

The maintenance of flower colour polymorphism in self-pollinating *Boechea stricta*

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Abstract

Pollinator-mediated selection cannot explain why flower colour polymorphisms exist in self-pollinating plants. There is a tight link between plant stress response and anthocyanin pigment production, which means that abiotic stress, such as UV radiation or drought, and biotic stress, such as herbivory or competition, can also affect flower colour selection indirectly. I used *Boechnera stricta* to test whether environmental factors are responsible for flower colour polymorphism in self-pollinating plants, and evaluate 1) the biotic and abiotic correlates of flower colour variation, 2) the fitness consequences of this polymorphism, and 3) whether flower colour variation and ultimately pigment production are plastic traits. I sampled at Gothic, Colorado along an elevational range of approximately 2700m to 3400m, where I visited 11 locations and sampled from six 15m transects randomly per location. I measured plant growth and reproduction, along with abiotic and biotic factors that may correlate with flower colour. I also analyzed data from the long-term common garden experiment run by the Anderson lab to determine plasticity of flower colour. The observational data are still under analyses, but we have analyzed some of the common garden data and can conclude that 1) lower elevation plants are more likely to produce pink or purple flowers, and 2) pink or purple flowered plants have lower herbivore damage than white flowered plants. Water availability and natural enemies may play important roles in the first pattern because lower elevations generally show lower precipitation and a greater influence of biotic interactions. Plants with pigmented flowers may also induce greater herbivore-resistance compounds due to the shared biosynthetic pathway of anthocyanin and other flavonoids. This may present indirect selection on flower colour if herbivores influence chemical resistance traits in these plants. However, we will need to analyze the observational data to determine how these abiotic and biotic factors correlate with flower colour, and analyze heritability from the common garden.

Introduction

Flower colour has evolved several times in angiosperms, and shows great variation between species, and even among populations and individuals (Rauscher 2008). A common theory for the presence of vast flower colour diversity is that pollinator preference imposes natural selection on flower colour. For example, hummingbirds and bumblebees discriminate against rare white-flower morphs of several wildflower species in the Colorado Rocky Mountains, resulting in lower fitness than blue-flowered conspecifics (Waser & Price 1981, 1983). Additionally, bumblebees prefer one colour morph of snapdragons (*Antirrhinum majus*) over another, leading to greater fitness of the successfully pollinated morph, along with assortative mating (Jones & Reithel 2001). Sobral et al. (2015) also observed a longitudinal cline in flower colour of *Gentiana lutea*, with a direct positive association with pollinator species composition.

Pollinator preference is also thought to drive the maintenance of flower colour polymorphism within populations. Negative frequency-dependent selection maintains flower colour polymorphism in rewardless angiosperm species (Gigord et al. 2001). This theory suggests that rare colour morphs are favoured because pollinators tend to visit novel phenotypes while foraging, and only learn of the rewardless trait once the morph becomes common in the population. However, several studies have revealed that the maintenance of flower colour polymorphism may be due to selective agents other than pollinators (Durmont et al. 2010, Frey 2004, Imbert et al. 2014, and Pellegrino et al. 2005). For example, in a greenhouse experiment,

Irwin & Strauss (2005) found pollinator-mediated selection on yellow morphs of the radish *Raphanus sativus*, but their field study showed no change in morph frequency in the next generation. If pollinators were the only selective force acting on flower colour of radish, yellow morphs would dominate under field conditions. Their contrary field results suggest other selective forces may be maintaining the flower colour polymorphism under natural conditions.

The hypothesis of pollinator-driven selection of flower colour also does not explain why colour variants exist in self-pollinating species. The production of anthocyanin pigments is metabolically costly (Chalker-Scott 1999); thus, maintaining flower pigmentation in the absence of animal-pollination may not confer a selective advantage. Alternatively, there may be other biotic and abiotic factors maintaining polymorphism for flower colour. For example, flower colour polymorphism can be maintained through pleiotropic effects or by indirect selection of correlated traits (Armbruster 2002, Coberly & Rausher 2003, Imbert et al. 2014, Irwin & Strauss 2005). Selection on vegetative traits, such as stem and leaf pigmentation can indirectly affect flower colour polymorphism, as seen in *Dalechampia* species (Armbruster 2002). Selection pressure from herbivores and pathogens can maintain polymorphism if it counteracts selection imposed by pollinators, or if there is spatial and temporal variation in selection via pollinators and antagonists (ex. Anderson et al. 2014, Frey 2004, Irwin et al. 2003, Irwin & Strauss 2005). Abiotic pressures such as temperature extremes and drought stress may also affect flower colour variation in temporally variable environments (Anderson et al. 2014, Arista et al. 2013, Strauss & Whittall 2006, Warren & Mackenzie 2001).

Biotic and abiotic factors likely interact to exert selection on flower colour, given that flower colour and stress tolerance are tightly linked through a common biosynthetic pathway (Coberly & Rausher 2003). The ability of plants to withstand environmental stress requires the production of stress tolerance compounds called flavonoids, which are involved in protection against UV and heat damage, hormone regulation, resistance against natural enemies, and even pollination and fertilization (Coberly & Rausher 2003). Anthocyanins are flavonoid derivatives involved in pink and purple pigmentation, which too can be induced through biotic and abiotic factors because of their shared biosynthetic pathway with other flavonoids (Chalker-Scott 1999, Dick et al. 2011, Guo et al. 2008). Anthocyanins are produced throughout the plant, so both reproductive and vegetative parts are pigmented to provide systemic protection (Streisfeld and Rausher 2011).

A shared biosynthetic pathway for stress tolerance and floral pigmentation suggests that flower colour may be a good indicator of plant adaptations to stressful environments. White-flowered morning glory (*Ipomoea purpurea*) mutants with inhibited anthocyanin production showed lower reproductive success at high temperatures (Coberly & Rausher 2003). Pink and purple morphs of five British floral species show greater drought resistance than white morphs (Warren & Mackenzie 2001). Irwin et al. (2003) demonstrated that herbivores prefer and perform better on yellow and white anthocyanin-recessive morphs of wild radish than anthocyanin-dominant pink and bronze morphs. This suggests that anthocyanins also provide several anti-herbivory functions, such as camouflage and aposematism (Lev-Yadun & Gould 2008). Anthocyanins are commonly associated with cold tolerance, UV protection, low pH, pathogen infection, and nutrient deficiencies (Chalker-Scott 1999), indicating a close link between pigmentation and environmental stress tolerance. However, these studies were not done on self-pollinating plants, which means that flower colour variation cannot be completely dissociated from their evolutionary history with pollinators.

The self-pollinating mustard species, *Boechna stricta* (*Brassicaceae*), is a good candidate for studying non-pollinator related factors that maintain flower colour polymorphism. In the absence of pollinator-driven selection, the maintenance of rare purple flowers along with common white morphs in *B. stricta* remains elusive. It is possible that flower colour polymorphism has evolved and is maintained in *B. stricta* through indirect selection on anthocyanin pigment production, as a response to biotic and abiotic stress. Morphs with pigmented flowers also consistently produce pigmented vegetative tissues (Anderson, observation), suggesting that systemic anthocyanin production involved in stress tolerance may indirectly influence flower colour variation. *Boechna stricta* populations are highly locally adapted, with elevational clines in morphological and physiological traits likely due to environmental heterogeneity (Anderson et al. 2014, Anderson & Gezon 2015, Song et al. 2006). Additionally, *B. stricta* has been historically exposed to a range of environmental stresses, including herbivory, pathogen infection, drought, and cold temperatures that can lead to the adaptation of tolerance traits (Rushworth et al. 2011). Pigmented morphs are commonly found at low pH and drought conditions, and greenhouse experiments explain that flower colour is a heritable trait (Anderson, Matton & McDurmon, unpublished data), which also presents potential for flower colour variation through indirect selection on stress tolerance.

A thorough analysis of the biotic and abiotic conditions, and flower colour variation is required to determine if environmental factors are responsible for flower colour polymorphism in *B. stricta*. To do so, I surveyed the native range of *B. stricta* in the Colorado Rocky Mountains along an elevational gradient to evaluate 1) the biotic and abiotic correlates of flower colour variation, 2) the fitness consequences of this polymorphism, and 3) whether flower colour variation and ultimately anthocyanin production are plastic traits. I tested the hypothesis that pink and purple flower coloration is more prevalent in populations subject to stress, such as herbivory, drought, or UV radiation. This study increases our understanding of why and how self-pollinating plants maintain flower colour polymorphisms, and illuminate the ecological and evolutionary consequences of flower colour in terms of plant-animal interactions and plant response to rapid climate change.

Methods

Study Organism

Boechna stricta is a perennial forb native to North America, and has evolved in a wide range of environmental conditions, ranging from arid to alpine environments (Rushworth et al. 2011). It is a diploid, self-pollinating species that is closely related to the model organism *Arabidopsis thaliana* (Rushworth et al. 2011). This species is also a good model for studying evolutionary ecology under natural conditions because populations typically grow in habitats that vary in abiotic and biotic conditions (Rushworth et al. 2011). *Boechna stricta*, and other species of *Boechna*, exhibit clear patterns of local adaption driven by natural enemies and climatic factors (Anderson et al. 2015, Knight et al. 2006, McKay et al. 2001, Rushworth et al. 2011, Wadgyamar et al. 2017).

Data Collection

I conducted an observational study that was similar to a previous student's, Ansley McDurmon, who also worked on the maintenance of flower colour polymorphism in *B. stricta*. I

sampled *B. stricta* populations along an elevational gradient in the Rocky Mountains of Colorado to determine environmental effects on flower colour polymorphism. The elevations ranged from approximately 2700m-3400m, where I visited 11 locations (Fig. 1) across the Gunnison National Forest and Rocky Mountain Biological Laboratory (RMBL). At each location, I sampled from six 15m transects by randomly choosing a point on a compass and the number of paces to take for the start of the transect. Labelled flags were used as markers to ensure that transects do not overlap. I surveyed *B. stricta* individuals that fall within 1m of each side of the transect line, so that the sampling area of one transect is 15m x 2m. I tagged the plants I have surveyed with a Plant ID that specifies the transect number, and the side of the transect where the plant was surveyed.

To determine the environmental conditions that may affect flower colour polymorphism in *B. stricta*, I measured a range of biotic and abiotic factors for each transect and plant. For each transect, I determined UV exposure indirectly through measuring elevation, slope, and aspect, where slope will be calculated by measuring and dividing elevational gain and horizontal distance travelled. I also collected soil cores at the beginning, middle, and end of each transect to analyze soil nutrient and water availability, and pH level. Water availability was determined by measuring initial weight of the soil and dry weight using the equation:

$$\% \text{ Soil Moisture} = \frac{\text{Wet weight} - \text{Dry weight}}{\text{Dry weight}} \times 100$$

Once initial soil mass was recorded, the samples were placed in an oven at 50°C for approximately 48-72 hours. The samples will be taken to the University of Georgia for nutrient and pH analysis in the lab.

For each plant, I measured flower colour and fitness traits, including the number of open flowers and fruit (siliques), plant height, herbivore damage, and estimated the percent relative cover around each individual. I measured flower colour using the colour-categorizing system developed by Anderson and Embick (Fig. 2), on a scale of 0 (white) to 5 (purple). Herbivore damage will be calculated using the protocol by Anderson et al. (2015), which requires total number of leaves, the number of damaged leaves, and an estimate of the percent leaf area removed. These data will be used for the equation:

$$\% \text{ herbivore damage} = \frac{\# \text{ leaves damaged} \times \% \text{ leaf area removed}}{\# \text{ total leaves}}$$

I surveyed each site twice; the first census was during flowering season in June and July, and the second census was when the plants set fruit at the end of July. Due to the physiological change at different elevations, I started sampling from the lower elevation sites first, then moved up to higher elevation sites. A total of 1019 plants were surveyed in the first census, and 941 plants in the second census because the Copper Creek site was not revisited due to conflicts with the US Forest Service.

I also collected tissue from natural and experimental populations to quantify anthocyanin production in fall 2017 at the University of Georgia. Anthocyanin production in both reproductive and vegetative tissue will be measured using high-performance liquid chromatography (HPLC) in the lab, which requires proper storage of plants until fall. The

samples will be dried immediately once picked so the herbarium tissue can be stored until needed for analysis (Harborne 1998).

Statistical Analysis

To model the effects of environmental variables and herbivore damage on flower color in natural populations, we used multinomial regression. We also evaluated the fitness consequences of flower color variation by analyzing fecundity as a function of flower color with plant size as a covariate.

Since fall 2013, the Anderson lab has planted approximately 22,000 juvenile rosettes into five experimental gardens arrayed across an elevational gradient around the Rocky Mountain Biological Laboratory (garden elevations: 2540m, 2710m, 2890m, 3133m, 3340m). These individuals originate from approximately 75 natural populations spread across this elevational range. The Anderson lab planted multiple full siblings per maternal family into each garden after having grown seeds for a generation in the greenhouse to generate the maternal families. Within each garden, half of the siblings are exposed to contemporary conditions and half are exposed to early snow removal, which simulates climate change in this region. From the summer of 2014 to the present, the Anderson lab has monitored flowering success in these plants every 2-5 days, recording flower color. We used a repeated measures logistic regression to model the probability of producing pink or purple flowers in our gardens across 3 years (2014-2016) for the 2013 cohort. We modelled the probability of producing pink or purple flowers as a function of garden environment, source elevation, and their interactions, as well as foliar damage from herbivores and its interaction with garden and treatment. This model included a random effect for plant family (genotype) to account for the non-independence of siblings. We used a binary distribution with a logit link in Proc Glimmix (SAS, ver. 9.2). This analysis determined the extent to which flower color is determined by plant genotype (source elevation), environment (garden and treatment) and genotype by environment interactions. We also estimated the heritability of flower color using these data.

Results

The observational data collected have not yet been analyzed because they will be compared to the previous student's results to determine temporal differences in flower colour frequency. Despite sampling heavily, I found only 5 individuals with pink or purple flowers this year, which is much lower than in previous years. The decline this year suggests that there is temporal variation, and likely plasticity, in this trait.

From the common garden analyses, we found clear evidence that flower color has both plastic and genetic components, along with both spatial and temporal variation (Table 1). The significant main effect of garden indicated a spatially plastic response to the environment, with a greater proportion of individuals producing pink or purple flowers in the low elevation gardens than the higher elevation gardens (Fig. 3). For temporal plasticity, we see that 2015 had the greatest probability of having pink or purple flowers relative to the other two years. There was no significant difference between 2014 and 2016. Precipitation in 2015 was greater relative to the other two years, which must be considered. Ansley did her study during 2015 when purple flowers were most prevalent in the gardens and in natural populations.

There is a source elevation by garden interaction in this model, where we see the same genetic cline emerge in two experimental gardens: Schofield (Fig. 4) and North Pole (Fig. 5). In both gardens, plants from low elevation populations have a greater probability of producing pink or purple flowers. There is also a negative relationship between the probability of making pigmented flowers and leaf damage (Fig. 6); thus, white flowers receive more damage than pigmented flowers.

Discussion

This study explored how flower colour polymorphisms are maintained in self-pollinating species that do not face direct selection from animal pollinators. Other biotic and abiotic factors may be influential in this maintenance, such as herbivory, drought and UV radiation. To test this, I used the self-pollinating species *Boechea stricta*, which has both white morphs and rare purple and pink morphs. I sampled *B. stricta* across an elevational gradient and determined which environmental factors influence flower colour. The results from these data are still under analyses, so they will not be presented in this report.

However, we also analyzed previous data from the long-term common garden experiment run by the Anderson lab. Our results show spatial and temporal plasticity in flower colour (Fig. 3), along with a genetic cline at the high elevation gardens (Fig. 4 & 5), and a negative relationship between flower pigmentation and herbivory (Fig. 6). The first two results indicate that lower elevation plants are more likely to produce pink or purple flowers, and that this is both environmentally and genetically determined. This suggests that individuals at lower elevations experience more stressful environmental conditions than those at higher elevations. McDougal & Parks (1986) showed that flavonoid compositions in red oak (*Quercus rubra* L.) changed across elevation, and too showed both plastic and genetic components to this composition. This suggests that flavonoid and pigment production may be tightly linked as predicted, and are induced through stress response at lower elevations. Specifically, water availability and natural enemies may play important roles in this pattern because lower elevations generally show lower precipitation and a greater influence of biotic interactions (Hulshof et al. 2013, McCain & Grytnes 2010).

Abscisic acid is a plant hormone commonly known to induce plant water stress response and tolerance (Shinozaki & Yamaguchi-Shinozaki 1997). However, the same review also suggests that pathways independent of the plant hormone may also contribute to water stress response. Thus, if the pattern of more pigmented *B. stricta* at lower elevations is an indicator of plant water stress, it is possible that the flavonoid and anthocyanin biosynthetic pathway may also be involved in water stress response. However, despite there being an indication of water stress in the spatial data, the temporal data show contradicting results. Plants in 2015 showed a significantly greater probability of producing pigmented flowers than in 2014 and 2016, and 2015 was recorded to be a relatively moist year. This suggests for the temporal data that flower colour does not correspond with water stress. It may be important to analyze other factors, such as herbivore activity across years to determine if they may have a greater effect on plant stress response. As for exposure to natural enemies, Reynolds & Crossley Jr. (1997) showed a decrease in percent leaf area removed as elevation increased, along with changes in the type of insect herbivores present across the gradient. This suggests that plants at lower elevations are exposed to greater herbivore stress relative to those at higher elevations, which may induce the flavonoid biosynthesis pathway, and indirectly influence anthocyanin pigment production. Competition

may not play as great a role as herbivory since it has been shown that vegetation at lower elevations is more sparse relative to higher elevations, suggesting less competition (Callaway 1998). However, to determine whether these factors truly correlate with flower colour, we will need to analyze soil moisture measurements, percent herbivore damage, and percent vegetative cover in natural populations from our observational experiments.

Our results did show a negative relationship between flower pigmentation and leaf herbivory, indicating that pink and purple flowered plants have lower herbivore damage than white flowered plants. These patterns were also seen in Ansley's study, which shows temporal consistency in the interactions between plant pigmentation and herbivory. This further suggests a tight link between plant stress response and anthocyanin pigment production because plants with pigmented flowers may induce greater herbivore-resistance compounds due to the shared biosynthetic pathway of anthocyanin and other flavonoids. Previous work suggests that anthocyanins may directly be involved in biotic stress response, and is shown to be effective for pathogens, but it is recently seen as an indirect correlation with insect herbivore response (Simmonds 2003). Flavonoids can be induced through herbivore damage and often deter further herbivory by insects (Treutter 2006). Thus, the induction of flavonoids by herbivory and the shared biosynthetic pathway with anthocyanin suggests an indirect influence of herbivory on flower colouration. If herbivores influence chemical resistance traits in *B. stricta*, this may present a potential for indirect selection on flower colour. However, to determine if this is true, we will need to analyze heritability of flower colour. Our Schofield and North Pole gardens show a significant genetic-cline in flower colour, suggesting a strong genetic basis for this trait. This along with a significant effect of genotype (Table 1), indicates that flower colour may be heritable. The Anderson lab also showed heritability for flower colour in a greenhouse experiment (Anderson, unpublished data), which also suggests potential for indirect selection through herbivory, but this is yet to be shown under natural conditions and is currently being analyzed for this study.

Alternatively, if *B. stricta* invest in hosting natural enemies that protect the plant by reducing herbivore damage, they may not invest as much into chemical defenses (Ode 2006). *B. stricta* produce extra-floral nectaries used to attract ants that defend the plants against herbivores. During data collection, I noted whether flowers have extra-floral nectar, which can be used to determine how extra-floral nectar production and flower colour are related. Further research on this work can also provide insight into the relative costs and benefits of investing into different defense strategies for self-pollinating species.

So far, our common garden work on *B. stricta* has shown both a genetic and plastic component to flower colour, along with a strong negative correlation between flower colouration and herbivory. The results from our observational studies will show which environmental correlates influence flower colour, and test my hypotheses that water stress and biotic interactions are most important based on the common garden results. They will also determine the fitness consequences of flower pigment production through analyzing growth and reproductive data. Together these results will help understand how flower colour polymorphisms are maintained and have potentially evolved in plants that do not require animal-pollination for reproduction.

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Tables & Figures

Table 1. Summary of the results from the mixed effects logistic regression. Significant p-values are bolded.

Source	F _{numerator d.f., denominator d.f.}	p-value
Garden	F _{4, 5312} =4.59	0.0011
Source elevation	F _{1, 5312} =4.08	0.0435
Source elevation × Garden	F _{4, 5312} =5.63	0.0002
Season	F _{2, 5312} =10.46	<0.0001
Foliar damage from herbivores	F _{1, 5312} =6.03	0.0141
Foliar damage from herbivores × Garden	F _{4, 5312} =1.67	0.15
Experimental block	$\chi^2=3.98$	0.023
Genotype	$\chi^2=889.96$	<0.0001

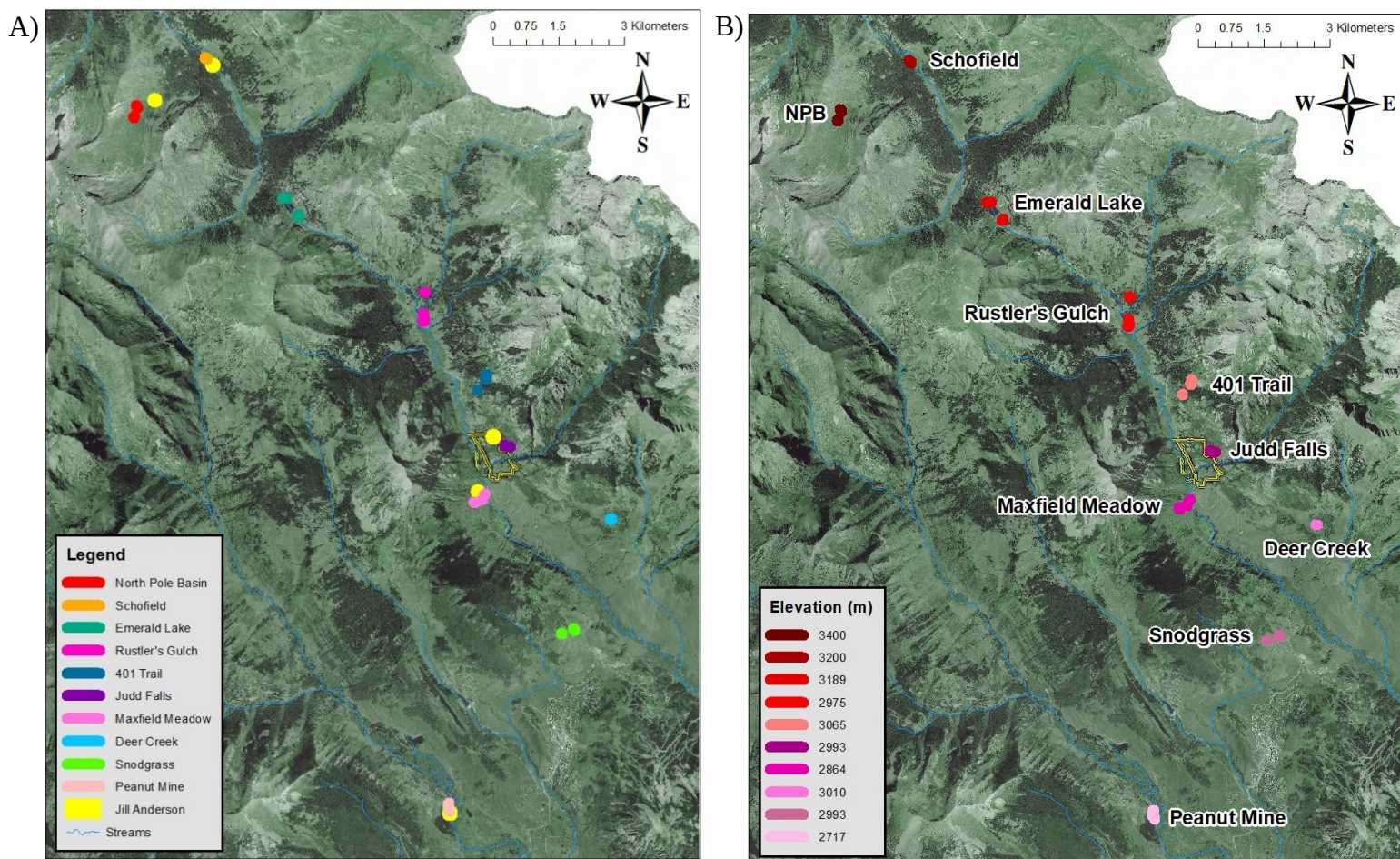


Figure 1. A vicinity map (A) and elevation map (B) of the 11 locations from which *Boechera stricta* were sampled across an elevational gradient. Each location has 3 colour coded points that are 15m transects.



Figure 2. Flower colour-categorizing system developed by Anderson and Embick for *Boechera stricta*.

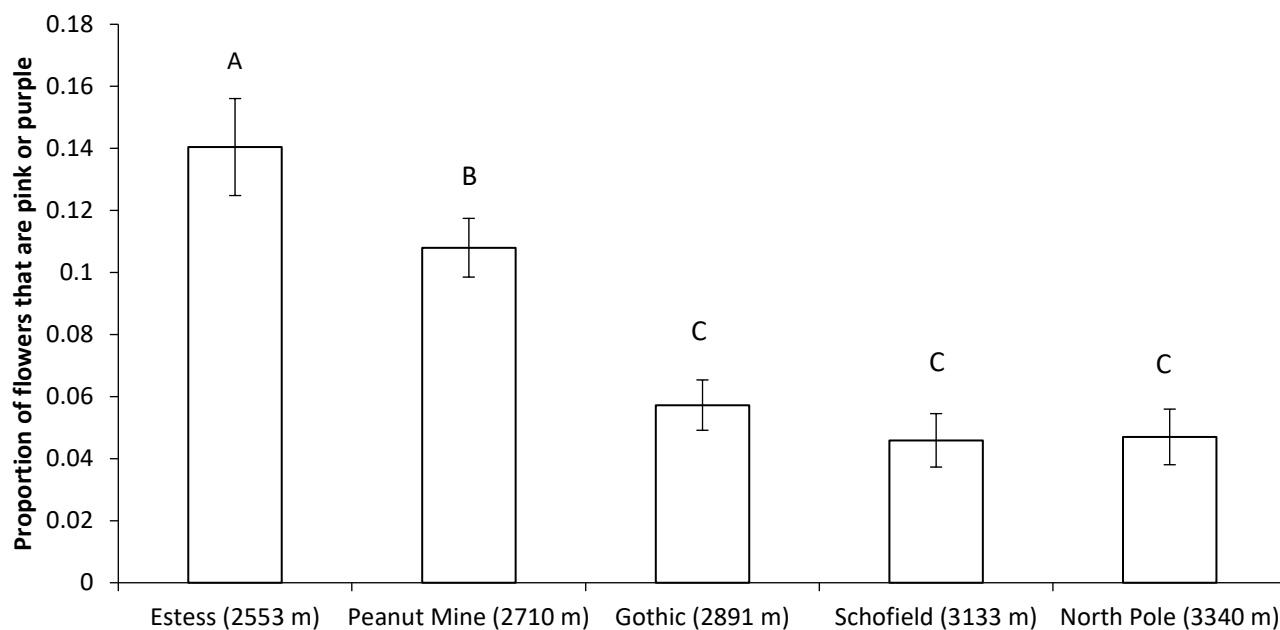


Figure 3. The proportion of pink or purple flowers across the 5 common garden treatments. Different letters represent significance among different gardens. The two lower elevation gardens, Estess and Peanut Mine, show a significantly greater proportion of pigmented flowers than the higher elevation gardens (Garden main effect: $F_{4, 3450}=2.51$, $P\text{-value}=0.04$).

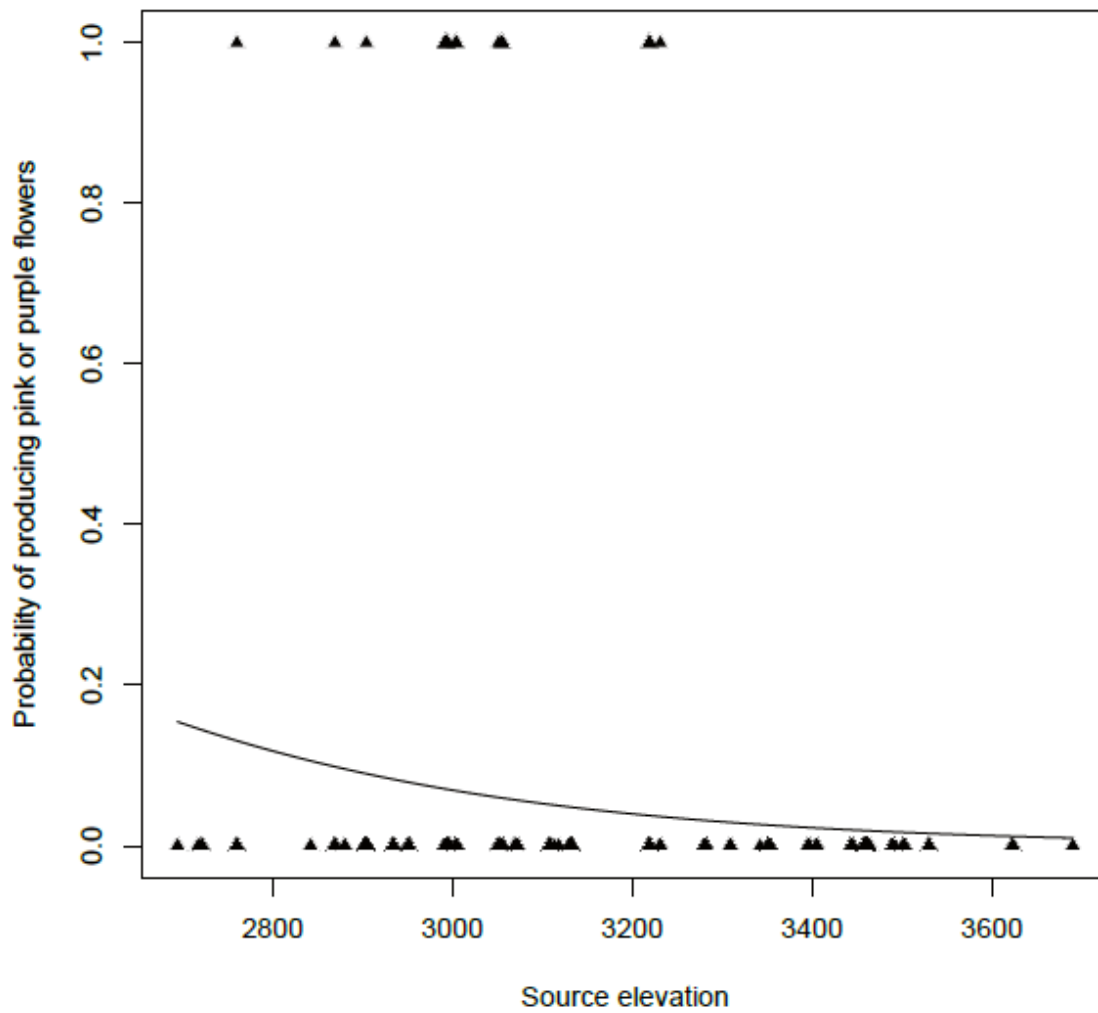


Figure 4. The probability of producing purple or pink flowers among different genotypes (i.e. source elevation) in the second highest elevation garden; Schofield. There is a significant genetically-based cline in flower color, showing a negative relationship between source elevation and the probability of pink or purple flowered plants ($t=-2.22$, $p=0.026$).

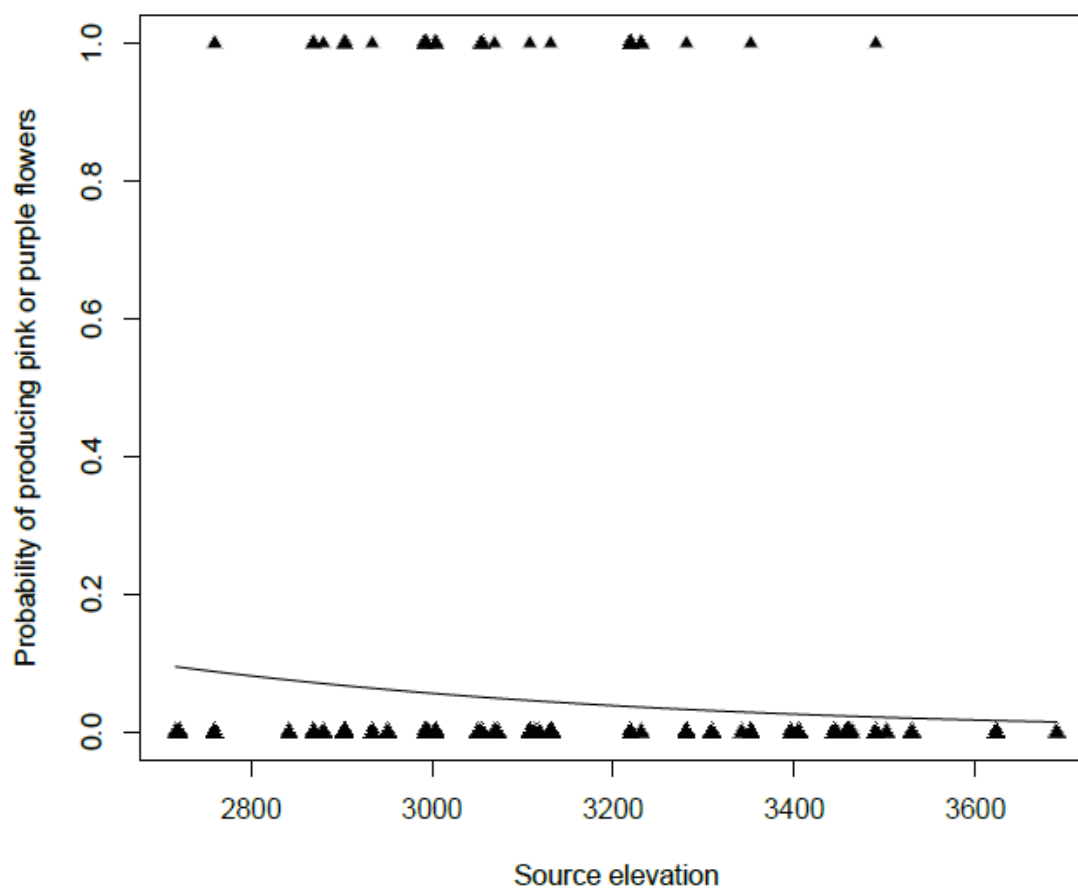


Figure 5. The probability of producing purple or pink flowers among different genotypes (i.e. source elevation) in the highest elevation garden; North Pole. There is a significant genetically-based cline in flower color, showing a negative relationship between source elevation and the probability of pink or purple flowered plants ($t=-3.47$, $p=0.0005$).

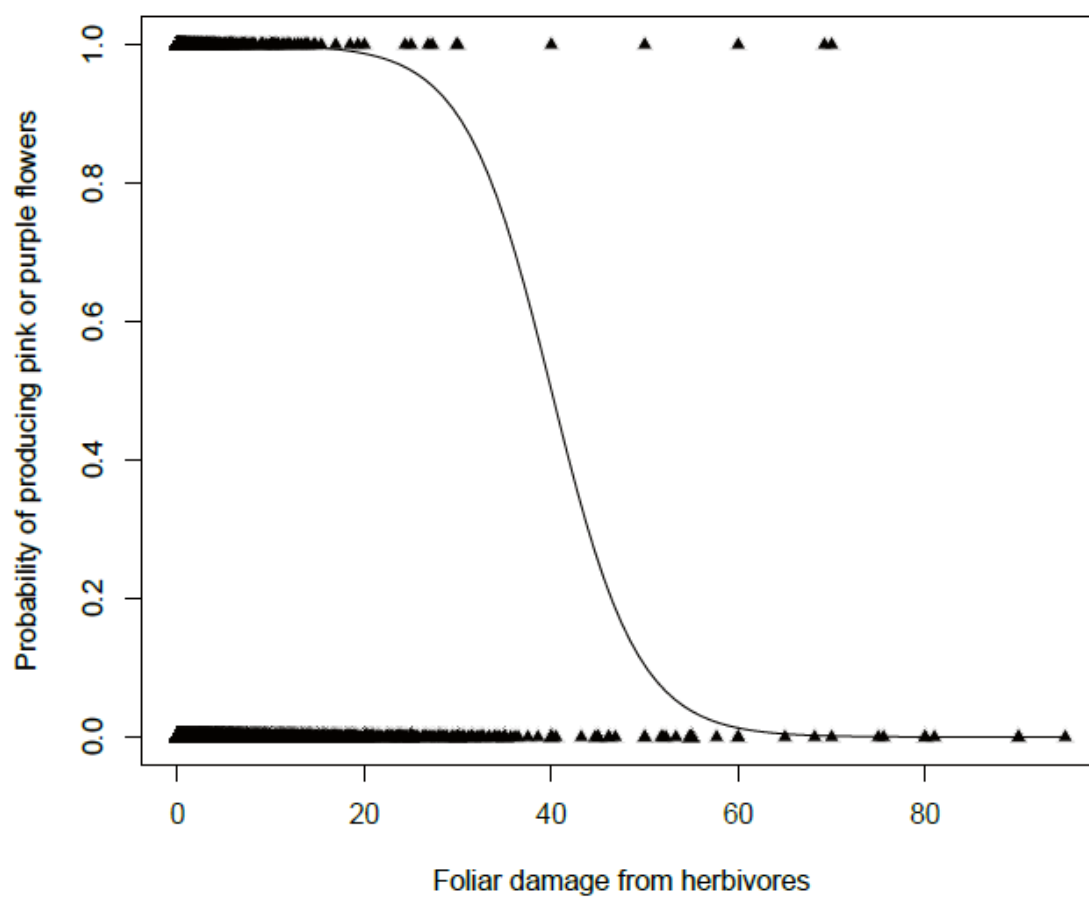


Figure 6. The probability of producing pink or purple flowers in relation to herbivory on leaves. There is a significant negative relationship, in which pigmented flowered plants received less foliar damage than white flowered plants ($F_{1, 5312}=6.03$, $P\text{-value}=0.0141$).