



CHICAGO JOURNALS

Betalain Color Morphs Exhibit Differential Growth, Defensive Ability, and Pollen Tube Growth Rates in *Mirabilis jalapa* (Nyctaginaceae)

Author(s): Andrea E. Berardi, Frank M. Frey, Elsie M. Denton and Jessica H. Wells,

Source: *International Journal of Plant Sciences*, (-Not available-), p. 000

Published by: [The University of Chicago Press](#)

Stable URL: <http://www.jstor.org/stable/10.1086/673245>

Accessed: 18/10/2013 20:15

Your use of the JSTOR archive indicates your acceptance of the Terms & Conditions of Use, available at <http://www.jstor.org/page/info/about/policies/terms.jsp>

JSTOR is a not-for-profit service that helps scholars, researchers, and students discover, use, and build upon a wide range of content in a trusted digital archive. We use information technology and tools to increase productivity and facilitate new forms of scholarship. For more information about JSTOR, please contact support@jstor.org.



The University of Chicago Press is collaborating with JSTOR to digitize, preserve and extend access to *International Journal of Plant Sciences*.

<http://www.jstor.org>

BETALAIN COLOR MORPHS EXHIBIT DIFFERENTIAL GROWTH, DEFENSIVE ABILITY, AND POLLEN TUBE GROWTH RATES IN *MIRABILIS JALAPA* (NYCTAGINACEAE)

Andrea E. Berardi,^{1,*} Frank M. Frey,^{2,*} Elsie M. Denton,^{3,*} and Jessica H. Wells^{4,*}

*Department of Biology, Colgate University, 13 Oak Drive, Hamilton, New York 13346, USA

Editor: Patrick S. Herendeen

Premise of research. Nonphotosynthetic pigments play significant roles in plant life history, including in pigmentation, pollinator attraction, antioxidants, and defense against pathogens and herbivores. However, little is known about the specific roles of the betalains, which are present in several families of the Caryophyllales. In this study, we took a phenotypic and experimental approach to investigate whether betalain pigments might play diverse roles in plant life history as their phylogenetic counterparts, the anthocyanins, do.

Methodology. We measured floral and leaf betalain concentrations and several phenological and performance traits in four floral color morphs of horticultural lines of *Mirabilis jalapa* (Nyctaginaceae). We also performed herbivore assays and measured pollen tube length in situ to investigate the role of betalain concentration in defense and prezygotic barriers among the floral color morphs.

Pivotal results. Our results show that betacyanin and betaxanthin concentrations in leaves are negatively associated with leaf damage by a generalist herbivore and that pollen performance is associated with betacyanin and betaxanthin levels in the donor or recipient flowers. We also suggest that the floral color morphs significantly differ with respect to phenotypic architecture, with the pink morphs being slightly larger but germinating later than the other color morphs.

Conclusions. Our experiments using these horticultural lines suggest that betalains and resulting floral color morphs of *M. jalapa* are associated with several plant life-history traits, such as growth rate, germination, defense against herbivory, and pollination success. It is likely that these roles are subject to selection, and further studies should be conducted along these lines.

Keywords: *Mirabilis jalapa*, betalains, pigments, plant defense, pollen tubes.

Introduction

Nonphotosynthetic pigments such as anthocyanins play diverse roles in plant life history. It is well known that the expression and localization of pigments in floral tissues can affect pollination success (Faegri and van der Pijl 1979), and a number of studies have demonstrated that pollinators are agents of directional selection on floral color (Campbell et al. 1997; Schemske and Bradshaw 1999; Frey 2004; Irwin and Strauss 2005). In addition, the presence of nonphotosynthetic pigments in vegetative tissues is well documented (Harborne 1991; Lee and Gould 2002), and their relative abundance and distribution have been ascribed a variety of functions includ-

ing, but not limited to, protection from UV radiation and herbivores (Gould 2004; Koes et al. 2005). Several studies investigating the maintenance of floral color variation have suggested that pigment concentration may be linked to defensive ability against natural enemies through the direct pleiotropic properties of the pigments themselves or through the effects of shared metabolic pathways (Fineblum and Rausher 1997; Armbruster 2002; Irwin et al. 2003; Frey 2004, 2007; Strauss and Whittall 2006). Finally, there is good evidence that the abundance and composition of anthocyanins and other flavonoids can directly impact pollen development and performance (Mo et al. 1992; Ylstra et al. 1992; Vogt and Taylor 1995; Frey 2007; Frey et al. 2011).

It is not surprising that much of the work investigating the roles of pigments in plant life history has been focused on anthocyanins, given their near ubiquitous distribution among flowering plants. In contrast, less is known about the roles of betalains, which are restricted to certain members of the Caryophyllales and are fundamentally different from anthocyanins (Brockington et al. 2011). Betalains are water-soluble “chromoalkaloids” that occur in floral and vegetative tissues and are broadly classified into the red/crimson betacyanins and the yellow betaxanthins, although there are multiple molecular

¹ Current address: Department of Biology, University of Virginia, Charlottesville, Virginia 22904, USA.

² Author for correspondence; e-mail: ffrey@colgate.edu.

³ Current address: Department of Biology, Colorado State University, Fort Collins, Colorado 80523, USA.

⁴ Current address: Department of Community Health, Brown University, Providence, Rhode Island 02903, USA.

Manuscript received March 2013; revised manuscript received June 2013; electronically published October 14, 2013.

species of each type (Mabry 1980; Clement and Mabry 1996). Anthocyanins are phenolics produced from the phenylalanine-derived flavonoid pathway, whereas betalains are produced from tyrosine through an entirely separate route. Betalamic acid, which is enzymatically derived from tyrosine-derived L-3,4-dihydroxyphenylalanine (L-DOPA), can spontaneously condense with *cyclo*-DOPA (and derivatives) via a cytochrome P450 enzyme to generate betacyanin or with a number of amino acids and amines in an unselective manner to generate betaxanthins (Tanaka et al. 2008; Hatlestad et al. 2012). Although the functional expressions of anthocyanins and betalains appear to be mutually exclusive in plant species, it is interesting to note that betalain-producing species have functional anthocyanidin synthases (Shimada et al. 2005) and that both betacyanin and betaxanthin production are possible in anthocyanin-producing species (Harris et al. 2012).

In this study, we used a common betalain-producing species, *Mirabilis jalapa* (four-o'clock; Nyctaginaceae), to investigate possible associations between pigment concentration and a suite of phenotypic and reproductive traits. Individuals produce a single floral color variant, and colors in this system include stable white, yellow, pink, red, and magenta morphs with many areas of gradation in between (Leal et al. 2001); however, there are also examples of floral color variegation (broken colors) in commercially cultivated lines. Betalains can also be found throughout the body of *M. jalapa*; betacyanin pigments are primarily present in epidermal cells and can diffuse between neighboring cells, whereas betaxanthin pigments are found in both epidermal and parenchyma cells (van Kester et al. 1975). Early work on *M. jalapa* and *Beta vulgaris* demonstrated the classic two-gene system for betalain production (Showalter 1934; Keller 1936), which has since been updated by describing pleiotropic effects in the inheritance of floral color (Engels et al. 1975; Spitters et al. 1975) and functional identification of many responsible loci (Hatlestad et al. 2012). Several forms of betacyanin and betaxanthin can be found in betalain-containing species at once, often accompanied by alkaloids such as tyramine and dopamine (Piatelli and Manale 1964; Piatelli et al. 1965; van Kester et al. 1975). Given the systemic presence of these pigments throughout the plant body, the mutual exclusiveness of anthocyanins and betalains in plant taxa, and the polyfunctional nature of anthocyanins in other systems, we hypothesized that betalain pigments might also play diverse roles in plant life history.

To investigate this question, we took a three-pronged approach using horticultural varieties of *M. jalapa*. Recognizing that cultivation of this species began as many as 600 years ago for medicinal purposes and showy flowers (Showalter 1934; Leal et al. 2001), we decided to perform two generations of haphazard outcrossing within and among varieties before starting our studies. Although the history of artificial selection limits the interpretation of our results to some extent, it is worth noting that many important results regarding the architecture and evolution of plant pigment pathways were revealed using agricultural species such as maize (Coe et al. 1981) and tomato (Butelli et al. 2008). We first used an observational study to document how betacyanin and betaxanthin concentrations in floral tissues were associated with floral color morph (in this study, yellow, pink, red, and magenta) and to determine whether these floral color morphs differed with re-

spect to the concentrations of leaf betalains between early and late life stages. Simultaneously, we used these plants to investigate whether variation in floral and leaf pigmentation was associated with a series of phenological and performance measures to investigate whether there may be trade-offs associated with pigment production. Second, we performed an experimental assay with a generalist herbivore to investigate whether leaf betalain concentration was associated with resistance to herbivory. Finally, we performed a crossing experiment within and between the floral color morphs to investigate whether pollen performance was associated with the floral color of the donor or recipient plant.

Methods

Study System

Mirabilis jalapa (Nyctaginaceae) is a tropical American herb that produces compact inflorescences of flowers with actinomorphic, corolla-like calyxes on slightly decumbent, branching light-green stalks that often display a red or yellow tinge along the veins (Vanvinckenroye 1993; Leal et al. 2001). Flowers open in the early evening, and stigmas remain receptive until the following morning when the flowers close, often brushing the dehiscent anthers across the stigma, inducing self-pollination. Selfing appears to be the primary mode of reproduction in the field (Cruden 1973; Leal et al. 2001), with some anthers even dehiscing in the bud before the flowers open in the greenhouse (A. E. Berardi, personal observation). Despite the high frequency of self-pollination, hawkmoths have been observed pollinating *M. jalapa* in the field (Cruden 1973; Leal et al. 2001). The ovary is unicarpellate, has a long style (3–4 cm), and produces black rounded achenes.

Pigmentation and Phenotypic Associations

We grew 160 plants in 1-L pots with I-PV Canadian growing mix (Fafard, Agawam, MA) at 21°C and 60% RH under a 10-h supplemental light schedule with sodium-halide lamps. We obtained seed stock from five commercial suppliers (Earthly Goods, New Albany, IN; Gurney's Seed and Nursery, Greendale, IN; Holland Wildflower Farm, Elkins, AK; Main Street Seed and Supply, Bay City, MI; and Veseys, York, Prince Edward Island, Canada). The seeds used in this study were the product of two generations of haphazard outcrossing among these original parental lines to try to maximize genetic variation and eliminate breeder-specific selection effects. Plants were watered daily and randomly rotated every week in the Colgate University Greenhouse.

We recorded days to germination, days to first true leaves, and days until first flower. We recorded weekly measures of plant height from germination through first flower (10 wk) and estimated the growth rate of each plant using a linear regression, which was a good fit for the pooled data ($R^2 = 0.976$). At flowering, we identified color morph by sight (yellow, pink, red, magenta) and took spectral data from replicate flowers at three random locations on different petals using a S2000 miniature fiber-optic spectrophotometer with a PX-2 pulsed-xenon lamp (Ocean Optics, Dunedin, FL). We recorded the percentage reflectance from 250–750 nm in 0.38-nm increments. Replicate measures of percentage reflectance were

highly correlated within and among flowers on individual plants ($r > 0.90$; $P < 0.001$), and we therefore pooled these measures to obtain a single estimate of percentage reflectance at each wavelength for each plant.

We collected two samples of leaf tissue from separate leaves using a standard single-hole punch at 2 wk postgermination and again at first flowering (10 wk postgermination). We also collected two samples of petal tissue from two different flowers per plant at first flowering. We then weighed the samples and placed them in a 1.5-mL microfuge tube on ice with 1.1 mL of betalain extraction solution (80% methanol and 50 mM ascorbic acid; Kobayashi 2002), where they were ground with a micropestle, shaken on ice for 10 min, and centrifuged at 14,000 rpm for 20 min at 4°C. We transferred 1 mL of supernatant to a cuvette and analyzed it with a DU 800 spectrophotometer (Beckman Coulter, Danvers, MA). We recorded absorbance values at 480 nm (betaxanthin) and 538 nm (betacyanin) for each sample. We used the absorbance values in combination with the weight data and appropriate molecular extinction coefficients in a modified Beer-Lambert's equation to calculate the amount of betaxanthin or betacyanin present in the sample in units of milligrams per 100 g leaf tissue (Girod and Zyrd 1991; Cai et al. 1998; Kugler et al. 2004). To estimate betacyanin concentration, we used the molecular weight (MW) and molecular extinction coefficient (ϵ) of betanin (MW = 550 g/mol; $\epsilon = 60000$ L/mol cm in H₂O; $\lambda = 538$ nm; Kugler et al. 2004). Betaxanthins were estimated using parameters from vulgaxanthin I (MW = 339 g/mol; $\epsilon = 48000$ L/mol cm in H₂O; $\lambda = 480$ nm; Girod and Zyrd 1991; Kugler et al. 2004). Replicate estimates of betacyanin (2 wk: $r = 0.99$; 10 wk: $r = 0.62$; flower: $r = 0.99$) and betaxanthin (2 wk: $r = 0.92$; 10 wk: $r = 0.72$; flower: $r = 0.54$) were highly correlated ($P < 0.001$) and averaged to obtain a single per-plant estimate of betacyanin and betaxanthin in leaves at weeks 2 and 10 and in flowers. Three weeks after flowering, we cut all plants at the base of the stem, measured total leaf area using a LI-3000 leaf area meter and LI-3050C transparent belt conveyor (LICOR Biosciences, Lincoln, NE), and separately dried the stems and leaves of each plant at 60°C for 10 d to obtain dry biomass.

We took multiple approaches to investigate how floral color morphs varied with respect to the phenotypic traits measured. First, we used one-way ANOVAs to examine whether the morphs differed with respect to petal betacyanin and betaxanthin concentrations. We used a MANOVA to investigate whether the other traits discriminated the four floral color morphs and calculated a Kendall coefficient of concordance for the leaf pigmentation data (betacyanin and betaxanthin concentrations at weeks 2 and 10), the phenological data (days to germination, first true leaves, first flower), and the growth/biomass data (growth rate, total leaf area, stem and leaf biomass) to see whether there was consistency with how individuals varied with respect to these traits (e.g., whether the rank order of individuals' leaf pigment concentrations at weeks 2 and 10 was consistent). We used a series of one-way ANOVAs to investigate the nature of among-morph variation for each of these traits alone.

We performed a simple correlation analysis with sequential Bonferroni correction to investigate how betacyanin and betaxanthin levels in leaves covaried with the phenological data

and the growth/biomass data. Given the strong correlations among total leaf area, stem biomass, and leaf biomass, we used the total leaf area data in this analysis and excluded the biomass data. To investigate whether the structure of covariation among these nine traits (betacyanin and betaxanthin concentrations in leaves at weeks 2 and 10, days to germination, first true leaves, first flower, growth rate, total leaf area) was maintained among the four color morphs, we used the common principal components (CPC) method (Flury 1988) and the CPC program (<http://pages.uoregon.edu/pphil/programs/cpc/cpc.htm>). This technique is commonly used to infer the degree of multivariate similarity among covariance matrices and uses a "jump-up" and "step-up" approach (Phillips and Arnold 1999). The jump-up technique utilizes log-likelihood ratio tests to compare a model that assumes complete heterogeneity among the color morph covariance matrices (i.e., unrelated) to progressively similar models (i.e., number of shared principal components to proportionality to equality) until a statistically significant deviation is encountered. The step-up approach instead tests the likelihood that a particular model is valid (e.g., two shared principal components among the morphs) against the next-lower model (e.g., one shared principal component among the morphs). Traits were standardized to units of within-trait standard deviations (zero mean, unit variance) prior to calculating the phenotypic variance-covariance matrices.

Pigmentation and Herbivory

In this second study, we planted 250 seeds that were the product of one generation of haphazard outcrossing among parental stocks obtained from four commercial suppliers (Earthly Goods, Gurney's Wildflower Farm, Main Street Seed and Supply, and Veseys). Plants were grown under the same conditions as described above.

To test whether leaf pigment concentration was associated with herbivore damage, we performed a leaf damage bioassay using *Popillia japonica* (Japanese beetles; Scarabaeidae) caught locally in Hamilton, New York, using traps (Tanglefoot, Grand Rapids, MI). We chose to use *P. japonica* because this species is a widespread generalist herbivore and was observed damaging *M. jalapa* in local gardens, although not to the extent of other plants (F. M. Frey, personal observation). Generalist herbivores should be prone to the general chemical defenses of plants, whereas specialist herbivores tend to be less sensitive to their host plant's defensive chemicals (Agrawal and Fishbein 2006). Therefore, if betalains serve as or are associated with defensive chemicals in *M. jalapa*, we predicted that we would be able to observe the effects of plant defense with these generalist herbivores. We housed the Japanese beetles in growth chambers at 23°–25°C with broad-spectrum fluorescent lighting from 0630 to 2100 hours to simulate summer day length and fed them lettuce leaves (*Lactuca sativa*; Asteraceae) ad lib. prior to the assays. At 4 wk postgermination, we randomly selected 93 plants to use in this study, measured betaxanthin and betacyanin concentrations (mg/100 g) from three separate leaves as described above, and pooled the measures to obtain a single estimate of betaxanthin and betacyanin for each plant. We cut newly opened leaves at the petiole and photographed them with a Nikon D100 camera on a white background with

a scale bar. We then placed each leaf in a 100 × 15-mm petri dish with a wet paper towel and one randomly selected Japanese beetle. We starved the Japanese beetles for at least 4 h prior to the feeding trial so that no food remained in their gut (Held and Potter 2003) and weighed them immediately before being placed in the petri dish with the leaf. All treatments were initiated between 1300 and 1500 hours and continued for a 24-h period in the growth chamber. At the conclusion of the trial, we weighed the beetles and photographed the leaves again on a white background with a scale bar. Each plant was tested three separate times with a different Japanese beetle.

We used ImageJ (<http://rsbweb.nih.gov/ij>) to calculate percent leaf removal as final leaf area/initial leaf area and calculated the percent change in beetle weight as (final weight – initial weight)/initial weight. There were a total of 78 plants for which we had complete replication and measures of leaf betaxanthin and betacyanin concentrations, and for each plant we averaged the three measures of percent leaf removal and percent weight change. The data for each of the four variables were not significantly different from a normal distribution (one-sample Kolmogorov-Smirnov tests, $P > 0.10$), and we used a simple correlation analysis to examine associations between them.

Pigmentation and Pollen Performance

To test whether floral color morphs differed with respect to pollen performance, we used the remaining plants from the second study (250 planted, one generation of haphazard outcrossing among parental lines) for a pollen tube growth experiment at flowering (yellow = 67, pink = 16, red = 19, magenta = 38). We divided plants by floral color and randomly designated 75 plants as pollen recipients and 65 plants as pollen donors, crossing them such that each pollen recipient plant received pollen from 2 to 4 pollen donor plants in duplicate. Given the timing of flowering among plants, it was not possible to have complete replication of pollen donor color morphs on all recipients. We emasculated flowers on pollen-recipient plants the morning prior to opening to prevent self-pollination. When the stigmatic surface was receptive (between 1700 and 2200 hours), we transferred a relatively high pollen load (20–30 grains; Niesenbaum 1999) to two separate flowers using pollen pooled from at least two flowers from the identified donor. We performed 196 outcross pollinations between the color morphs (392 crosses) and 39 self-pollinations (78 crosses). One hour following pollination, we collected the styles and fixed them in 70% ethanol. After rinsing with distilled water, we softened and cleared styles with 1 M NaOH and then rinsed them again with distilled water before staining with 0.1% aniline blue in 0.1 M K_3PO_4 (Niesenbaum 1999). We measured the length of each style with digital calipers and then examined them at 100× under UV light. The number of pollen tubes per style ranged from 0 to 8, and the length of the longest pollen tube per style was measured from the stigmatic surface to the tip of the pollen tube using SPOT Imaging Solutions (Sterling Heights, MI). We averaged the two style length measures and pollen tube lengths from each pollen recipient-donor combination.

We used a one-way ANOVA to investigate whether variation in style length was associated with the floral color of the re-

ipient plant. To analyze the pollen tube data, we first used a series of log-likelihood tests (“G-tests”) to see whether the proportion of crosses that resulted in pollen tube penetration of the style was associated with self- or outcross pollination, the floral color of the pollen recipient, and the floral color of the pollen donor ($n = 235$). We then looked at those data for which pollen tubes penetrated the style ($n = 178$) and used a one-way ANOVA to examine whether variation in pollen tube length was associated with self- or outcross pollination. Because this analysis showed that pollen tube lengths were not statistically different between outcross and self-treatments, we then used a two-way ANOVA to see whether variation in pollen tube length was associated with the floral color of the pollen donor or recipient ($n = 178$). We also performed this same analysis on the subset of data that resulted from outcross pollination ($n = 143$). With the exception of the CPC analyses, all statistics were performed using SPSS for Mac OSX (SPSS v18, Chicago, IL).

Results

Pigmentation and Phenotypic Associations

Of the total 160 plants grown, there were 18 yellow, 10 pink, 95 red, and 37 magenta morphs (fig. 1A). The spectral measures indicate that these are relatively discrete floral color morphs, with a large amount of variation among floral color morphs between 400 and 650 nm and relatively little variation within floral color morphs (fig. 1B). In addition, the four color morphs varied predictably with respect to the petal concentration of betaxanthin ($F_{3,156} = 23.53$, $P < 0.001$) and betacyanin ($F_{3,156} = 38.26$, $P < 0.001$), with yellow morphs having a large concentration of betaxanthin, pink morphs having little betaxanthin and betacyanin, red morphs having high concentrations of both pigments, and magenta morphs having a large concentration of betacyanin (fig. 1C).

The phenotypic traits measured (leaf betaxanthin and betacyanin at weeks 2 and 10; days to germination, first true leaves, and first flower; growth rate; leaf area; stem and leaf biomass) significantly discriminated the four floral color variants (Wilk’s $\lambda = 0.66$, $F_{33,401} = 1.83$, $P < 0.01$). As would be expected given the nature of the traits measured, there was significant consistency in how these 11 traits varied among individuals in the data set (Kendall coefficient of concordance: $W = 0.85$, $df = 10$, $P < 0.001$; fig. 2). This consistency was strongest for the phenological traits (days to germination, first true leaves, and first flower; $W = 1.0$, $df = 2$, $P < 0.001$) and more moderate for the growth/biomass traits (growth rate, leaf area, stem and leaf biomass; $W = 0.62$, $df = 3$, $P < 0.001$) and pigmentation traits (leaf betaxanthin and betacyanin concentrations at weeks 2 and 10; $W = 0.39$, $df = 3$, $P < 0.001$). There was significant variation among the floral color morphs for leaf betacyanin concentration (mg/100 g) at 2 wk postgermination (yellow: 1.33 ± 0.17 SE; pink: 3.96 ± 0.71 SE; red: 6.80 ± 0.45 SE; magenta: 6.90 ± 0.76 SE; $F_{3,156} = 10.45$, $P < 0.001$) but not for betaxanthin at 2 wk postgermination ($F_{3,156} = 0.58$, $P > 0.10$). At 10 wk postgermination, the leaf betacyanin concentration trend at 2 wk persisted but was not statistically significant ($F_{3,156} = 2.55$, $P = 0.058$), and the floral color morphs did not vary for betaxanthin concen-

A

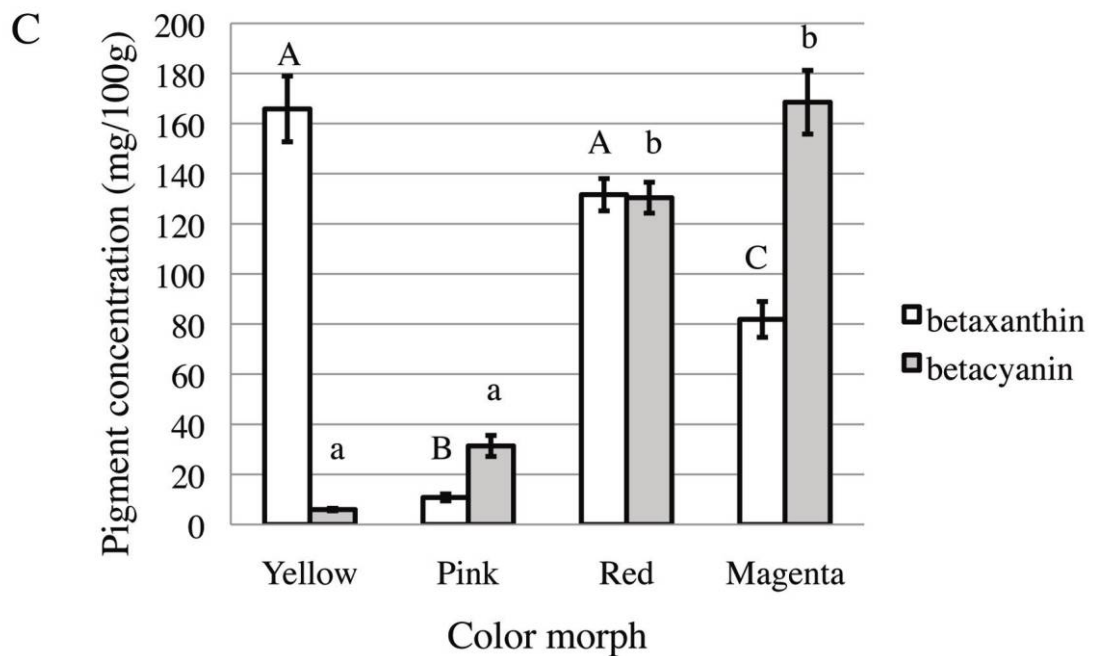
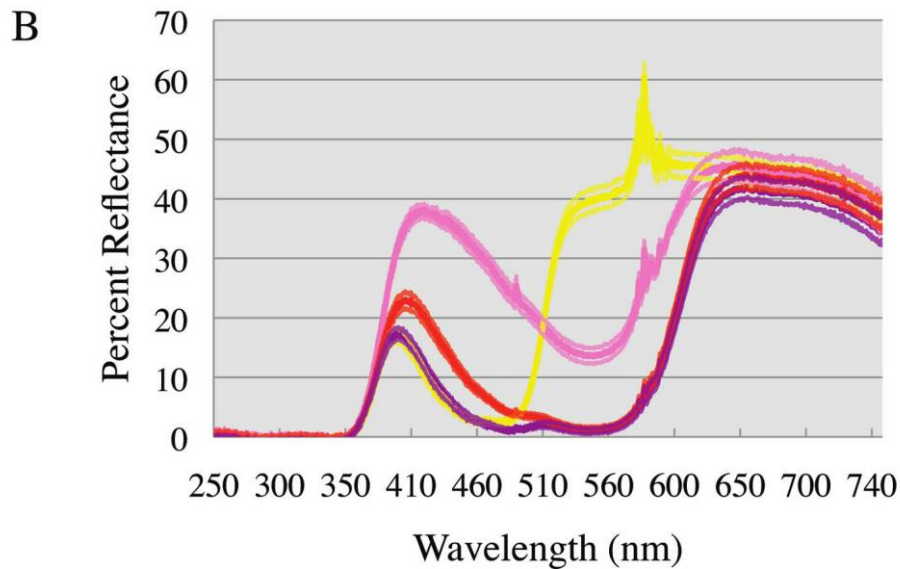


Fig. 1 A, Yellow, pink, red, and magenta floral color morphs in *Mirabilis jalapa*. B, Average percent reflectance from 250 to 750 nm for each of the floral color morphs \pm 1 SE; there is substantial variation among color morphs and little variation within color morphs. C, Average concentration (mg/100 g) of betaxanthin (open bars) and betacyanin (filled bars) in petals \pm 1 SE. Uppercase letters denote homogeneous subsets among the morphs for betaxanthin ($F_{3,156} = 23.53$, $P < 0.001$), and lowercase letters denote homogeneous subsets for betacyanin ($F_{3,156} = 38.26$, $P < 0.001$).

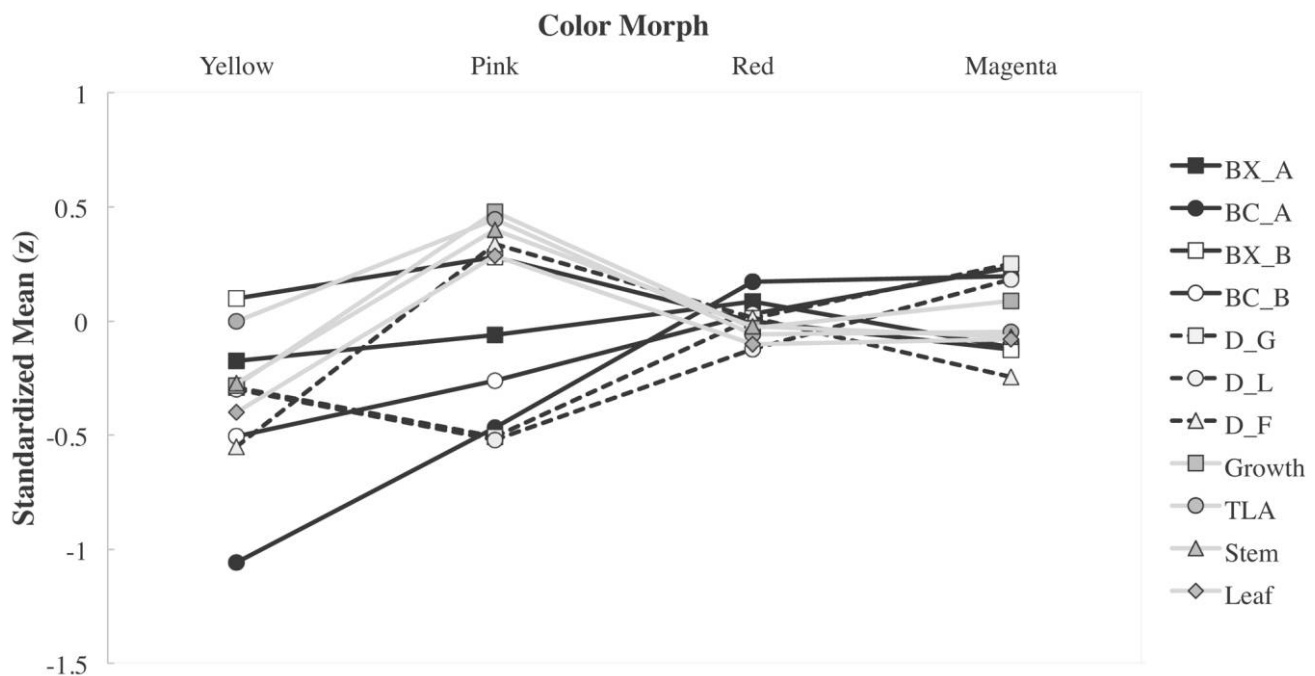


Fig. 2 Means of standardized phenotypic traits (zero mean, unit variance) for four floral color variants of *Mirabilis jalapa*. Leaf pigment concentrations (solid line) at 2 wk (black symbols) and 10 wk (white symbols), phenological traits (dashed line, light gray symbols), and growth/biomass traits (gray line, dark gray symbols) are shown. Legend abbreviations are as in table 1.

tration ($F_{3,156} = 0.51, P > 0.10$). In addition, there was no significant variation among the floral color morphs for days to germination ($F_{3,156} = 2.19, P > 0.05$), days to first true leaves ($F_{3,154} = 1.94, P > 0.10$), days to first flower ($F_{3,154} = 0.58, P > 0.10$), growth rate ($F_{3,156} = 1.38, P > 0.10$), total leaf area ($F_{3,155} = 0.78, P > 0.10$), stem biomass ($F_{3,153} = 1.10, P > 0.10$), or leaf biomass ($F_{3,153} = 1.21, P > 0.10$).

Leaf betacyanin and betaxanthin concentrations were strongly correlated with each other at weeks 2 and 10 post-germination, and leaf betacyanin concentration was strongly correlated between the 2- and 10-wk measures (table 1). Not surprisingly, days to germination, first leaf, and first flower were highly correlated, and total leaf area, stem biomass, and leaf biomass were highly correlated (table 1). Stem biomass was positively correlated with growth rate and negatively correlated with days to first flower; interestingly, leaf betaxanthin concentration at week 10 was positively correlated with days to first flower (table 1).

The CPC analyses showed that there was significant heterogeneity among the color morphs with respect to the structure of the phenotypic covariance matrices (table 2). In the jump-up approach, the null hypothesis assuming complete heterogeneity among the four color morphs was indistinguishable from a model that assumed that one common principal component was weakly rejected ($\chi^2_{24} = 37.1, P < 0.05$). The null hypothesis assuming complete heterogeneity among the four color morphs was indistinguishable from a model that assumed that three principal components were more strongly rejected ($\chi^2_{63} = 97.5, P < 0.01$). Likewise, the step-up technique rejected the model assuming that one shared principal component was

indistinguishable from complete heterogeneity ($\chi^2_{24} = 37.1, P < 0.05$) and more strongly rejected the model assuming that three shared principle components were indistinguishable from the model assuming two shared principle components ($\chi^2_{18} = 35.3, P < 0.01$). Taken together, these results indicate that the structure of the phenotypic covariation matrices for these traits is rather unrelated; at most, color morphs share a common orientation for only two of the nine axes of variation.

Pigmentation and Herbivory

At 4 wk postgermination, leaf betaxanthin (mean: 29.41 mg/100 g \pm 1.00 SE) and betacyanin (mean: 6.61 mg/100 g \pm 0.35 SE) were highly correlated ($r = 0.74, P < 0.001$) and negatively associated with percent leaf removal by Japanese beetles (betaxanthin: $r = -0.32, P < 0.01$; betacyanin: $r = -0.29, P < 0.01$; fig. 3). Percent leaf removal was positively associated with percent beetle weight change ($r = 0.47, P < 0.001$).

Pigmentation and Pollen Performance

Style length did not vary among the floral color morphs (mean: 3.79 cm \pm 0.15 SE; $F_{3,231} = 0.173, P > 0.10$). There was a greater proportion of pollen tubes penetrating the style in self-pollinations compared to outcross pollinations (90% vs. 73%; $G = 5.79, P < 0.05$) but no evidence that this difference was associated with the floral color of the donor or recipient plant ($P > 0.10$). There was no evidence that pollen tube length was associated with self- or outcross pollination ($F_{1,176} = 0.19, P > 0.10$); however, there was a strong effect of

Table 1
Phenotypic Correlations in *Mirabilis jalapa*

	BX_A	BC_A	BX_B	BC_B	D_G	D_L	D_F	Growth	TLA	Stem	Leaf
BX_A	...	<u>.54</u>	.06	.05	-.18	-.02	-.04	-.01	-.11	-.07	-.11
BC_A	<.01	...	-.02	<u>.47</u>	.06	.13	-.08	.10	-.07	.02	-.05
BX_B	ns	ns	...	<u>.40</u>	.10	.03	<u>.47</u>	-.20	.19	.05	.09
BC_B	ns	<.01	<.0103	.01	.03	-.04	.01	.09	.07
D_G	<.05	ns	ns	ns	...	<u>.89</u>	<u>.25</u>	<u>.30</u>	.05	.02	.05
D_L	ns	ns	ns	ns	<.01	...	<u>.24</u>	<u>.32</u>	-.02	-.07	-.01
D_F	ns	ns	<.01	ns	<.01	<.01	...	<u>-.26</u>	-.04	<u>-.24</u>	-.05
Growth	ns	ns	<.05	ns	<.01	<.01	<.0114	<u>.30</u>	.16
TLA	ns	ns	<.05	ns	ns	ns	ns	ns	...	<u>.64</u>	<u>.73</u>
Stem	ns	ns	ns	ns	ns	ns	<.01	<.01	<.01	...	<u>.83</u>
Leaf	ns	ns	ns	ns	ns	ns	ns	ns	<.01	<.01	...

Note. Traits measured are leaf betaxanthin and betacyanin concentration (mg/100 g) at 2 wk (BX_A, BC_A, respectively) and 10 wk (BX_B, BC_B, respectively) postgermination, days to germination (D_G), days to first true leaf (D_L), days to first flower (D_F), growth rate (Growth), total leaf area (TLA), stem biomass (Stem), and leaf biomass (Leaf). Pearson's correlation coefficients (above diagonal) and *P* values (below diagonal) are shown. Underlined correlation coefficients are those considered statistically significant following a Bonferroni correction.

the floral color of the pollen donor and an interaction between the floral color of the pollen donor and recipient (recipient: $F_{3,162} = 1.04$, $P > 0.10$; donor: $F_{3,162} = 3.86$, $P < 0.05$; interaction: $F_{9,162} = 3.62$, $P < 0.001$). When only pollen tubes resulting from outcross pollination were considered, the effect of the pollen donor floral color was even stronger (recipient: $F_{3,127} = 1.07$, $P > 0.10$; donor: $F_{3,127} = 4.21$, $P < 0.01$; interaction: $F_{9,127} = 3.60$, $P < 0.001$). Pollen tubes from yellow and pink donor morphs were shorter than the mean, and pollen tubes from red and magenta donor morphs were longer than the mean, with pollen from yellow and red donor morphs performing best on yellow-flowered recipients, pollen from pink donor morphs performing equally well on all recipient morphs, and pollen from magenta donor morphs performing least well on yellow-flowered recipients (fig. 4).

Discussion

In this study, we investigated whether betalain pigments might play diverse roles in plant life history using a phenotypic and experimental approach with horticultural lines of *Mirabilis jalapa*. *Mirabilis jalapa* produces several color morphs, including white flowers lacking betalain pigments and broken color patterns. We observed four discrete floral color morphs in this study: yellow, pink, red, and magenta. These morphs differed predictably with petal betaxanthin and betacyanin concentrations: yellow petals had high levels of betaxanthin, magenta petals had high levels of betacyanin, red petals had high and roughly equal concentrations of both pigments, and pink petals had low levels of betacyanin and very little betaxanthin. Floral color morphs also differed with respect to leaf betacyanin concentration at 2 wk postgermination, with red- and magenta-flowering morphs having higher concentrations of leaf betacyanin than yellow-flowering or pink-flowering morphs. These differences between the morphs with respect to leaf betacyanin concentration persisted but weakened by 10 wk postgermination. The color morphs did not significantly vary with respect to leaf betaxanthin concentration.

The differences among floral color morphs in leaf betacyanin concentration at the seedling stage led us to ask whether leaf pigment concentration might be associated with (1) a stress response at the seedling stage or (2) defense against herbivory at the seedling stage. Anthocyanins, the analog to betalains in

Table 2
Comparison of Phenotypic Covariance Matrices among the Four Floral Color Morphs of *Mirabilis jalapa*

	χ^2	df	<i>P</i>
Jump-up:			
Hypothesis:			
Equality	418.8	135	.0001
Proportionality	417.3	132	.0001
Full model	313.8	108	.0001
CPC (7)	307.8	105	.0001
CPC (6)	232.3	99	.0001
CPC (5)	202.6	90	.0001
CPC (4)	137.2	78	.0001
CPC (3)	97.5	63	.0035
CPC (2)	62.2	45	.0451
CPC (1)	37.1	24	.0423
Unrelated
Step-up:			
Model:			
Equality vs. proportionality	1.4	3	.6977
Proportionality vs. full CPC	103.5	24	.0001
Full CPC vs. CPC (7)	6.0	3	.1121
CPC (7) vs. CPC (6)	75.6	6	.0001
CPC (6) vs. CPC (5)	29.7	9	.0005
CPC (5) vs. CPC (4)	65.4	12	.0001
CPC (4) vs. CPC (3)	39.7	15	.0005
CPC (3) vs. CPC (2)	35.3	18	.0088
CPC (2) vs. CPC (1)	25.1	21	.2434
CPC (1) vs. unrelated	37.1	24	.0423
Unrelated

Note. We followed the jump-up and step-up procedures of Phillips and Arnold (1999). CPC = common principal components analysis.

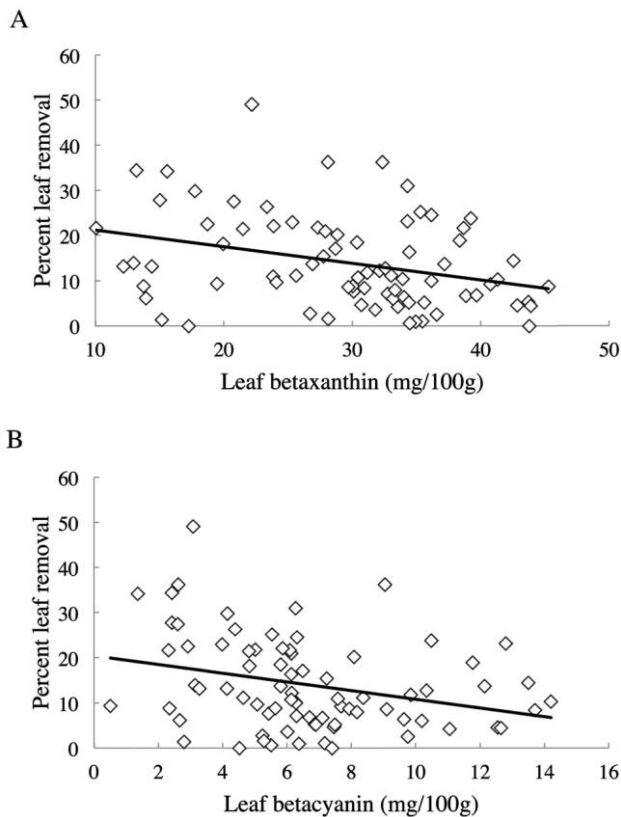


Fig. 3 Percent leaf removal is associated with leaf betalain concentration (mg/100 g). Plants with greater betaxanthin (A) and betacyanin (B) concentrations experienced less herbivore damage (A, $r = -0.32$, $P < 0.01$; B, $r = -0.29$, $P < 0.01$; trend line shown).

the plant kingdom, have been shown to accumulate in vegetative tissues under stressful situations in plants to act as free-radical scavengers, to reduce photoinhibition, to act as thermoregulators or osmoregulators, or even to serve as an antiherbivory signal (Gould 2004). Betalains themselves are chromoalkaloids and may act as part of the generalized defense system in *M. jalapa*. However, given that other alkaloids have been detected in *M. jalapa* and that betalain expression may be linked to alkaloid production, we suggest that betalains may act as a part of the *M. jalapa* defense syndrome or a suite of quantitative defensive traits (Agrawal and Fishbein 2006). We tested our hypothesis with generalist herbivores, which should be sensitive to the general chemical defenses of plants (Agrawal and Fishbein 2006). Our results showed that as betalain concentration increased, percent leaf removal decreased, although it was surprising to us that the association was stronger for betaxanthin concentration than betacyanin concentration. Clearly, further studies are needed to parse out the exact function of leaf betalain expression in herbivore defense, but these data suggest that some floral color morphs may be better defended against herbivores than others, providing a possible pleiotropic nature of pigment expression that is in line with work on other systems (Irwin et al 2003; Frey 2004; Strauss et al. 2004).

In addition to measuring leaf pigment concentration at 2

and 10 wk postgermination, we also took phenological data (days to germination, first true leaves, first flower) and growth/biomass data (growth rate, total leaf area, stem and leaf biomass) to investigate patterns of phenotypic variation among the color morphs and make inferences about the phenotypic architecture. Although these phenotypic patterns likely do not reflect the exact nature of the underlying genetic structure (Willis et al. 1991), data such as these are useful for understanding how the expression of floral color might be linked to other traits and how the evolution of floral color might take place in light of multiple selection pressures (i.e., whether betalain expression is associated to varying degrees with targets of fecundity, fertility, and viability selection). In a multivariate sense, the leaf pigmentation, phenological, and growth/biomass data significantly discriminated among the four color morphs, suggesting that the flower color morphs differ broadly in phenotypic architecture and perhaps targets of selection. Interestingly, pink-flowering morphs tended to be about a half standard deviation above the mean with respect to growth/biomass traits and about a half standard deviation below the mean with respect to days to germination and first true leaves (fig. 2), although in a strictly univariate sense color morphs did not significantly differ with respect to these six individual traits. These data may suggest a possible cost to betalain production in the form of a trade-off between allocation to the production of betalain pigments and aspects of life history

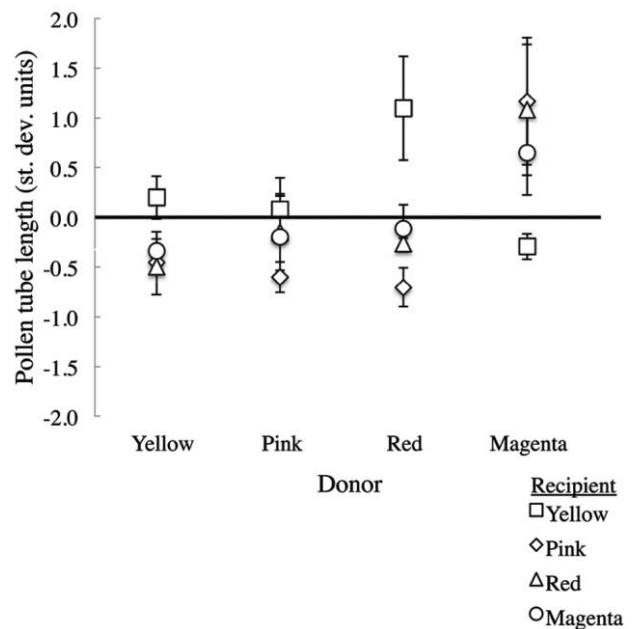


Fig. 4 Pollen tube length resulting from different donor and recipient floral color morph combinations. Data shown are for out-crossed combinations only and have been converted into z scores to more clearly illustrate differences among the combinations. Yellow-flowered donors are more successful on yellow-flowered recipients, pink-flowered donors are equally successful on all recipients, red-flowered donors are more successful on yellow-flowered recipients, and magenta-flowered donors are least successful on yellow-flowered recipients.

(Agrawal 2011); perhaps increased betalain production results in longer times to germination/first true leaf and slower growth rates, resulting in lower total leaf area and biomass. Taken together, these data suggest a possible pleiotropic link between betalain expression and life-history traits.

Floral color morphs did not vary with respect to average style length, and the proportion of pollen tubes that penetrated the style was consistently greater in self-pollinations compared to outcross pollinations, regardless of the floral color of the donor or recipient plant. These results are in line with other work on pollen competition in *M. jalapa* (Niesenbaum and Schueller 1997; Niesenbaum 1999). Interestingly, when pooled across all pollen recipients, pollen tubes from red- and magenta-flowered plants were longer than pollen tubes from yellow- and pink-flowered plants. There were also strong interactions between the floral color of the pollen donor and that of the pollen recipient with respect to average pollen tube length. When outcrossed, pollen from magenta-flowered morphs performed the poorest on yellow-flowered recipients, whereas pollen from yellow- and red-flowered morphs performed the best on yellow-flowered recipients. Although we have no data on betacyanin and betaxanthin concentrations in tapetal cells or stylar tissues, these structures are pigmented in the same fashion as the petals and could contain similar levels of betacyanins and betaxanthins. If so, it is possible that betacyanin production is positively associated with pollen development, and perhaps there is some relationship between betaxanthin level in the style and the performance of outcrossed pollen developed in high- or low-betaxanthin environments. These interactions between pollen donor and recipient floral color morphs may also contribute to the maintenance of high-betacyanin-containing and high-betaxanthin-containing floral color morphs in *M. jalapa*.

Our results suggest that betalain pigments have multiple ecological and evolutionary roles in plant life history. Betacyanin and betaxanthin expression might be indirect or direct

targets of sexual, viability, and fecundity selection (Arnold 1994). Clearly, flower color might influence rates of pollinator visitation (sexual selection), and there is some evidence to suggest hawkmoth preferences for yellow flowers over red flowers (Hirota et al. 2012). Our results show that betacyanin and betaxanthin concentrations in leaves were negatively associated with leaf damage by a generalist herbivore (viability selection) and that pollen performance was associated with betacyanin and betaxanthin levels in the donor or recipient flowers (fecundity selection). In addition, the phenotypic analyses suggest that the floral color morphs significantly differ with respect to phenotypic architecture. Not only did the measured traits significantly discriminate the floral color morphs but also little of the multivariate structure of the phenotypic covariance matrices was shared among the floral color morphs. If the patterns revealed in these phenotypic analyses reflect underlying genetic covariance structures, it would appear that the color morphs have different genetic architectures and therefore may follow different evolutionary courses when exposed to potential indirect selection pressures on betacyanin and betaxanthin expression.

Acknowledgments

We thank R. Stagnaro for excellent greenhouse care for the research plants. S. Forster, J. LaValle, A. Lopez, K. Soule, S. Stolz, A. Watkins, and A. White assisted in data collection. We also thank J. Antonovics, P. Fields, E. Schultheis, and D. Taylor for their comments on early versions of the manuscript. The comments of two anonymous reviewers greatly improved the quality and clarity of the work. Funding was provided through a Carter-Wallace Research Fellowship (F. M. Frey), the Colgate University Research Council (F. M. Frey), and the Sherman-Fairchild Research Fellowship (A. E. Berardi and J. H. Wells).

Literature Cited

- Agrawal AA 2011 Current trends in the evolutionary ecology of plant defence. *Funct Ecol* 25:420–432.
- Agrawal AA, M Fishbein 2006 Plant defense syndromes. *Ecology* 87: S132–S149.
- Armbruster WS 2002 Pleiotropy and the evolution of flower colour. *J Evol Biol* 15:468–486.
- Arnold SJ 1994 Is there a unifying concept of sexual selection that applies to both plants and animals? *Am Nat* 144(suppl):S1–S12.
- Brockington SF, RH Walker, BJ Glover, PS Soltis, DE Soltis 2011 Complex pigment evolution in the Caryophyllales. *New Phytol* 190:854–864.
- Butelli E, L Titta, M Giorgio, H-P Mock, A Matros, S Peterek, EGWM Schijlen, et al 2008 Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. *Nat Biotechnol* 26:1301–1308.
- Cai Y, M Sun, H Wu, R Huang, H Corke 1998 Characterization and quantification of betacyanin pigments from diverse *Amaranthus* species. *J Agric Food Chem* 46:2063–2070.
- Campbell DR, NM Waser, EJ Melendez-Ackerman 1997 Analyzing pollinator-mediated selection in a plant hybrid zone: hummingbird visitation patterns on three scales. *Am Nat* 149:295–315.
- Clement JS, TJ Mabry 1996 Pigment evolution in the Caryophyllales: a systematic overview. *Bot Acta* 109:360–367.
- Coe EH, SM McCormick, SA Modena 1981 White pollen in maize. *J Hered* 72:318–320.
- Cruden RW 1973 Reproductive biology of weedy and cultivated *Mirabilis* (Nyctaginaceae). *Am J Bot* 60:802–809.
- Engels JMM, WNM van Kester, CJT Spitters, L Vosselman, AC Zeven 1975 Investigations of the inheritance of flower variegation in *Mirabilis jalapa* L. 1. General introduction and 2. Inheritance of colour in uniformly coloured flowers. *Euphytica* 24:1–5.
- Faegri K, L van der Pijl 1979 The principles of pollination ecology. Pergamon, Oxford.
- Fineblum WL, MD Rausher 1997 Do floral pigmentation genes also influence resistance to enemies? the *W* locus in *Ipomoea purpurea*. *Ecology* 78:1646–1654.
- Flury B 1988 Common principal components and related multivariate models. Wiley, New York.
- Frey FM 2004 Opposing natural selection from herbivores and pathogens may maintain floral-color variation in *Claytonia virginica* (Portulacaceae). *Evolution* 58:2426–2437.
- 2007 Phenotypic integration and the potential for independent color evolution in a polymorphic spring ephemeral. *Am J Bot* 94:437–444.
- Frey FM, J Dunton, K Garland 2011 Floral color variation and as-

- sociations with fitness-related traits in *Malva moschata* (Malvaceae). *Plant Species Biol* 26:235–243.
- Girod PA, JP Zyrd 1991 Secondary metabolism in cultured red beet (*Beta vulgaris* L.) cells: differential regulation of betaxanthin and betacyanin biosynthesis. *Plant Cell Tissue Organ Cult* 25:1–12.
- Gould KS 2004 Nature's Swiss Army knife: the diverse protective roles of anthocyanins in leaves. *J Biomed Biotechnol* 2004:314–320.
- Harborne JB 1991 Flavonoid pigments. Pages 389–426 in GA Rosenthal, MR Berenbaum, eds. *Herbivores: their interactions with secondary plant metabolites*. Vol 1. Academic Press, San Diego, CA.
- Harris NN, H Javellana, KM Davies, DH Lewis, PE Jameson, SC Deroles, KE Calcott, KS Gould, KE Schwinn 2012 Betalain production is possible in anthocyanin-producing plant species given the presence of DOPA-dioxygenase and L-DOPA. *BMC Plant Biol* 12: 34.
- Hatlestad GJ, RM Sunnadaniya, NA Akhavan, A Gonzalez, IR Goldman, J Mitchell McGrath, AM Lloyd 2012 The beet R locus encodes a new cytochrome P450 required for red betalain production. *Nat Genet* 44:816–819.
- Held DW, DA Potter 2003 Characterizing toxicity of *Pelargonium* spp. and two other reputedly toxic plant species to Japanese beetles (Coleoptera: Scarabaeidae). *Environ Entomol* 32:873–880.
- Hirota SK, K Nitta, Y Kim, A Kato, N Kawakubo, AA Yasumoto, T Yahara 2012 Relative role of flower color and scent on pollinator attraction: experimental tests using F1 and F2 hybrids of daylily and nightlily. *PLoS ONE* 7:e39010.
- Irwin RE, SY Strauss 2005 Flower color microevolution in wild radish: evolutionary response to pollinator-mediated selection. *Am Nat* 165:225–237.
- Irwin RE, SY Strauss, S Storz, A Emerson, G Guibert 2003 The role of herbivores in the maintenance of a flower color polymorphism in wild radish. *Ecology* 84:1733–1743.
- Keller W 1936 Inheritance of some major color types in beets. *J Agric Res* 52:27–38.
- Kobayashi N 2002 Contributions to betalain biochemistry: new structures, condensation reactions, and vacuolar transport. PhD diss. Universität Halle (Saale), Germany.
- Koes R, W Verweij, F Quattrocchio 2005 Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends Plant Sci* 10:236–242.
- Kugler F, FC Stintzing, R Carle 2004 Identification of betalains from petioles of differently colored Swiss chard (*Beta vulgaris* L. ssp. *cicla* [L.] Alef. Cv. Bright Lights) by high-performance liquid chromatography-electrospray ionization mass spectrometry. *J Agric Food Chem* 52:2975–2981.
- Leal AA, Y Terada, M de Fátima Pires da Silva Machado 2001 Floral biology of a population of *Mirabilis jalapa* L. (Nyctaginaceae) from southern Brazil. *Maringa* 23:587–591.
- Lee DW, KS Gould 2002 Anthocyanins in leaves and other vegetative organs: an introduction. Pages 1–16 in KS Gould, DW Lee, eds. *Anthocyanins in leaves: advances in botanical research*. Vol 37. Academic Press, London.
- Mabry T 1980 Betalains. Pages 513–533 in E Bell, B Charwood, eds. *Encyclopedia of plant physiology, secondary plant products*. Springer, Berlin.
- Mo Y, C Nagel, LP Taylor 1992 Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. *Proc Natl Acad Sci USA* 89:7213–7217.
- Niesenbaum RA 1999 The effects of pollen load size and donor diversity on pollen performance, selective abortion, and progeny vigor in *Mirabilis jalapa* (Nyctaginaceae). *Am J Bot* 86:261–268.
- Niesenbaum RA, SK Schueller 1997 Effects of pollen competitive environment on pollen performance in *Mirabilis jalapa* (Nyctaginaceae). *Sex Plant Reprod* 10:101–106.
- Phillips PC, SJ Arnold 1999 Hierarchical comparison of genetic variance-covariance matrices. I. Using the Flury hierarchy. *Evolution* 53:1506–1515.
- Piatelli M, L Minale 1964 Pigments of the Centrospermae. II. Distribution of betacyanins. *Phytochemistry* 3:547–557.
- Piatelli M, L Minale, RA Nicolaus 1965 Pigments of the Centrospermae. V. Betaxanthins from *Mirabilis jalapa* L. *Phytochemistry* 4:817–823.
- Schemske DW, HD Bradshaw Jr 1999 Pollinator preferences and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proc Natl Acad Sci USA* 96:11910–11915.
- Shimada S, YT Inoue, M Sakuta 2005 Anthocyanidin synthase in non-anthocyanin-producing Caryophyllales species. *Plant J* 58:950–959.
- Showalter HM 1934 Self flower-color inheritance and mutation in *Mirabilis jalapa* L. *Genetics* 19:568–580.
- Spitters CJT, L Vosselman, JMM Engels, WNM van Kester, AC Zeven 1975 Investigations of flower variegation in *Mirabilis jalapa* L. 6. Genetic system of flower variegation and speculation about its existence. *Euphytica* 24:323–332.
- Strauss SY, RE Irwin, VM Lambrix 2004 Optimal defence theory and flower petal colour predict variation in the secondary chemistry of wild radish. *Ecology* 92:132–141.
- Strauss SY, JB Whittall 2006 Non-pollinator agents of selection on floral traits. Pages 120–138 in LD Harder, SCH Barrett, eds. *The ecology and evolution of flowers*. Oxford University Press, Oxford.
- Tanaka Y, N Sasaki, A Ohmiya 2008 Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *Plant J* 54:733–749.
- Van Kester WNM, CJT Spitters, L Vosselman, JMM Engels, AC Zeven 1975 Investigations of the inheritance of floral variegation in *Mirabilis jalapa* L. 3. Somatic chromosome number, 4. Distribution of the pigments, and 5. Chromatographic studies. *Euphytica* 24:6–12.
- Vanvinckenroye PE, LP Cresens, R Decraene, E Smets 1993 A comparative floral developmental study in *Pisonia*, *Bougainvillea*, and *Mirabilis* (Nyctaginaceae) with special emphasis on the gynoeceum and floral nectaries. *Bull Jard Bot Nat Belg* 62:69–96.
- Vogt T, LP Taylor 1995 Flavonol 3-O-glycosyltransferases associated with *Petunia* pollen produce gametophyte-specific flavonol diglycosides. *Plant Physiol* 108:903–911.
- Willis JH, JA Coyne, M Kirkpatrick 1991 Can one predict the evolution of quantitative characters without genetics? *Evolution* 45: 441–444.
- Ylstra B, RM Touraev, B Moreno, E Stoger, AJ van Tunen, O Vicente, JNM Mol, E Heberle-Bors 1992 Flavonols stimulate development, germination, and tube growth of tobacco pollen. *Plant Physiol* 100: 902–907.