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Research

Ecological causes and consequences of flower color polymorphism in a self-pollinating plant (*Boechera stricta*)

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Summary

- Intraspecific variation in flower color is often attributed to pollinator-mediated selection, yet this mechanism cannot explain flower color polymorphisms in self-pollinating species. Indirect selection mediated via biotic and abiotic stresses could maintain flower color variation in these systems.
- The selfing forb, *Boechera stricta*, typically displays white flowers, but some individuals produce purple flowers. We quantified environmental correlates of flower color in natural populations. To disentangle plasticity from genotypic variation, we performed a multiyear field experiment in five gardens. In controlled conditions, we evaluated herbivore preferences and the effects of drought stress and soil pH on flower color expression.
- In natural populations, purple-flowered individuals experienced lower foliar herbivory than did their white-flowered counterparts. This pattern also held in the common gardens. Additionally, low-elevation environments induced pigmented flowers (plasticity), and the likelihood of floral pigmentation decreased with source elevation of maternal families (genetic cline). Viability selection favored families with pigmented flowers. In the laboratory, herbivores exerted greater damage on tissue derived from white- vs purple-flowered individuals. Furthermore, drought induced pigmentation in white-flowered lineages, and white-flowered plants had a fecundity advantage in the well-watered control.
- Flower color variation in selfing species is probably maintained by herbivory, drought stress, and other abiotic factors that vary spatially.

Introduction

Angiosperms display impressive variation in flower color across taxa and even within species (Gigord *et al.*, 2001; Rausher, 2008; Hopkins & Rausher, 2012; Koski & Ashman, 2016). Macroevolutionary transitions between flower colors are often accompanied by shifts in pollinators (Wessinger & Rausher, 2012), and single quantitative trait loci (QTLs) can underlie both flower color variation and pollinator visitation (Bradshaw & Schemske, 2003). Additionally, interspecific competition for pollinators can cause species living in sympatry to diverge in flower color (Muchhala *et al.*, 2014). Flower color can be a reliable indicator of the presence of nectar, thereby directly influencing pollinator visitation rates (Kantsa *et al.*, 2017). Pollinator behavior clearly imposes strong natural selection on flower color (Waser & Price, 1983; Fenster *et al.*, 2004; Rausher, 2008), and this pollinator-mediated selection has evolutionary implications. For example, pollinator preference for specific flower colors can reinforce reproductive isolation between sister species (Hopkins & Rausher, 2011, 2012). Furthermore, spatial variation in pollinator communities can contribute to local adaptation and genetically based clines in flower color (Streisfeld & Kohn, 2005; Sobral *et al.*, 2015).

Nevertheless, pollinator-mediated selection is not the only mechanism underlying flower color polymorphisms (Frey, 2004; Strauss & Whittall, 2006; Caruso *et al.*, 2010; Dick *et al.*, 2011; Arista *et al.*, 2013; Imbert *et al.*, 2014). Indeed, the pigments that produce flower color variants and their biosynthetic precursors serve numerous physiological functions (Winkel-Shirley, 2002; Grotewold, 2006; Strauss & Whittall, 2006; Truetter, 2006; Lev-Yadun & Gould, 2009). Abiotic factors such as temperature, drought stress, and exposure to ultraviolet radiation influence flower color variation, and individuals with pigmented flowers can have a fitness advantage under heat and drought stress (Schemske & Bierzychudek, 2001; Warren & Mackenzie, 2001; Coberly & Rausher, 2003; Arista *et al.*, 2013). Recent work

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suggests that flower color polymorphisms can evolve through indirect selection on correlated traits (Simms & Bucher, 1996; Armbruster, 2002; Simmonds, 2003; Irwin & Strauss, 2005; Hanley *et al.*, 2009; Lev-Yadun & Gould, 2009; Dick *et al.*, 2011; Arista *et al.*, 2013; Carlson & Holsinger, 2013; Imbert *et al.*, 2014). For example, floral pigmentation in *Dalechampia* (Euphorbiaceae) may have evolved as an indirect response to selection favoring anthocyanin production in stem and leaf tissue to ameliorate stress from drought, high light intensities, or natural enemies (Armbruster, 2002). Irwin *et al.* (2003) demonstrated that herbivores have reduced performance on anthocyanin-dominant pink and bronze morphs than on yellow and white anthocyanin-recessive morphs of wild radish. Shared biosynthetic pathways or pleiotropy could generate both secondary defensive compounds and floral pigments, resulting in a correlation between flower color and resistance against insect herbivory (Strauss *et al.*, 2004). Additionally, compounds such as flavonoids that confer floral pigmentation act as antioxidants, protecting against the reactive oxygen species generated by exposure to UV-B radiation (Agati & Tattini, 2010). Thus, in some systems, flower color may not be the primary target of natural selection, nor pollinators the primary agent.

Here, we investigate the ecological causes and consequences of flower color variation in the self-pollinating mustard, *Boechera stricta* (Brassicaceae). As pollinator-mediated selection probably plays a minimal role in floral evolution in this primarily selfing species, the maintenance of rare purple flower morphs alongside the common white morphs in *B. stricta* remains elusive (Fig. 1). We hypothesize that this flower color polymorphism has evolved through selection as a response to abiotic (drought and UV-B exposure) and biotic (herbivory) factors.

We tested this hypothesis through a series of experimental and observational studies in populations spanning elevational gradients in the US Rocky Mountains. A complex suite of biotic and abiotic environmental conditions change continuously across elevational gradients, making montane systems ideal for investigations of the ecological and evolutionary processes that contribute to phenotypic variation (Körner, 2007). Nevertheless, little is known about how these suites of interacting factors influence flower color (Arista *et al.*, 2013; Shrestha *et al.*, 2014). Globally, UV radiation increases with elevation (Koski & Ashman, 2016). Elevated UV-B radiation can increase floral pigmentation in the visible (Zhao & Tao, 2015) and UV spectra (Koski & Ashman, 2016) and the secondary metabolites associated with floral pigmentation can protect plant tissues from UV radiation (Truetter, 2006; Agati & Tattini, 2010). Biotic stresses also vary across elevation. For example, a recent analysis found a reduction in herbivore pressure with increasing elevation for woody and deciduous plants worldwide (Galmán *et al.*, 2018). In *B. stricta*, resistance to insect herbivory declines with source elevation in multiple common gardens, suggesting that low-elevation populations have evolved in response to a more abundant herbivore community than have high-elevation populations (Anderson *et al.*, 2015). If pigmentation confers protection against UV radiation, we expect pigmented flowers to be more frequent in high-elevation populations (e.g. Koski & Ashman, 2016). By contrast, if pigmentation serves either an antiherbivore role or a drought-tolerance role, we would expect a greater frequency of pigmented plants at lower elevations. UV exposure, herbivory, and drought stress could interact to maintain rare purple floral morphs within natural populations, in which case we might fail to find a signal of elevation in the expression of flower color.

Edaphic conditions like soil pH and mineral nutrient concentration can also induce variation in flower color (Shaked-Sachray *et al.*, 2002; Zhao & Tao, 2015) and can vary across elevational gradients (Mayor *et al.*, 2017). Horticulturalists modify mineral nutrients and soil pH levels to regulate flower color (Zhao & Tao, 2015). Flower color variation could be maintained as a phenotypically plastic trait driven by environmentally variable conditions. Nevertheless, examinations of flower color in relation to soil characteristics come primarily from the horticultural literature (Kondo *et al.*, 1992; Shaked-Sachray *et al.*, 2002; Yoshida *et al.*, 2003) and we know little about how soil properties influence flower color in nature.

To evaluate the maintenance of flower color variation in this selfing species, we conducted four complementary studies. We first quantified the frequency of rare flower color morphs and examined the environmental correlates of flower color variation in natural populations. We then sought to disentangle the contributions of phenotypic plasticity and genetic variation to this flower color polymorphism in a multiyear field experiment in five common gardens. This experiment tested whether flower color variation is driven by spatial and temporal plasticity, and examined genetically based clines, which signal an evolutionary response to long-term spatial variation in selection across environmental gradients (Kooyers *et al.*, 2015). These first two studies in natural and experimental populations identified putative causal environmental factors associated with flower color variation, which we then manipulated in our two final experiments. Specifically, we tested mechanistic connections between flower color variation and drought and soil pH in a glasshouse experiment and floral and foliar herbivory in a laboratory experiment. These abiotic and biotic factors also influence floral pigmentation in other systems (Warren & Mackenzie, 2001). Our studies sought to illuminate the ecological and evolutionary processes that contribute to the maintenance of flower color polymorphisms in self-pollinating plants.

Materials and Methods

Focal species and study location

Boechera stricta (Graham) Al-Shehbaz is a perennial forb native to the Rocky Mountains in North America, where it inhabits a diversity of environments in elevations ranging from 700 to 3900 m and latitudes from Utah to Alaska (Al-Shehbaz & Windham, 2010; Rushworth *et al.*, 2011). *B. stricta* populations are highly inbred, with an average F_{IS} of 0.89 (Song *et al.*, 2006). This diploid self-pollinating species exhibits local adaptation to spatial variation in natural enemies and climatic factors (Song *et al.*, 2009; Lee & Mitchell-Olds, 2013; Anderson *et al.*, 2015; Wadgymar *et al.*, 2017). The vast majority of *B. stricta*

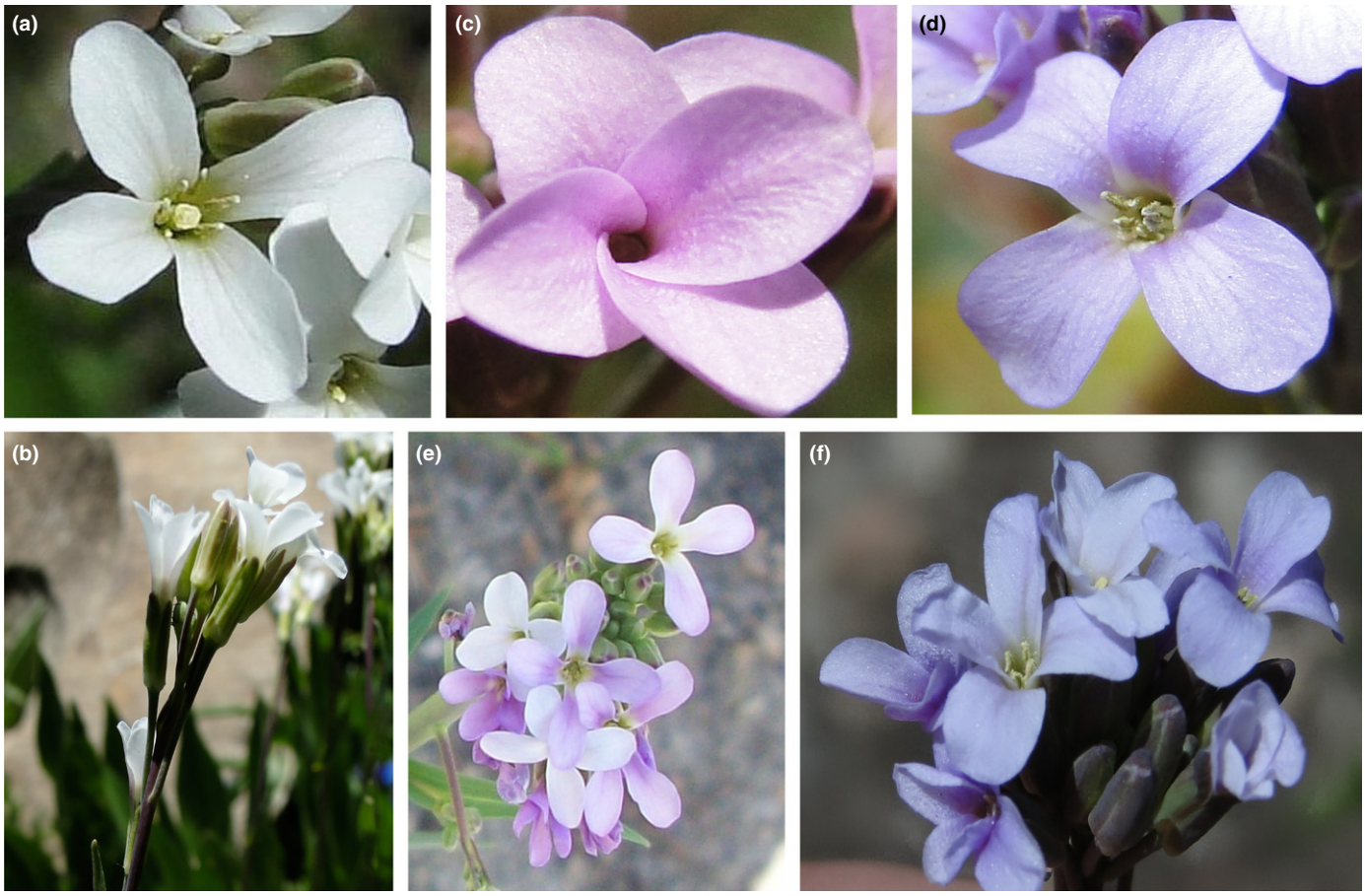

Fig. 1 *Boechera stricta* flower color variation. Most *B. stricta* individuals have exclusively white flowers (a, b), but a small proportion of individuals in natural populations have flowers ranging from pink to purple (c–f). The extent of pigmentation can even vary within a plant (e). In our system, *B. stricta* petals average 3.21 (± 0.79 SD) mm ($n = 1052$) in length, and petals of pigmented flowers are slightly longer (LSMeans ± SE = 3.5 ± 0.13 mm) than those of white flowers (LSMeans ± SE = 3.05 ± 0.06; $F_{1,1002} = 13.09$, $P = 0.0003$; J. Anderson, unpublished).

individuals have small white flowers with petals 1–3 mm long, but we have also observed individuals with lavender to purple flowers in natural populations (Fig. 1). Within the genus *Boechera*, flower colors typically range from white to purple, and intraspecific variation in flower color is common. For example, sister species to *B. stricta* include *B. spatifolia* and *B. fendleri* (Alexander *et al.*, 2013); whereas *B. stricta* and *spatifolia* typically have white flowers with rare lavender morphs, the flowers of *B. fendleri* are generally lavender with rare white morphs. Phylogeographic analysis of flower color has not yet been undertaken in this genus.

We carried out fieldwork in subalpine meadows around the Rocky Mountain Biological Laboratory (RMBL; Gothic, CO). In this region, temperatures and drought stress decrease with elevation (Dunne *et al.*, 2003; Anderson & Gezon, 2015).

Environmental correlates of flower color variation

As a first step to evaluating the ecology and evolution of this flower color polymorphism, we quantified the frequency of purple flower morphs in natural populations, examined abiotic and biotic correlates of flower color variation, tested whether plants with pigmented flowers experience reduced herbivory relative to white-flowered plants, and investigated the fitness consequences of flower color variation. To do so, we established three 5 × 5 m plots in natural populations at nine sites near the RMBL in the summer of 2015 (Supporting Information Table S1). We selected locations across a broad elevational range (2706–3288 m) to maximize environmental variation. Within each plot, we identified and labeled all naturally recruiting *B. stricta* individuals and quantified plant-level traits and plot-level environmental factors. We monitored a total of $n = 551$ flowering *B. stricta* plants in this study. During the peak flowering season, we recorded the color of newly opened flowers as a binary variable (exclusively white flowers vs one or more pigmented flowers) for each individual plant. We estimated foliar herbivory by counting total number of leaves (L), the number of leaves with evidence of herbivore damage (DL), and the average proportion of leaf area removed by herbivores on the damaged leaves (Prop_dam). Following Anderson *et al.* (2015), we calculated leaf area removed by herbivores as (Prop_dam × DL)/L. We returned in July and August to quantify fecundity as the total number of fruits.

At each plot, we recorded the elevation and quantified plant species cover (abundance) by identifying each flowering species present within a 1 × 1 m subsection of each plot during the first census and scoring the percentage cover. We collected three soil

samples from each plot, dried them in an oven at 50°C, pooled the three samples per plot, and sent them to the Clemson University extension laboratory (https://www.clemson.edu/public/regulatory/ag-srvc-lab/soil-testing/index.html) for analysis of soil pH and mineral nutrients (P, K, Ca, Mg, Na, Al). The phosphorus value for one plot was clearly inaccurate (2425.5 kg ha^{-1} relative to the 100.9–210.7 values of the other plots at that site, and an average of 134.8 across all sites). We excluded that data point from analysis.

To evaluate the environmental correlates of flower color variation, we tested whether the frequency of plants with pigmented flowers within a plot (no. of plants with pigmented flowers/total no. of flowering individuals) varied as a function of plot-level environmental conditions using a generalized linear model with fixed effects for abiotic (elevation, soil pH, soil P, K, Ca, Mg, Na, Al) and biotic (percentage plant cover) factors. We included a random effect for site and used a binomial distribution with a logit link (PROC GLIMMIX, SAS v.9.4; SAS Institute, Cary, NC, USA). We used the R package VISREG (v.2.3-0; R Foundation for Statistical Computing, Vienna, Austria) to plot partial residuals from multiple regressions while holding other explanatory variables at their median value (conditional plots). As multicollinearity could influence the results, we conducted complementary univariate analyses of each predictor separately, using the Benjamini & Hochberg (1995) procedure to correct for multiple testing.

We then examined plant-level characteristics to test the prediction that plants with pigmented flowers experienced reduced foliar herbivory. We used a zero-inflated beta regression and implemented the R package GAMLSS (BEINF0 family in version 5.0-5, Rigby & Stasinopoulos, 2005) to analyze leaf area damaged by herbivores (a proportion) as a function of flower color (pigmented vs white), plant size (number of leaves) and elevation, with a random effect for site. Zero-inflated beta regression analyzes proportions as a mixture of Bernoulli and beta distributions. These models simultaneously estimate two parameters: the probability that the proportion has a value of 0 (*nu*), which has a logit link; and the expected value for the beta component (values between 0 and 1, *mu*), which has a log link.

Finally, to test the fitness consequences of flower color variation, we modeled total fruit number as a function of site, flower color and the interaction, with covariates for plant size and foliar damage and a random effect for plot nested within site (negative binomial distribution with a log link; PROC GLIMMIX). We predicted that purple-flowered plants would have greater fitness in sites where purple flowers predominate.

Common garden experiment

We leveraged data from a multiyear field experiment to examine the contributions of plasticity and genotypic variation to this flower color polymorphism, to quantify genetically based clines (in reference to source elevation), to test the hypothesis that floral pigmentation is associated with reduced rates of herbivory, to estimate heritability in flower color, and to quantify selection on flower color. In October 2013, we initiated a field experiment by transplanting $n = 3334$ juvenile *B. stricta* individuals into five common gardens (elevations: 2553, 2710, 2890, 3133, 3340 m). Experimental individuals originated from 43 natural populations spread across a broad elevational range (source elevations: 2694–3690 m). We transplanted multiple full siblings from each of 104 maternal families into each garden after having grown seeds for a generation in the glasshouse to generate the maternal families.

We visited each plant every 2–5 d across three summers (2014–2016), recording flower color as white, pink or purple. Once per summer, one of us (J.T.A.) quantified leaf damage produced by herbivores as described earlier. At the end of the growing season, we quantified the number of mature fruits on each plant.

We tested the extent to which flower color is determined by the evolutionary history of a plant (genetically based cline relative to source elevation) and plasticity (garden environment and growing season year), as well as the hypothesis that pigmented flowers receive lower amounts of herbivory than do white flowers. We used a repeated-measures logistic regression to model the probability of producing pigmented flowers as a function of growing season, garden environment, source elevation, foliar damage from herbivores and interactions. We performed this analysis at the family level; the response variable was the number of individuals with pigmented flowers/the number of individuals that flowered successfully for each family in each garden and growing season. This model included a random effect for plant family nested within population of origin to account for the non-independence of families planted into separate gardens and a repeated effect for season with an autoregressive covariance structure (AR(1)). We used a binomial distribution with a logit link (PROC GLIMMIX SAS v.9.4). Full models with interactions between growing season and garden failed to converge. As we had no *a priori* expectation that genetically based clines would vary across seasons or gardens, we present a reduced model.

As a first pass at evaluating natural selection on flower color variation, we conducted genotypic selection analyses using two fitness components: survival to flowering, and fecundity (number of fruits) among individuals that successfully flowered. By analyzing data at the family level, we could link flower color (an adult trait) with survival to flowering (a juvenile fitness component). We coded each family in the study as having exclusively white flowers or as having at least one individual that produced pigmented flowers. We recognize that this binary flower color variable is coarse. Finer-scale analyses of the fitness consequences of flower color await future studies. We ran a logistic regression to evaluate the probability of flowering as a function of growing season, garden, the binary flower color variable, and two-way interactions. The model did not converge when we included the three-way interaction. We included a random effect for plant family nested within source population and a repeated effect for season, using an autoregressive covariance structure (AR(1)). Flowering success was a binomial variable (number of individuals that flowered/number of individuals that were alive at the beginning of each growing season). If purple flower color results from indirect selection on foliar traits, we would expect that families with the propensity to produce pigmented flowers would have

greater survival, especially in environments where pigmented flowers are more frequent. We conducted a Poisson regression to evaluate whether the number of fruits produced varied with the same predictors, again with a random effect for genotype as a repeated effect accounting for measurements of the same families across years (PROC GLIMMIX).

We estimated broad-sense heritability (H^2) of flower color using restricted maximum likelihood as the genetic variance (V_G) divided by phenotypic variance (V_P = family variance + family by garden variance + block variance + error variance) using a binary distribution and logit link in a model that included fixed effects of season and garden, and random effects for family, family by garden, and block and repeated effects for plant identity across seasons (PROC GLIMMIX, SAS v.9.4). Broad-sense heritability is appropriate for selfing species (Roughgarden, 1979).

Glasshouse experiment: soil pH, drought, and genetic background

To evaluate the role of abiotic conditions in inducing pigmented flowers in plants from different genetic backgrounds, we conducted a fully factorial glasshouse experiment at Duke University (September 2012–April 2013) in which we manipulated drought stress and soil pH. In July 2011, we collected seeds from white- and purple-flowered individuals that we had previously tagged in four populations along a hiking trail. By collecting from multiple populations at approximately the same elevation (average = 2999 m, range 2962–3034 m), we minimized the relatedness of individuals while maximizing similarity in the environmental conditions of the source populations. We grew seeds for a generation in well-watered glasshouse conditions to minimize maternal effects and generate full-sibling families via self-fertilization. For this experiment, we used a total of 12 full-sib families, six from purple- and six from white-flowered grandmothers.

In September 2012, we planted 35 individuals per family into each of two treatments: high and low soil pH. We generated the low-pH soil by combining 5 l of peat moss, 3 g of gypsum, 1.25 l of perlite and 100 g of soda lime. For the high soil pH, we used 200 g of soda lime with the same quantities of other substances. At planting, we determined the pH of each soil treatment by pouring water through a pot of the soil, collecting the water runoff, and testing this water sample using an electronic pH meter. The pH of the high soil pH treatment was 7.2 and the low soil pH treatment was 5.9; this range of pH values is similar to what is found in the native field environment of *B. stricta* (Table S1).

In November 2012, we randomized the experimental plants into two high vs low soil water treatments. We delayed the water treatments until plants were 2 months old, because exposing young seedlings to drought stress could have greatly decreased their survival. Additionally, this procedure reflects water stress under field conditions, when drought is more common in summer after seedlings have established. We watered the high soil water content treatment daily, and restricted the low soil water content plants to receiving water once a week. Surviving plants of each genotype were evenly divided into a fully factorial design cross high/low pH and high/low water availability. Of these, 326 individuals flowered during the course of the experiment (see Table S2 for exact sample sizes). We categorized the color of each flower as white or purple and we counted all fruits produced by each individual.

To test the effects of treatment and genetic background on the expression of flower color, we performed a logistic regression modeling the proportion of flowers on an experimental individual that were pigmented as a function of soil pH treatment, soil water treatment, grandparental flower color, and all two- and three-way interactions (binomial distribution, logit link, PROC GLIMMIX). We included random effects for block nested within treatment and maternal family nested within grandparental flower color. To examine the fitness consequences of flower color variation, we analyzed fruit number as a function of soil pH treatment, soil water treatment, the proportion of flowers that were pigmented, and all two- and three-way interactions (Poisson distribution, log link, PROC GLIMMIX). We modeled the same random effects for maternal family nested in grandparental flower color and block nested within treatment, and used plant height as a covariate.

Laboratory experiment: herbivory and flower color

We hypothesize that plants with pigmented flowers experience lower amounts of herbivory than do those with white flowers. To test the mechanistic link between flower color variation and herbivory, we conducted choice and no-choice experiments in June 2015 in a laboratory at the RMBL. In both experiments, we exposed larvae of two species of lepidopteran herbivores to leaves and flower petals derived from *B. stricta* plants with white vs purple flowers. We used a generalist, *Tricoplusia ni* (Noctuidae), and a Brassicaceae specialist, *Plutella xylostella* (Plutellidae). We acquired herbivores from a commercial retailer (Benzon Research, Carlisle, PA, USA) to ensure that all individuals were of the same developmental stage (first instar) and size. Although these caterpillars are not native herbivores of *B. stricta*, researchers have relied on them to study herbivore preferences, and to evaluate induced and constitutive defenses in *Boechera* and other systems (e.g. Agrawal, 2000; Foggo *et al.*, 2007; Zhang *et al.*, 2008; Manzaneda *et al.*, 2010). Rearing native *B. stricta* herbivores is complicated by incomplete identifications and lack of information on growth requirements. In the field, we have observed various herbivores consuming *Boechera* tissue, including beetles, lepidopterans, weevils, aphids, and leaf hoppers (L. Carley *et al.*, unpublished).

For both experiments, we collected intact, undamaged flowers and leaves from plants with white and dark purple flowers originating from a single natural population near the RMBL. We established each experiment immediately after harvest to ensure that herbivores had access to fresh tissue. Hereafter, we will refer to purple vs white leaves to indicate that leaves came from plants with purple vs white flowers, respectively. Leaves used in these experiments were robust, green, mature, cauline leaves of approximately the same size (*c.* 1.3 cm^2) that had not previously experienced herbivory.

The choice experiment examined herbivore preferences. For this test, we established separate experimental arenas (48 mm Petri dishes with filter paper) for each tissue type (petal vs leaf)

and herbivore species. Within each arena, we exposed one larva per herbivore species to white and purple leaves in equal proportions (either one or two leaves of each flower color; 24 arenas for *T. ni* and nine for *P. xylostella*) or white vs purple petals in equal proportions (two to three petals of each color; 25 arenas for *T. ni* and 25 for *P. xylostella*). We allowed the larvae to consume petals for 20 h and leaves for 48 h before quantifying damage. We generated images of all tissues using a Canon image class scanner (model MF4570dn; Canon, Tokyo, Japan). In ImageJ (Schneider *et al.*, 2012), we quantified leaf area before and after herbivory to calculate the proportion of leaf area removed by herbivores. Owing to the small size of *B. stricta* flower petals and the difficulty of capturing white petals against white filter paper, we estimated petal damage in 5% increments using a visual scale we created.

We ran a no-choice experiment to further evaluate differential damage to white vs purple flower color morphs. In this test, we presented each species of herbivore with leaves or flowers from one color morph only. We established 200 arenas (48 mm Petri dishes with filter paper) divided evenly across two herbivore species, two flower color morphs and two plant tissue types (petals vs leaves). As with the choice experiment, we exposed one larva to four flower petals or two leaves and allowed it to consume tissue for 20 or 48 h, respectively. We quantified tissue damage using methods described earlier for the choice experiment.

To test whether the color of the flower influenced the extent of herbivory, we analyzed the proportion of leaf tissue removed by herbivores using generalized linear models with a quasi-binomial distribution and logit link (PROC GLIMMIX). We included herbivore species (*T. ni* vs *P. xylostella*), flower color (purple vs white), and tissue type (leaf vs petals), and all two- and three-way interactions as predictors. We analyzed choice vs no-choice experiments separately because of differences in the experimental design. For the choice experiment, we also incorporated an R-sided random effect (a repeated effect) for Petri dish to account for nonindependence of purple and white tissue samples within each dish. As herbivores were exposed to petals and leaves for different periods of time (20 and 48 h, respectively), any main effect of tissue type is probably an experimental artefact. Here, we are specifically testing whether one flower color morph incurred greater damage from herbivores than the other. This pattern would be evident through main effects of flower color on damage levels or interactions between flower color and other fixed effects.

Data accessibility

We archived our data in Dryad Digital Repository (doi: 10.5061/dryad.q0032).

Results

Environmental correlates of flower color variation

Across natural populations around the Rocky Mountain Biological Laboratory in 2015, the frequency of pigmented flowers varied from 0 to 0.80 (mean = 0.18, SD = 0.20; $n = 26$ plots with a total of 122 plants with pigmented flowers out of 547 plants that flowered). Multivariate plot-level analyses indicated that the odds of purple floral pigmentation declined by 2% for every kg ha^{-1} increase in soil potassium (odds ratio (OR) = 0.98, 95% confidence limits (CL): 0.97–0.99, $F_{1,7} = 17.14$, $P = 0.0043$; range of soil K values in the study, 97–801 kg ha^{-1}; Fig. 2). Univariate analysis of soil K showed nearly identical results (Table S3). Furthermore, multivariate models revealed that the probability of pigmentation declined by 0.06% for every kg ha^{-1} increase in soil calcium ($F_{1,7} = 5.64$, $P = 0.049$, Table S3) and increased by

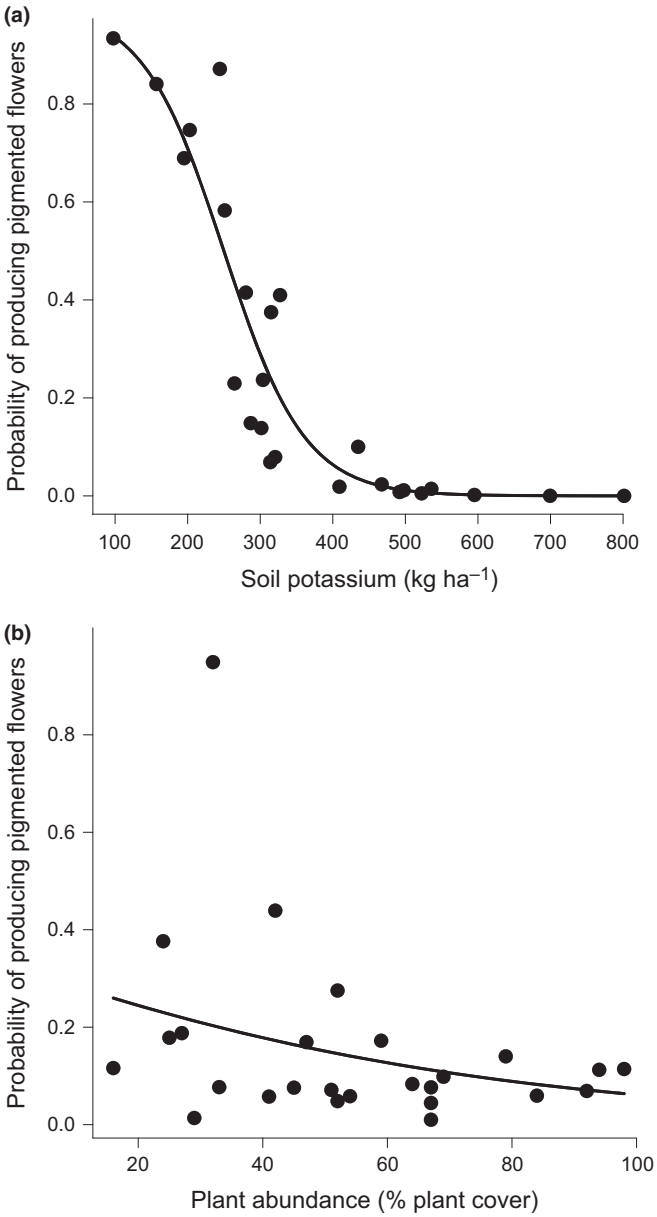

Fig. 2 Environmental correlates of flower color variation. In natural *Boechera stricta* populations, the probability of producing pigmented flowers (pink to purple) declined with: (a) soil potassium in univariate and multivariate models ($F_{1,7} = 13.3$, $P = 0.0082$); and (b) plant abundance in univariate models ($F_{1,16} = 11.46$, false discovery rate-corrected $P = 0.028$). We generated partial residual plots in the R package VISREG to account for other factors in the model, including the random effect for plot.

1.8% and 12%, respectively, for every 1 kg ha^{-1} increase in soil magnesium ($F_{1,7} = 6.62$, $P = 0.037$; Table S3) and sodium ($F_{1,7} = 6.38$, $P = 0.039$; Table S3). As univariate analyses did not uncover significant relationships between floral pigmentation and these soil cations (Ca, Mg, Na; Table S3), we included figures from the multivariate analyses in the supplemental file only (Fig. S1). Multivariate models showed no relationship between floral pigmentation and plant abundance (percentage cover), but in univariate models, the probability of pigmentation declined by 2% for every 1% increase in abundance (OR = 0.98, 95% CL: 0.97–0.99, $F_{1,16} = 11.85$, false discovery rate (FDR)-corrected $P = 0.028$; Fig. 2). Floral pigmentation did not vary with other environmental factors (Table S3).

Plants with white flowers experienced significantly greater foliar damage from insect herbivores than did those with pigmented flowers (Fig. 3; Table S4). The odds that a plant with pigmented flowers received no foliar damage were 208% greater than the odds that a white-flowered plant received no damage (*nu* component of analysis; OR (95% CI) of zero damage comparing pigmented vs white plants = 2.08 (1.29–3.37), $t = 2.98$, $P = 0.003$). For the component of the analysis that excluded plants with no foliar damage, pigmented plants experienced half the amount of foliar herbivory of white-flowered plants (*mu* component of analysis for proportional damage on the range (0, 1); exponentiated parameter estimate (95% CI) comparing damage on plants with pigmented vs white flowers = 0.532 (0.388–0.729), $t = -3.9$, $P = 0.000\,106$).

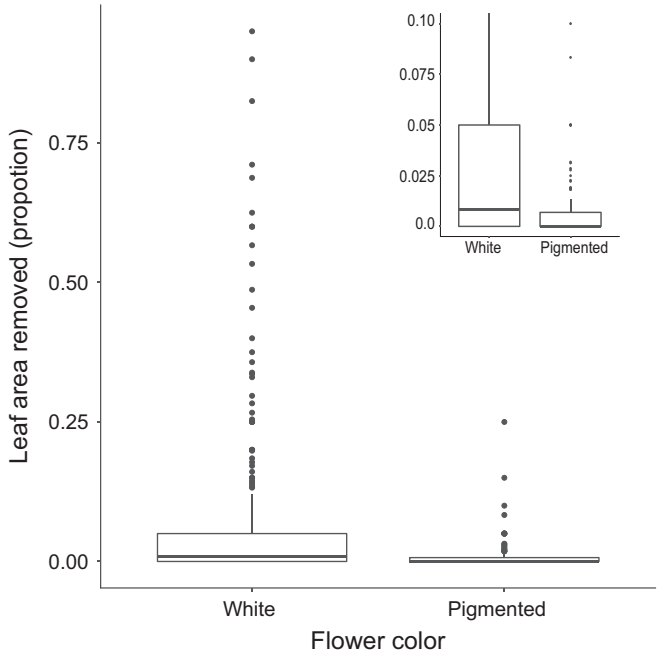

Fig. 3 Foliar herbivory varies with flower color. In natural *Boechera stricta* populations, white-flowered plants experienced more foliar herbivory than did plants with pigmented flowers. Herbivory is quantified as the proportion of leaf area removed by insect herbivores. Box plots indicate the median, interquartile range (hinges showing 25th and 75th percentiles), 1.5 times the interquartile range (whiskers) and the outliers. This difference in foliar damage is easier to visualize in the inset panel, which excludes the outliers for white-flowered plants.

Fecundity varied by site ($F_{8,17} = 7.48$, $P = 0.0003$) and increased with plant size ($F_{1,503} = 75.31$, $P < 0.0001$), but did not change with foliar herbivory ($F_{1,503} = 2.2$, $P = 0.14$). Importantly, there was no indication that flower color influenced fecundity ($F_{1,503} = 0.34$, $P = 0.56$) nor did we find a site × flower color interaction for fecundity ($F_{6,503} = 1.23$, $P = 0.29$).

Common garden experiment

We recorded a total of 2464 plants in flower across the three growing seasons of this experiment. Of these, 208 individuals produced pigmented flowers and the remainder produced white flowers; thus, pigmented flowers occurred on *c.* 8.4% of flowering individuals. Flower color in this experiment was highly heritable ($H^2 = 0.60 \pm 0.066$, $\chi^2 = 64.8$, $P < 0.0001$ in a model that excluded two gardens; the model failed to converge if those two gardens were included. Separate heritability models using data from those gardens also failed to converge).

As was true for natural populations, plants with pigmented flowers received significantly lower amounts of foliar damage from herbivores; the odds that a plant produced pigmented flowers declined by 12% for every 1% increase in foliar damage (OR = 0.88, 95% CL: 0.78–0.99, $F_{1,667} = 4.38$, $P = 0.037$; Fig. 4a). There was no evidence that the relationship between flower color and foliar damage differed across gardens or seasons (Table S5).

Our analyses revealed significant spatial plasticity as well as genetic clines in flower color variation. Pigmented flowers have the greatest probability of forming in the most arid low-elevation garden ($F_{4,667} = 2.42$, $P = 0.047$; Fig. 4b). Genetically based clines were consistent with this plasticity. In the two higher-elevation gardens (Fig. 4c), the odds of producing pigmented flowers declined by 1% with every 1 m increase in the source elevation of transplanted families (garden at elevation 3133 m: OR = 0.99, 95% CL: 0.985–0.997, $t_{667} = -2.79$, $P = 0.0055$; garden at elevation 3340: OR = 0.995, 95% CL: 0.991–0.999, $t_{667} = -2.71$, $P = 0.0070$). Somewhat surprisingly, we found no evidence for this genetically based cline in the three lower-elevation gardens. Finally, family-level flower color did not vary across growing seasons ($F_{2,667} = 1.54$, $P = 0.21$); thus, our analyses did not find temporal plasticity in floral pigmentation.

Genotypic selection analysis revealed a significant survival advantage to families with purple flowers (LSMeans of survival probability ± SE: white-flowered families, 0.47 ± 0.02; purple-flowered families, 0.55 ± 0.045; $F_{1,667} = 3.96$, $P = 0.042$), but no evidence of spatial or temporal variation in this advantage (no garden × flower color or season × flower color interaction; Table S6). Fecundity did not differ between maternal families that produce white vs pigmented flowers (Table S7).

Glasshouse experiment: soil pH, drought, and genetic background

We quantified the color on a total of 2550 flowers from 326 individuals that reproduced in this glasshouse study. The grandparental flower color significantly influenced flower color

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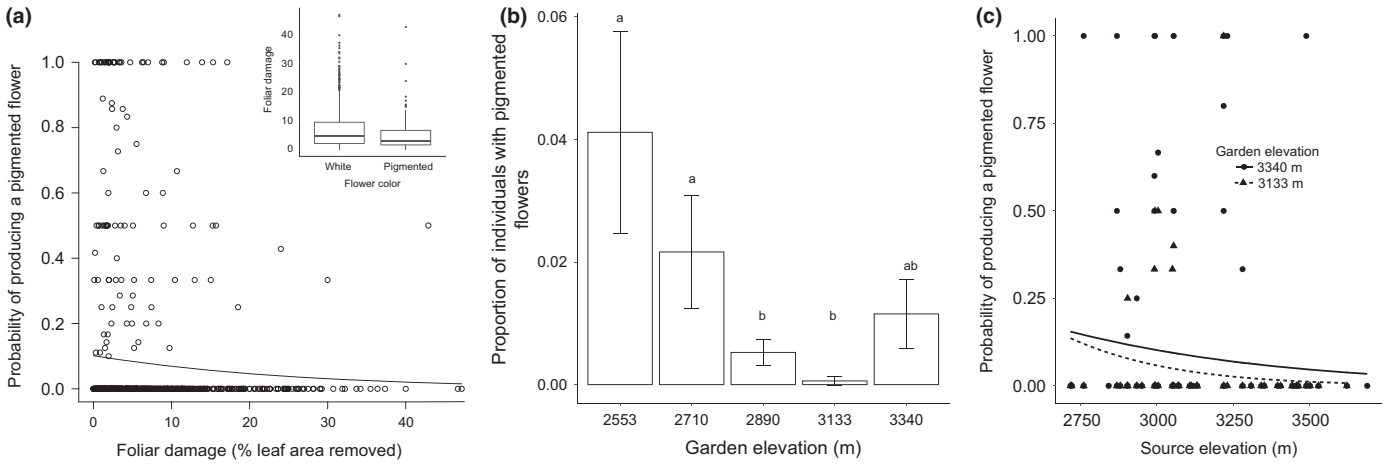

Fig. 4 Herbivory, plasticity, and genetic clines in the common garden experiment with *Boechera stricta*. We conducted a single multivariate mixed-effects logistic regression to evaluate flower color variation (probability of producing pigmented flowers) in response to foliar damage, garden environment, and source elevation. (a) The common garden experiment confirmed that white-flowered individuals experienced elevated foliar herbivory relative to purple-flowered plants ($F_{1,667} = 4.38$, $P = 0.037$). The inset panel depicts box plots with flower color on the *x*-axis to enable comparison with flower color variation in natural populations presented in Fig. 3. Box plots indicate the median, interquartile range (hinges showing 25th and 75th percentiles), 1.5 times the interquartile range (whiskers) and outliers. (b) Low-elevation environments induce pink or purple flower color. Plotted are LSMeans (± SE) generated from the overall model ($F_{4,667} = 2.42$, $P = 0.047$). Different letters represent significantly different LSMeans after Tukey–Kramer correction for multiple testing. (c) Genetic clines in flower color emerged in the two highest-elevation gardens, with the probability of producing pigmented flowers declining with source elevation (garden at elevation 3133 m, $t_{667} = -2.79$, $P = 0.0055$; garden at elevation 3340, $t_{667} = -2.71$, $P = 0.0070$).

expression in the experimental individuals, as purple-flowered lineages were > 39 times more likely to produce pigmented flowers than were white-flowered lineages (OR = 39.7, 95% CI: 1.8–854.0, $F_{1,10} = 7.14$, $P = 0.023$; Fig. 5). A significant interaction between grandparental flower color and drought revealed that individuals from white-flowered lineages were three times more likely to produced pigmented flowers under drought than under well-watered conditions (OR = 3.03, 95% CI: 1.074–8.53, $F_{1,10} = 7.14$, $t_{304} = 2.76$, Tukey–Kramer adjusted *P*-value = 0.031), whereas the flower color expressed by purple-flowered lineages did not vary across treatments ($t_{304} = -0.26$, Tukey–Kramer adjusted *P*-value = 0.99).

Drought significantly depressed fecundity, and there was a marginal trend for reduced fecundity under low soil pH (Table S8). We found a significant interaction between the proportion of flowers that were pigmented and water treatment ($F_{1,301} = 5.39$, $P = 0.021$). Within the drought treatment, there was no significant relationship between flower color variation and fitness ($t_{301} = 0.16$, $P = 0.87$); however, in the well-watered control, fitness declined with increasing pigmented proportion ($t_{301} = -2.42$, $P = 0.016$), indicating that white flower color had a fitness advantage under ample soil moisture (Fig. 5).

Laboratory experiment: herbivory and flower color

Choice experiment Herbivores consumed a greater proportion of white than of purple flower petals ($t_{79} = -5.30$, $P < 0.0001$), but they did not discriminate between leaves originating from plants with white vs purple flowers ($t_{79} = 0.68$, $P = 0.91$; Fig. 6; tissue × flower color interaction in Table S9). The two species of herbivores did not differ in their extent of consumption of tissue from white- vs purple-flowered plants (Table S9).

No-choice experiment When herbivores were not presented with a choice, the generalist herbivore, *T. ni*, consumed a greater proportion of floral and leaf tissues from plants with white flowers than from plants with purple flowers ($t_{192} = -1.17$, $P = 0.65$), whereas the Brassicaceae specialist, *P. xylostella*, consumed equal proportions of white and purple tissues ($t_{192} = -7.39$, $P < 0.0001$; herbivore species × flower color interaction in Table S10; Fig. 6). We also found a significant tissue type × flower color interaction (Table S10). In this case the difference across tissue types was a matter of degree: herbivore damage was proportionally larger for white than for purple petals relative to the damage incurred by leaves of white-flowered vs purple-flowered plants (Fig. 6).

Discussion

Flower color is a remarkably evolutionarily labile trait with vast diversity between and within species (Muchhala *et al.*, 2014). Historically, pollinators were considered the primary agent of selection operating on this trait. Researchers now recognize that various biotic and abiotic factors exert selection on flower color, and that flower color and stress tolerance are tightly linked through common biosynthetic pathways (Coberly & Rausher, 2003; Irwin *et al.*, 2003; Strauss *et al.*, 2004; Strauss & Whittall, 2006; Truetter, 2006; Dick *et al.*, 2011).

Here, we asked how variation in flower color is maintained in a self-fertilizing angiosperm that probably experiences minimal pollinator-mediated selection. To answer this question, we conducted four complementary studies to test whether flower color variation can evolve in response to abiotic and biotic factors other than pollinators. We found that an average of 80% of *B. stricta*

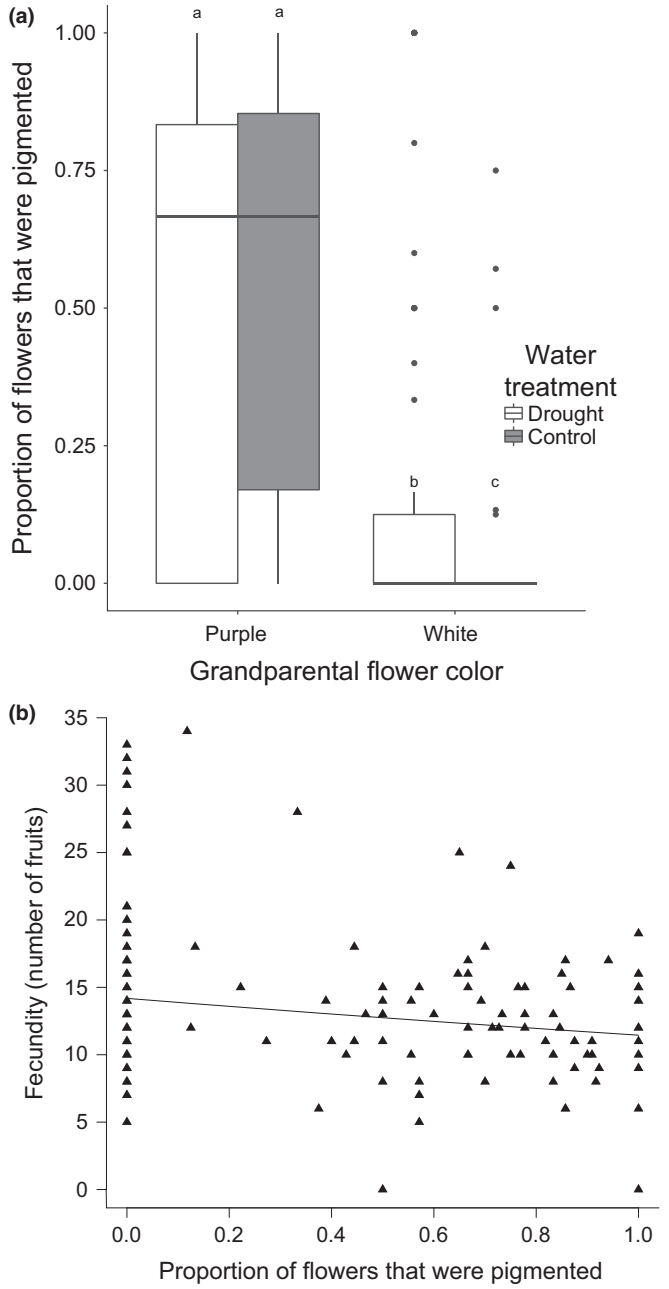

Fig. 5 Drought stress and flower color variation in the glasshouse experiment with *Boechera stricta*. (a) In the glasshouse, drought stress induced purple floral pigmentation in individuals from white-flowered lineages (with white-flowered grandparents; $t_{304} = 2.76$, Tukey–Kramer adjusted *P*-value = 0.031), but water treatment did not alter the flower color expression of pigmented lineages ($t_{304} = -0.26$, Tukey–Kramer adjusted *P*-value = 0.99). In both water treatments, individuals whose grandparent had pigmented flowers in the field were significantly more likely to produce pigmented flowers than were individuals from white-flowered lineages. (b) Selection favored white-flowered individuals under control (well-watered) conditions, and fecundity declined for individuals that produced a large proportion of pigmented flowers ($t_{301} = -2.42$, $P = 0.016$). In both panels, box plots indicate the median, interquartile range (hinges showing 25th and 75th percentiles), 1.5 times the interquartile range (whiskers) and outliers. Different letters represent significantly different LSMeans after Tukey–Kramer correction for multiple testing.

individuals display white flowers, with the remaining plants producing pink to lavender to dark purple flowers. Our common garden experiment revealed that flower color is highly heritable. One of the most pronounced results to emerge from our series of studies is the relationship between flower color and herbivory. Across observational and experimental studies in field and controlled settings, plants with pigmented flowers experienced lower herbivory than did plants with white flowers. In natural populations, floral pigmentation was higher in stressful microsites with low vegetative cover where plants are sparse. Similarly, arid lower elevation environments induced floral pigmentation in our common garden experiment. Additionally, genetically based clines were concordant with phenotypic plasticity: the probability of purple flowers declined with source elevation in two of five gardens. These results all point to increased rates of floral pigmentation under biotic and abiotic stresses. We hypothesize that flower color variation in *B. stricta* results indirectly from selection for enhanced stress tolerance. Given that purple-flowered plants receive lower rates of herbivory, the question remains: why are they so infrequent? It is possible that plants with pigmented flowers may experience fitness costs at certain life-history stages or during benign years with ample rain and few herbivores. Alternatively, genetic tradeoffs between flower color and other traits subject to selection could constrain the evolution of pigmented flowers.

Floral pigmentation and herbivory

In our field experiment and observational study, *B. stricta* individuals with lavender or purple flowers experienced lower rates of foliar herbivory than did individuals with the more typical white flowers. We hypothesize that flower color variation is maintained via selection for antiherbivore defenses. Herbivory has profound fitness consequences for *B. stricta* (Prasad *et al.*, 2012) and other species (e.g. Frey, 2004), and secondary compounds that confer resistance to herbivores can be subject to strong selection (Mauricio & Rausher, 1997; Stowe, 1998). Foliar herbivory begins early in the growing season in our system, well before individuals flower, and insect herbivores are likely to be deterred by foliar compounds instead of floral color. Indeed, when presented with detached tissue in Petri dishes, a generalist lepidopteran herbivore consumed a greater proportion of leaves and petals from white-flowered than from purple-flowered *B. stricta* (no-choice experiment). In the choice experiment, the specialist and generalist herbivores discriminate against purple petals and preferentially consumed white petals. Thus, pigmentation may also confer protection against florivory.

Irwin *et al.* (2003) found a similar link between foliar damage and flower color in the outcrossing wild radish (*Raphanus sativus*). In that system, herbivores preferred the anthocyanin recessive yellow and white floral morphs over the dominant pink or bronze floral morphs (Irwin *et al.*, 2003). Furthermore, pink or bronze morphs were better defended against herbivores, producing a higher concentration of chemical defenses (indole glucosinolates) than those produced by the recessive morphs. In this

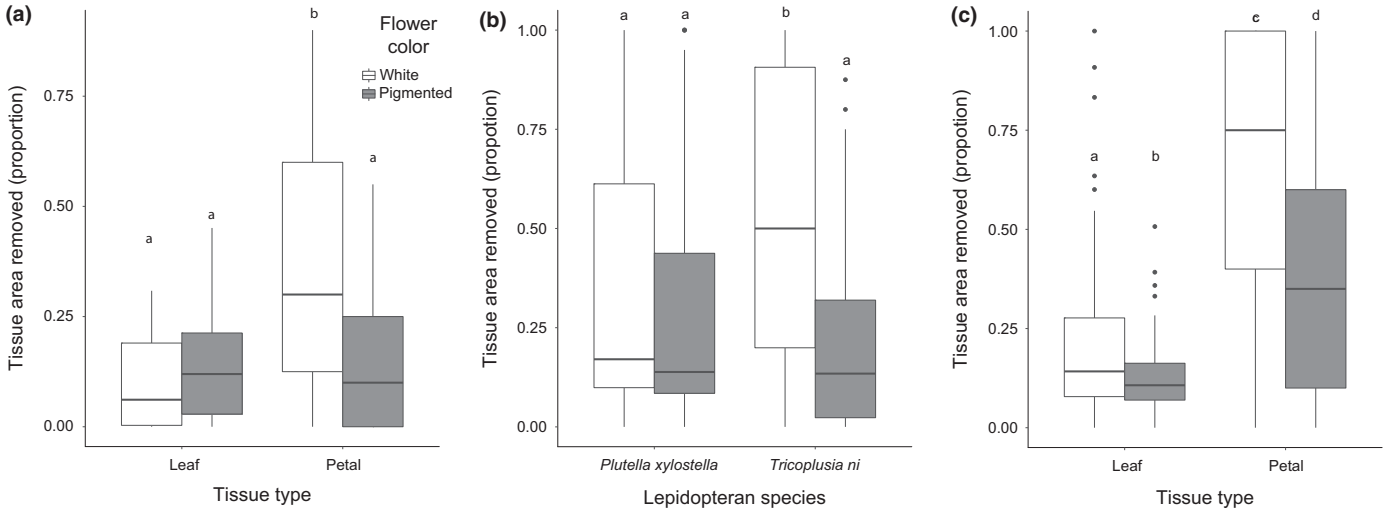

Fig. 6 Herbivore preference under controlled conditions in *Boechera stricta*. (a) In a choice experiment, herbivores preferred white to purple floral tissue ($t_{79} = -5.30$, $P < 0.0001$), but showed no difference in consumption of leaves from purple- vs white-flowered plants ($t_{79} = 0.68$, $P = 0.91$). (b) In a no-choice experiment, the generalist lepidopteran (*Tricoplusia ni*) herbivore consumed more floral and foliar tissue from white-flowered than from purple-flowered plants ($t_{192} = -7.39$, $P < 0.0001$), whereas the specialist herbivore (*Plutella xylostella*) consumed equal proportions of tissue from the two flower color morphs ($t_{192} = -1.17$, $P = 0.65$). (c) The no-choice experiment also showed that tissue from white-flowered plants experienced greater herbivory than tissue from purple-plants. A tissue × flower color interaction ($F_{1,79} = 5.28$, $P = 0.023$) revealed that white flowers receive proportionally more damage (relative to purple flowers) than white leaves (relative to purple leaves). All three panels display box plots, indicating the median, interquartile range (hinges showing 25th and 75th percentiles), 1.5 times the interquartile range (whiskers) and outliers. Different letters represent significantly different LSMeans after Tukey–Kramer correction for multiple testing.

case, selection against the recessive morphs imposed by herbivores counteracted pollinator-mediated selection (Stanton, 1987), maintaining flower color variation within the species (Irwin *et al.*, 2003). Colorful flowers can attract both pollinators and natural enemies (herbivores, predispersal seed predators, pathogens), setting the stage for conflicting selection (Irwin *et al.*, 2003; Frey, 2004; Strauss *et al.*, 2004; Irwin & Strauss, 2005; but see Caruso *et al.*, 2010). Conflicting selection could operate in selfing species as well, if natural enemies differ in their preferences, or selection imposed by natural enemies counters selection imposed by abiotic factors.

The physiological mechanism underpinning the relationship between floral pigmentation and foliar herbivory remains unresolved. One hypothesis is that shared biosynthetic pathways between floral pigments and flavonoids could generate flower color while reducing palatability of leaf tissue and enhancing stress tolerance (Chalker-Scott, 1999; Winkel-Shirley, 2002; Dick *et al.*, 2011). Flavonoids are secondary metabolites that enable plants to withstand abiotic and biotic stresses such as ultraviolet radiation, heat damage, drought stress, nutrient deficiencies, and elevated salinity, and can confer resistance against natural enemies such as herbivores, seed predators, and pathogens (Chalker-Scott, 1999; Winkel-Shirley, 2002; Coberly & Rausher, 2003; Strauss & Whittall, 2006; Truetter, 2006; Caruso *et al.*, 2010; Wessinger & Rausher, 2012; Koski & Ashman, 2016). The principal role of flavonoids in stress tolerance is probably related to their antioxidative capacity to scavenge free radicals (Simmonds, 2003).

Anthocyanins are flavonoid derivatives responsible for floral pigmentation in the vast majority of angiosperms (Wessinger & Rausher, 2012). Other pigment-producing compounds include the carotenoids, which typically generate yellow to orange and red pigmentation, and the betalains, which are restricted to the Caryophyllales; these compounds are much less prevalent than anthocyanins in vascular plants (Grotewold, 2006). The primary flavonoids involved in flower color are derived from precursors generated along the three branches of the anthocyanin pathway: pelargonidin (red flowers), cyanidin (red to purple to blue flowers), and delphinidin (blue/purple flowers) (Wessinger & Rausher, 2012). While many species express anthocyanins derived from all of these compounds in floral tissue, Wessinger & Rausher (2012) found that *c.* 89% of surveyed species produced only cyanidin in vegetative tissue. *B. stricta* morphs with pigmented flowers also produce pigmented vegetative tissues (J. T. Anderson, pers. obs.), as is true in other systems (Warren & Mackenzie, 2001; Armbruster, 2002), suggesting that systemic anthocyanin production involved in stress tolerance may indirectly influence flower color variation. Owing to the antiherbivore role that flavonoids and anthocyanins can play (Truetter, 2006; Lev-Yadun & Gould, 2009), we propose that the flower color variation in our system is maintained as a byproduct of systemic up-regulation of flavonoids and anthocyanins in response to herbivore stress and drought.

Floral pigmentation and abiotic conditions

The common garden experiment revealed that low-elevation environments induce purple coloration in flowers. Similarly, in two gardens, we detected genetically based clines in flower color,

with the probability of flowers being pigmented declined with source elevation. These current results are not consistent with a role for ultraviolet radiation in this visible flower color polymorphism, as we would have expected floral pigmentation to increase with elevation in that case. However, it is possible that the signal of UV radiation is swamped out by the countervailing effects of herbivory and drought stress. Our current observational and experimental studies suggest that phenotypic plasticity and genetically based clines may primarily result from selection favoring pigmentation in hot, arid, low-elevation environments where rates of herbivory are high. In morning glory (*Ipomoea purpurea*), white-flowered mutants with inhibited anthocyanin production showed lower reproductive success at high temperatures than did pigmented wild-type plants (Coberly & Rausher, 2003), and pink and purple morphs of five British species show greater drought resistance compared with white morphs (Warren & Mackenzie, 2001).

Purple flowers did not confer a fecundity advantage or experience a fecundity cost in our study of natural populations or in our common gardens. However, in our common garden experiment, purple-flowered maternal families had greater probability of flowering than did white-flowered families. This result suggests that a fitness advantage arises before reproduction, pointing to a potential role of anthocyanins – or their precursors – in vegetative tissue. In the glasshouse, drought induced greater rates of purple pigmentation among lineages derived from white-flowered grandparents. Additionally, we found that selection favored white-flowered individuals in well-watered conditions. Taken together, field and glasshouse results implicate drought stress as a potential causal factor in the maintenance of flower color variation in *B. stricta*. These results are consistent with other systems in which drought stress increases floral pigmentation (Chalker-Scott, 1999; Zhao & Tao, 2015). Elevational clines in flower color in our system are probably related to both drought stress and herbivore pressure.

In natural populations, the probability of producing purple flowers declined with soil potassium and plant abundance. These correlative results require experimental verification, but they suggest that other abiotic factors could influence the eco-evolutionary dynamics of flower color variation in *B. stricta* and probably other species. Schemske & Bierzychudek (2007) found that soil K was higher in areas with blue morphs of *Linanthus parryae* vs areas with white morphs, but their studies point to a greater role of drought stress in maintaining flower color polymorphisms in that system. Application of potassium to lily leaves before harvest improved flower color quality for horticulturalists (Burchi *et al.*, 2010). Whether soil K contributes noticeably to flower color variation in natural populations depends on the range and scale of edaphic variation and the mechanism by which K influences flower color, both of which remain to be explored. We also found a correlation between plant cover and flower color: in areas where the plant community is not abundant, *B. stricta* has increased rates of purple floral pigmentation. Areas with low plant abundance could represent local microsites that are abiotically stressful, where flavonoids could confer stress tolerance.

Conclusions

Our results indicate that flower color variation can be maintained by factors other than pollinator-mediated selection, such as herbivore pressure. Across observational and experimental studies in natural and controlled settings, we consistently found that purple-flowered plants experienced lower rates of herbivory than did white-flowered plants. We hypothesize that herbivory induces up-regulation of genes in the anthocyanin and glucosinoate pathways in some genetic backgrounds. Pigmented flowers may therefore be a natural consequence of systemic production of anthocyanins. That is, flower color may evolve via indirect selection operating on vegetative tissue.

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Author contributions

All authors (P.V., A.M., E.M., M.K., L.C., C-R.L., R.B. and J.A.) designed and conducted the studies. P.V., A.M., E.M. and M.K. did initial literature reviews and wrote reports and theses based on preliminary results. J.A. performed analyses and wrote the manuscript. All authors read the first draft and provided comments.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Multivariate analyses revealed that the probability of floral pigmentation was influenced by soil calcium, magnesium and sodium.

Table S1 Attributes of plots from the study of environmental correlates of flower color variation in natural populations

Table S2 Sample sizes of individuals that flowered in each treatment in the glasshouse experiment in which we manipulated soil pH and water level

Table S3 Results of multi- and univariate analyses examining the relationships between environmental factors and the probability of pink and purple floral pigmentation in natural populations

Table S4 Results of a zero-inflated beta regression to evaluate the extent to which foliar damage in natural populations varied with flower color, elevation, and plant size (number of leaves)

Table S5 Results of a multivariate, mixed-effect logistic regression evaluating plasticity and genetic clines in flower color variation from the common garden experiment

Table S6 Genotypic selection analysis of survival on flower color variation in the common garden experiment, indicating a slight – but significant – survival advantage of purple-flowered maternal families over white-flowered maternal families in all sites

Table S7 Selection via fecundity on flower color variation from the common garden experiment

Table S8 Results of our multifactorial manipulation of soil pH and water level in the glasshouse experiment, where the response variable was the number of pigmented to total number of flowers produced by an individual plant; fitness varies with treatment and flower color in the glasshouse experiment

Table S9 Results from the choice experiment where herbivores were presented with tissue derived from purple- and white-flowered plants

Table S10 Results from the no-choice experiment, which evaluated herbivore consumption of tissue derived from either purple- or white-flowered plants in isolation

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