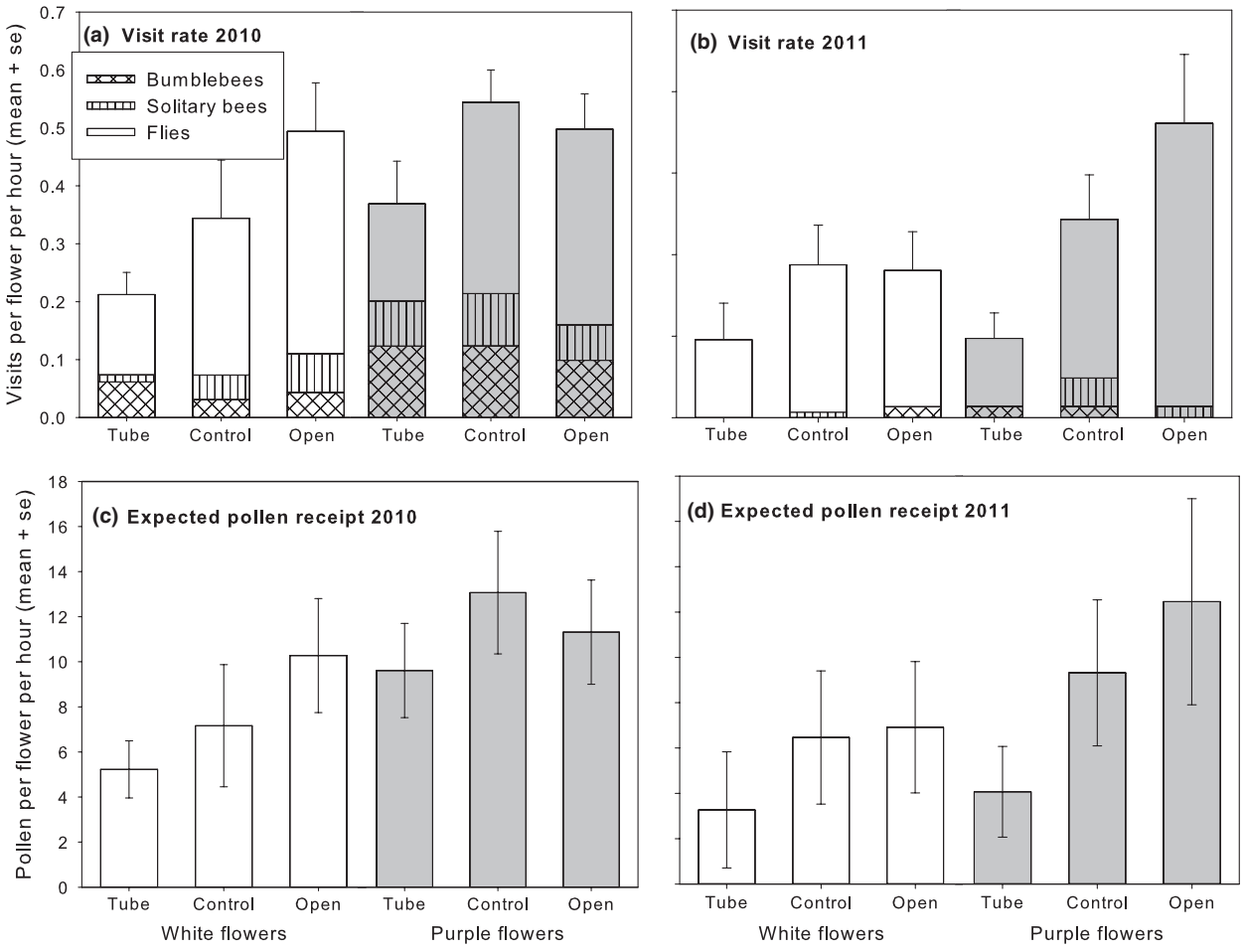

Fig. 3 Effects of manipulating colour and shape in factorial arrays on aspects of fitness. (a) and (b) Visits per flower per hour by bumblebees, solitary bees, flies and all three types of insects combined in 2010 and 2011. (c) and (d) Expected deposition of pollen per hour based on weighting visitation by single-visit pollinator effectiveness in 2010 and 2011.

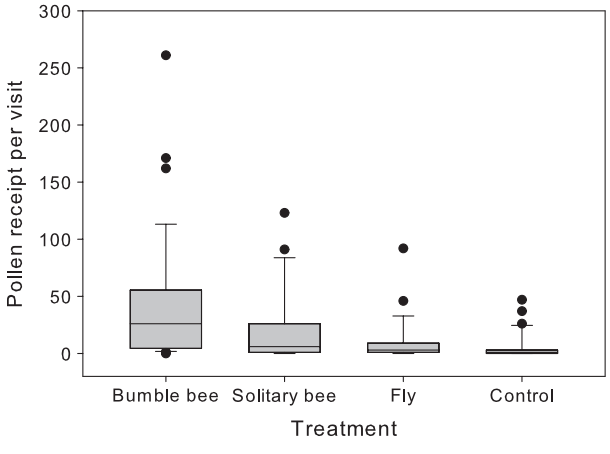

Fig. 4 Box plots for amount of conspecific pollen received on the stigma in one visit by a bumblebee, solitary bee, or fly, or no visit (control).

2010, bumblebees did 64% of the pollination of *Polemonium foliosissimum* at this site, solitary bees did 12% and flies were responsible for 24%. During our 2011 observations, however, flies performed the vast majority of the visits (87.9%) and pollination (85.8%), consistent with the general scarcity of bumblebees at RMBL that year. The majority of these fly visits were by Anthomyiidae even though they were rare visitors the previous years (only 2 flower visits in the factorial arrays). Over all observation periods in both years combined, we estimate that bumblebees did 58% of the pollination, solitary bees did 11% and flies were responsible for 31%.

We also used the mean single-visit effectiveness in combination with rates of visits per flower per hour to predict the expected amount of pollen delivered per hour by all types of insects combined to each of the six floral treatments in a double manipulation array (Fig. 3c,d). In 2010, there was a trend for open flowers

to receive the most estimated pollen if flowers were painted white but for control flowers to receive the most pollen if flowers were painted purple, but standard errors were largely overlapping. In that year, with bumblebees common, the experimental phenotype that matched the natural flowers (painted purple with a control shape) was predicted to receive the most pollen (Fig. 3c). In 2011, bees also visited that phenotype the most just as they had in 2010 (Fig. 3a,b). However, flies dominated the visits in 2011, with the net result that flowers painted purple with an open shape were actually predicted to receive the most pollen (Fig. 3d). In neither year could t tests detect differences between flower treatments based on standard errors obtained using the delta method.

Discussion

Responses to colour

Flower visitation by insects responded to independent manipulation of both petal colour and shape in *Polemonium foliosissimum*. When only petal colour was manipulated, both bumblebees and solitary bees overvisited purple flowers in comparison with white, whereas flies showed no preference. Bees have highly conserved colour photoreceptors with peak sensitivities of three receptors in the UV, blue and green (Briscoe & Chittka, 2001) and would be easily able to distinguish the white (bee blue green) and purple (bee blue) we used in our study. Colour vision of flies is less well characterized and appears more variable across species (Briscoe & Chittka, 2001), but in two previous behavioural tests, flies have responded differently to flowers (or flower parts) that appear white vs. blue to purple (Hansen *et al.*, 2011; Campbell *et al.*, 2012). The preference of bumblebees for purple in our study is consistent with the colour association postulated in pollinator syndromes, but the lack of fly preference for human-visible white is not (Faegri & van der Pijl, 1966). Bees, however, might have overvisited purple simply as a consequence of foraging experience, because the site contained mostly blue/purple and yellow flowers during the periods of observation. Bumblebees can readily learn to associate particular colours with rewards (Dyer & Chittka, 2004). Nevertheless, the higher overall visitation rate to purple flowers in the factorial arrays suggests the potential for selection maintaining this naturally occurring petal colour.

Responses to shape

Bumblebees, solitary bees and flies infrequently visited the flowers that were manipulated to have narrow tubes. In the arrays where only shape was manipulated, all three types of insects discriminated against tube flowers, whereas in the arrays where colour was also manipulated only the flies did so detectably. This apparent difference in bee behaviour has at least two potential explanations. First, it could partly reflect a difference in the species of solitary bees visiting the two sets of arrays. Most of the solitary bees at the shape arrays were in the family Andrenidae (74% of 19 foraging bouts), whereas most of the 22 solitary bee bouts in 2010 at the factorial arrays belonged to other families (3% Andrenidae, 14% Halictidae, 23% Colletidae, 27% Megachilidae and the remainder unclassified). Second, bumblebees and solitary bees might be less choosy about shape when they are also choosing on the basis of colour. Despite the weaker preference in the 2010 factorial arrays, flower shape still influenced the overall visitation rate of pollinators, suggesting the potential for selection favouring the unmanipulated shape or more open flower over the tube shape.

Multiple mechanisms could in principle explain the undervisitation of tube flowers. First, tube flowers might appear smaller to insects. Bumblebees foraging at another species of *Polemonium* (*P. viscosum*) preferentially visit more broadly flared flowers (Galen & Newport, 1987). These flowers have a greater distance separating the tips of the opposite corolla lobes, as do the open flowers used in this study, but also are larger overall. Moreover, they produce more nectar (Cresswell & Galen, 1991), and bumblebees quickly learn associations with sugar rewards (Makino & Sakai, 2007), suggesting a second possibility that experienced bumblebees in our study may have associated more open flowers with more nectar. Third, extraction of nectar might require greater handling time from a flower with a constricted tube, even for those insects whose tongue can reach the bottom of the tube (Harder, 1983). Fourth, the anthers of tubular flowers protruded less above the floral plane than did those of more open flowers. If anthers are a visual or olfactory attractant, this difference could have contributed to low visitation to the tube flowers. Finally, flies often landed on flowers, and the open flowers may have provided a more stable landing platform, helping to explain the exceptionally high visitation by flies to open/purple flowers in 2011.

Selection of combinations of floral traits

This study represents one of only two manipulative studies of correlational selection (the other being Sletvold & Ågren, 2011), where both floral traits manipulated are known to affect a component of fitness, in this case either the rate of pollinator visitation or expected pollen receipt. We saw no evidence for correlational selection based on either measure in 2010; however, in 2011, we detected correlational selection for fly visit rate (although not overall visit rate) favouring a mutant-like phenotype with purple flowers that were more open than the natural shape. Thus, patterns

of visitation alone can give rise to correlational selection.

One caveat about our predictions of pollen receipt is that we did not have effectiveness measures for all of the species of insects that visited our arrays and instead lumped insects into three categories. Lumping the bumblebees is justified in part by finding no difference in effectiveness between the two species with sufficient sample sizes to compare. Our effectiveness measure for solitary bees was based largely on Andrenidae, which were common visitors to the double manipulation arrays, but so were Halicitade and Megachilidae, and it is possible that they differ in effectiveness. For the flies, we had no measures of effectiveness for Sarcophagidae, which made a plurality of visits to those double manipulation arrays in 2010, nor for Bombyliidae, even though different species of flies can differ in single-visit pollen deposition (King *et al.*, 2013). Additional measurements of pollinator effectiveness for these other insects would help to refine predictions of pollen receipt as a function of floral traits and would be useful more generally as so few comparisons of a wide variety of insects exist for any plants (but see Robertson & Leavitt, 2011; King *et al.*, 2013).

A potential explanation for nondetection of correlational selection is low statistical power. Power is generally lower for detecting an interaction than a main effect in ANOVA, which could partly explain our failure to detect correlational selection for any visitation measure in 2010. However, power analyses with Proc Glmpower in SAS (ver 9.2) indicates we had higher statistical power for detecting an interaction effect size equivalent to that we observed for visitation than did the study by Sletvold & Ågren (2011) based on reported results for fruit mass in their Fig. 2 and Table 1. Furthermore, we did detect correlational selection for fly visitation in 2011 with fewer hours of observation. Low statistical power is a tenable explanation for failure to detect correlational selection based on expected pollen receipt in 2011. Whereas the pattern of means resembled that for fly visitation, expected pollen receipt incorporated additional variance.

Although fly visitation in 1 year showed a pattern that could generate correlational selection of flower colour and shape, that does not imply that overall selection of those traits takes the same form. We did not detect correlational selection based on total visitation or expected pollen receipt. Furthermore, several additional mechanisms could alter the strength of correlational selection if other contributors to fitness are also considered. First, flower shape might turn out to influence the single-visit pollinator effectiveness. Bees did not appear to enter the tubular flowers as far as the other shapes. If this behaviour resulted in, for example, deposition of only half as much pollen on a tube flower as it did on other flowers, the total pollen received by a purple tube flower and by a white tube flower would each be cut in half from our predictions, but this decrease would be greater in absolute terms for the purple tube flower, contributing to a greater difference between tube and other shapes for purple than for white and thus correlational selection. Second, we have assumed that expected pollen receipt is a product of number of visits and single-visit pollinator effectiveness. This may not be strictly true; for example, pollen receipt might eventually saturate with a large number of visits if the stigmatic surface becomes completely covered. Similarly, lifetime seed production may not be linearly related to pollen receipt, unless seed set is highly pollen-limited (Ashman *et al.*, 2004) as it is in *Polemonium viscosum* (Galen, 1985). Third, the floral traits we examined might affect the male fitness components of pollen removal and export differently from pollen receipt (Campbell, 1989; Kulbaba & Worley, 2012). Fourth, flower colour or shape might affect components of fitness unrelated to pollination, such as viability prior to trait expression (Mojica & Kelly, 2010) or consumption of seeds by the anthomyiid fly *Delia* sp. (Brody & Waser, 1995). This last mechanism could be especially important in a year like 2011 when many floral visitors were flies in the Anthomyiidae. Although we could not identify all of those on the wing, some individuals were likely *Delia* sp. These flies oviposit on elongated buds, and on another plant species (*Ipomopsis aggregata*), oviposition rate increases with corolla width, suggesting potential for response to the flower shape trait we manipulated here (Zimmerman & Brody, 1998; Campbell *et al.*, 2002). In theory, directional selection on one trait due to a pollinator (e.g. colour) and directional selection on a second trait (e.g. tube vs. open) due to a predator on seeds can give rise to correlational selection, as these two kinds of selection affect different components of fitness (e.g. seeds formed and undamaged seeds per seed formed) that multiply together to give overall fitness (McGlothlin, 2010).

Overlooked pollinators and temporal variation in selection

A surprising result of our study was that the presumed major pollinators of this plant species (bumblebees) accounted only for an estimated 58% of pollination, with solitary bees and flies also making substantial contributions in both years. In fact, flies may be critical agents of selection favouring the flower shape in this species, as they responded more strongly to shape manipulations than did bees. This result indicates the importance of studying the variety of flower visitors and not just ones that conform to a presumed syndrome (see also Mayfield & Waser, 2001).

In 2010, overall visitation patterns selected for maintenance of the current phenotype, with control-shaped purple flowers of this naturally purple flower receiving the highest expected pollen receipt (Fig. 3c). Our

phenotypic manipulations in that year suggested that mutants with white flowers or more tubular shape would be selected against and not able to invade a natural population unless there are counterbalancing selective pressures on these traits not mediated through pollinator visitation. Bumblebees were common in 2010, have historically been considered the main pollinators of this species, and were also frequent visitors in other years of observation, including 1977, 1978 and 1994 (Zimmerman, 1980; Brody, 1997), so there is reason to think this pattern of selection maintaining the natural phenotype could have been the historical pattern. Few other manipulative experiments have demonstrated the selective advantage maintaining multiple traits of a flower. One notable exception blocked expression of genes for two floral volatiles and demonstrated both floral traits were required to maximize fruit production and seed siring (Kessler *et al.*, 2008).

Although the current phenotype was favoured by pollinator visitation in 2010, that was not the case in 2011 when selection specifically favoured a mutant phenotype with more open flowers. This difference between years could be the result of a shift in pollinator assemblages, as bumblebees were scarce in 2011. Visitation by the relatively more common flies that year generated a pattern of correlational selection in which the shape was more critical for purple than for open flowers. Bumblebees were scarce at the RMBL not only in 2011 but also in 2012, both years with unusual snowmelt (late in 2011 and very early in 2012). If bumblebees continue in low abundance in future years, that shift might potentially select for evolution of more open flowers in *P. foliosissimum* in the RMBL area.

Conclusions

Pollinators generally visited the natural petal colour of *Polemonium foliosissimum* over flowers manipulated to produce artificial mutants with paler colour and the natural flower shape over more open or more tubular flower. When both colour and shape were manipulated in combination, selection favoured both traits in additive fashion in a year when bumblebees were common so that the natural phenotype combination was favoured. In 2011, bumblebees were scarce, flies accounted for most pollination, and visitation favoured a mutant phenotype rather than the natural one. These results show that flower colour and shape are subject to pollinator-mediated selection. Furthermore, in 1 year, flower shape had a larger effect on fly visitation if flowers were also purple, showing that it is possible to generate correlational selection during a single selective episode. Aspects of selection would have been missed if the study had focused solely on the presumed pollinators (bumblebees) without considering contributions of previously overlooked insects to pollen receipt. Tests of other potential mechanisms of correlational selection will require also measuring how other components of fitness besides pollinator visitation rate, such as pollinator effectiveness or seed predation, change as a function of the floral traits.

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References

Alarcón, R., Waser, N.M. & Ollerton, J. 2008. Year-to-year variation in the topology of a plant-pollinator interaction network. *Oikos* **117**: 1796–1807.

Armbruster, W.S. & Schwaegerle, K.E. 1996. Causes of covariation of phenotypic traits among populations. *J. Evol. Biol.* **9**: 261–276.

Ashman, T., Knight, T.M., Steets, J.A., Amarasekare, P., Burd, M., Campbell, D.R. *et al.* 2004. Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. *Ecology* **85**: 2408–2421.

Benitez-Vieyra, S., Medina, A.M., Glinos, E. & Cocucci, A.A. 2006. Pollinator-mediated selection on floral traits and size of floral display in *Cyclopogon elatus*, a sweat bee-pollinated orchid. *Funct. Ecol.* **20**: 948–957.

Benitez-Vieyra, S., Glinos, E., Medina, M. & Cocucci, A.A. 2012. Temporal variation in the selection on floral traits in *Cyclopogon elatus* (Orchidaceae). *Evol. Ecol.* **26**: 1451–1468.

Boberg, E. & Ågren, J. 2009. Despite their apparent integration, spur length but not perianth size affects reproductive success in the moth-pollinated orchid *Platanthera bifolia*. *Funct. Ecol.* **23**: 1022–1028.

Briscoe, A. & Chittka, L. 2001. The evolution of color vision in insects. *Annu. Rev. Entomol.* **46**: 471–510.

Brody, A.K. 1997. Effects of pollinators, herbivores, and seed predators on flowering phenology. *Ecology* **78**: 1624–1631.

Brody, A.K. & Waser, N.M. 1995. Oviposition patterns and larval success of a pre-dispersal seed predator attacking two confamilial host plants. *Oikos* **74**: 447–452.

Campbell, D.R. 1989. Measurements of selection in a hermaphroditic plant: variation in male and female pollination success. *Evolution* **43**: 318–334.

Campbell, D.R. 2009. Using phenotypic manipulations to study multivariate selection of floral trait associations. *Ann. Bot.* **103**: 1557–1566.

Campbell, D.R., Crawford, M., Brody, A.K. & Forbis, T.A. 2002. Resistance to pre-dispersal seed predators in a natural hybrid zone. *Oecologia* **131**: 436–443.

Campbell, D.R., Bischoff, M., Lord, J. & Robertson, A. 2010. Flower color influences insect visitation in alpine New Zealand. *Ecology* **91**: 2638–2649.

Campbell, D.R., Bischoff, M., Lord, J. & Robertson, A. 2012. Where have all the blue flowers gone: pollinator responses

and selection on flower colour in New Zealand *Wahlenbergia albomarginata*. *J. Evol. Biol.* **25**: 352–364.

Cresswell, J. & Galen, C. 1991. Frequency dependent selection and adaptive surfaces for floral trait combinations: the pollination of *Polemonium viscosum*. *Am. Nat.* **138**: 1342–1353.

Cuartas-Dominguez, M. & Medel, R. 2010. Pollinator-mediated selection and experimental manipulation of the flower phenotype in *Chloraea bletioides*. *Funct. Ecol.* **24**: 1219–1227.

Dudash, M.R., Hassler, C., Stevens, P.M. & Fenster, C.B. 2011. Experimental floral and inflorescence trait manipulations affect pollinator preference and function in a hummingbird-pollinated plant. *Am. J. Bot.* **98**: 275–282.

Dyer, A.G. & Chittka, L. 2004. Biological significance of distinguishing between similar colours in spectrally variable illumination: bumblebees (*Bombus terrestris*) as a case study. *J. Comput. Phys. A* **190**: 105–114.

Faegri, K. & van der Pijl, L. 1966. *The Principles of Pollination Ecology*. Pergamon, Oxford.

Galen, C. 1985. Regulation of seed-set in *Polemonium viscosum*: floral scents, pollination, and resources. *Ecology* **66**: 792–797.

Galen, C. & Newport, M.E.A. 1987. Bumble bee behavior and selection on flower size in the sky pilot, *Polemonium viscosum*. *Oecologia* **74**: 20–23.

Gómez, J.M., Perfectti, F. & Camacho, J.P.M. 2006. Natural selection on *Erysimum mediohispanicum* flower shape: insights into the evolution of zygomorphy. *Am. Nat.* **168**: 531–545.

Hansen, D.M., Van der Niet, T. & Johnson, S.D. 2011. Floral signposts: testing the significance of visual 'nectar' guides for pollinator behaviour and plant fitness. *Proc. R. Soc. B* **279**: 634–639.

Harder, L.D. 1983. Flower handling efficiency of bumble bees: morphological aspects of probing time. *Oecologia* **57**: 274–280.

Herrera, C.M. 2001. Deconstructing a floral phenotype: do pollinators select for corolla integration in *Lavandula latifolia*? *J. Evol. Biol.* **14**: 574–584.

Jones, A.G., Arnold, S.J. & Burger, R. 2003. Stability of the G-matrix in a population experiencing pleiotropic mutation, stabilizing selection, and genetic drift. *Evolution* **57**: 1747–1760.

Kessler, D., Gase, K. & Baldwin, I.T. 2008. Field experiments with transformed plants reveal the sense of floral scents. *Science* **321**: 1200–1202.

King, C., Ballantyne, G. & Willmer, P.G. 2013. Why flower visitation is a poor proxy for pollination: measuring single-visit pollen deposition, with implications for pollination networks and conservation. *Methods Ecol. Evol.* **4**: 811–818.

Kingsolver, J.G., Hoekstra, H.E., Hoekstra, J.M., Berrigan, D., Vignieri, S.N., Hill, C.E. *et al.* 2001. The strength of phenotypic selection in natural populations. *Am. Nat.* **157**: 245–261.

Kulbaba, M.W. & Worley, A.C. 2012. Selection on floral design in *Polemonium brandegeei* (*Polemoniaceae*): female and male fitness under hawkmoth pollination. *Evolution* **66**: 1344–1359.

Leonard, A.S., Dornhaus, A. & Papaj, D.R. 2011. Forget-me-not: complex floral displays, inter-signal interactions, and pollinator cognition. *Curr. Zool.* **57**: 215–224.

Maad, J. 2000. Phenotypic selection in hawkmoth-pollinated *Platanthera bifolia*: targets and fitness surfaces. *Evolution* **54**: 112–123.

Makino, T.T. & Sakai, S. 2007. Experience changes pollinator responses to floral display size: from size-based to reward-based foraging. *Funct. Ecol.* **21**: 854–863.

Mayfield, M. & Waser, N.M. 2001. Exploring the 'most effective pollinator principle' with complex flowers: Bumblebees and *Ipomopsis aggregata*. *Ann. Bot.* **88**: 591–596.

McGlothlin, J.W. 2010. Combining selective episodes to estimate lifetime nonlinear selection. *Evolution* **64**: 1377–1385.

Mojica, J.P. & Kelly, J.K. 2010. Viability selection prior to trait expression is an essential component of natural selection. *Proc. R. Soc. Lond. Ser. B* **277**: 2945–2950.

Ne'eman, G., Juergens, A., Newstrom-Lloyd, L., Potts, S.G. & Dafni, A. 2010. A framework for comparing pollinator performance: effectiveness and efficiency. *Biol. Rev.* **85**: 435–451.

O'Connell, L.M. & Johnston, M.O. 1998. Male and female pollination success in a deceptive orchid: a selection study. *Ecology* **79**: 1246–1260.

Pérez, F., Arroyo, M.T.K. & Medel, R. 2007. Phylogenetic analysis of floral integration in *Schizanthus* (*Solanaceae*): does pollination truly integrate corolla traits? *J. Evol. Biol.* **20**: 1730–1738.

Phillips, P.C. & Arnold, S.J. 1989. Visualizing multivariate selection. *Evolution* **43**: 1209–1222.

Pohl, N.B., Van Wyk, J. & Campbell, D.R. 2011. Butterflies show flower colour preferences but not constancy in foraging at four plant species. *Ecol. Entomol.* **36**: 290–300.

Price, M.V., Waser, N.M., Irwin, R.E., Campbell, D.R. & Brody, A.K. 2005. Temporal and spatial variation in pollination of a montane herb: a seven-year study. *Ecology* **86**: 2106–2116.

R Core Team 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Raguso, R. 2008. Wake up and smell the roses: the ecology and evolution of floral scent. *Ann. Rev. Ecol. Syst.* **39**: 549–569.

Raguso, R. & Willis, M.A. 2005. Synergy between visual and olfactory cues in nectar feeding by wild hawkmoths, *Manduca sexta*. *Anim. Behav.* **69**: 407–418.

Rausher, M.D. 1992. The measurement of selection on quantitative traits: biases due to environmental covariances between traits and fitness. *Evolution* **46**: 616–626.

Reynolds, R.J., Dudash, M.R. & Fenster, C.B. 2010. Multiyear study of multivariate linear and nonlinear phenotypic selection on floral traits of hummingbird-pollinated *Silene virginica*. *Evolution* **64**: 358–369.

Robertson, I.C. & Leavitt, H. 2011. Relative contributions to seed production by floral visitors of slickspot peppergrass, *Lepidium papilliferum* (*Brassicaeae*). *Arthropod Plant Interact.* **5**: 379–389.

Roff, D. & Fairbairn, D.J. 2012. A test of the hypothesis that correlational selection generates genetic correlations. *Evolution* **66**: 2953–2960.

Sletvold, N. & Ågren, J. 2011. Nonadditive effects of floral display and spur length on reproductive success in a deceptive orchid. *Ecology* **92**: 2167–2174.

Waser, N.M. & Price, M.V. 1983. Pollinator behavior and natural selection for flower colour in *Delphinium nelsonii*. *Nature* **302**: 422–424.

Waser, N. & Price, M. 1985. The effect of nectar guides on pollinator preference - experimental studies with a montane herb. *Oecologia* **67**: 121–126.

Wilson, P. 1995. Selection for pollination success and the mechanical fit of *Impatiens* flowers around bumblebee bodies. *Biol. J. Linn. Soc.* **55**: 355–383.

Worley, A.C., Ghazvini, H. & Schemske, D.W. 2009. A phylogeny of the genus *Polemonium* based on amplified fragment length polymorphism (AFLP) markers. *Syst. Bot.* **34**: 149–161.

Zimmerman, M. 1980. Reproduction in *Polemonium*: competition for pollination. *Ecology* **61**: 497–501.

Zimmerman, M. & Brody, A.K. 1998. Choices and consequences of oviposition by *Hylemya* (*Delia*) sp. (*Diptera: Anthomyiidae*). *J. Insect Behav.* **11**: 371–381.

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