

**Ecological causes and consequences of flower color polymorphism in
Boechera stricta.**

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INTRODUCTION:

Heritable phenotypic variation is the basis of evolution by natural selection. If natural selection favors phenotypes with the greatest fitness, then why is there so much phenotypic variation within species? Evolutionary biologists have long been invested in answering this question and in studying the processes that maintain variation within populations (Mitchell-Olds et al. 2007). Polymorphisms can be maintained by selection if rare morphs are beneficial (e.g frequency-dependent selection) or if different morphs are advantageous in spatially or temporally contrasting environmental conditions (e.g balancing selection or divergent selection). Alternatively, polymorphisms can be neutral and retained, for example, as a phylogenetic artifact. Finally, disadvantageous polymorphisms arising from novel mutations may simply not yet have been eliminated by selection. In general, we lack a fundamental understanding of the evolutionary processes that influence and maintain phenotypic variation (Mitchell-Olds et al. 2007).

Flower color is an excellent example of a trait that can vary within populations. Waser and Price (1981, 1983 & 1985) found that albino-flowered *Delphinium nelsonii* are at a selective disadvantage in the form of reduced seed-set relative to their normal, blue flowered counterparts because pollinators discriminate against them. Furthermore, flower color can influence adaptive evolution in plant, pollinator and herbivore communities (Bradshaw and Schemske 2003; Strauss et al. 2004). Anthocyanins, the most common flower pigments in angiosperms, serve as an excellent experimental model because they retain a well-understood, phylogenetically conserved biosynthetic pathway (Rausher 2008). Furthermore, anthocyanin pigments in vegetative tissues may attract or deter different pollinators and herbivores, and anthocyanin pigmentation can enhance plant fitness and survivorship under environmental stresses (Strauss & Whittall, 2006). While pollinators are often regarded as the strongest force of selection on floral traits (Waser and Price 1981 and 1983; Fenster et al. 2004), abiotic and biotic non-pollinator agents can also influence floral characteristics, including petal color (reviewed in Strauss & Whittall, 2006). Anthocyanin pigments can be expressed in vegetative tissues where they can enhance herbivore resistance and other related traits (Streisfeld and Rausher, 2011). Thus, variation in flower color could be related to systemic anthocyanin production throughout the plant for increased herbivore resistance.

Other abiotic factors also influence flower color. For example, the flowers of hydrangea cultivars (a common household shrub) can shift from red to blue as soil pH decreases, which increases Al^{3+} availability (Schreiber et al. 2010). Low temperatures have also been shown to induce anthocyanin synthesis in seedlings of *Arabidopsis* (Graham 1998 and Lyva et al. 1995) and Krol et al (1995) believe that higher levels of anthocyanins shield the mesophyll of *Pinus* saplings from low-temperature photoinhibition. Indeed, anthocyanin-rich species such as *Photinia* have extended growing periods compared to other shrubs because of increased tolerance to cool temperatures (Krol 1989). Additionally, experimental evidence suggests that in some species, episodes of heat or drought favor pink or purple flowered individuals over white-flowered ones because the associated anthocyanins in vegetative tissues improve stress tolerance (Strauss and Whittall 2006). For example, Schemske and Bierzychudek (2001) established that blue-flowered morphs of *Linanthus parryae* have greater fitness than white-flowered morphs during years of drought, whereas white morphs are more fit during years of increased precipitation. This is due to the anthocyanin pigmentation of the blue-flower morph, which is expressed in the vegetative tissue of *L. parryae*, enhancing its water-use efficiency.

Understanding which environmental factors affect flower color and yield phenotypic variation can be accomplished by studying plant populations in different environments. Using an elevational gradient to quantify environments varying in temperature and water availability is a powerful method of executing such investigations (Arnold et al 2009). For example, plants at higher elevations are subject to temperature extremes, decreased water availability and increased ultraviolet exposure, which can influence flower color (Arnold et al 2009). Indeed, Arnold et al (2009) studied flower color variation across an elevational gradient in response to different pollinator communities at different altitudes, and their approach laid the foundation for examining abiotic and biotic non-pollinator causes of flower color polymorphism at different altitudes and their environments.

Boechea stricta (Brassicaceae) is a great species for studying adaptive flower color polymorphism. *Boechea stricta* is a primarily self-pollinating, genetically well-defined perennial species that is closely related to the model organism *Arabidopsis thaliana*

(Song et al. 2006; Rushworth et al., in revision). *Boechera stricta* inhabits a large elevational gradient, from 1500 m to 3700 m (Anderson et al., 2010) and occurs in various habitats, such as meadows and forested areas (Song et al. 2006). Most *B. stricta* flowers have white petals; however some populations maintain a low-frequency purple flower morph (Anderson and Mitchell Olds, unpublished data). Very little is known about the nature of the purple morph or its genetic underpinnings, and the vibrancy of the purple petals can range from deep purple to pale purple-pink. Because *B. stricta* is predominantly self-pollinating and does not rely on pollinators for pollen dispersal (Rushworth et al., in revision), the reasons for maintaining a low-frequency flower color morph remain unclear. It may be in response to the local abiotic and biotic environmental conditions, and thus the purple morph could be a plastic response to the environment.

We conducted an observational field study to: (1) compare key phenotypic traits and fitness components of white vs. purple flowered plants, including foliar herbivore damage, plant size, and fruit production, and (2) assess whether flower color in *B. stricta* is associated with environmental factors (elevation, soil pH and soil water content). Establishing if flower color varies with environmental conditions will provide a strong foundation for manipulative experiments, which can establish causation and verify if the purple morph experiences selective disadvantage.

METHODS

We selected 10 sites that represent the environmental conditions *B. stricta* inhabits around the Rocky Mountain Biological Laboratory (RMBL) in west-central Colorado, and created two to four 5 m × 5 m representative plots at each site. The 10 sites were distributed along an elevational gradient (2,945 m-3,235 m) and contained a variety of habitats, including wet and dry meadows and forested environments (**Table 1**). In total, we established and monitored 30 plots.

To assess variation in flower pigmentation, we created a color-categorization system by collecting different colored flowers, photographing them, and giving them a score (on a scale of 0-5, from white to dark purple). We used these photographs as a guide to categorize the color of the flowers in our plots (**Figure 1**).

Quantifying Differences in Fitness Traits Between Morphs

To determine the relative fitness of the different flower color morphs, we measured various fitness-traits including herbivore damage, plant size (number of leaves and height), number of flowers and fruits (siliques), and length of the longest silique on each plant in each of the 30 plots. Plants were individually labeled and monitored three times throughout the summer. For the first plant census, we measured herbivore damage, number of leaves and height, number of flowers, buds, siliques and longest silique per plant. We calculated herbivore damage by counting the number of leaves on each individual with herbivory and the percent damage of each leaf. We averaged the percent damage for all herbivorized leaves to estimate the total herbivory for the plant. For the second census, we quantified the number of flowers in each color category, and for the final census we measured plant height, flower number and color, the number of siliques and the length of the longest silique. To establish whether the purple flowered plants are at a selective disadvantage in terms of reduced seed set and have higher levels of foliar herbivory, we performed five separate ANOVAs with leaf damage, initial and final plant height, and silique number and length as response variables (Proc Glimmix, SAS ver. 9.2, NC). Because the plant measurements were not collected within the same week for the first census, we corrected the census data for day of measurement by dividing the measurements by Julian day of the year. We incorporated plot nested within site as a random effect to account for non-independence of plants in the same plot and site. We used the proportion of flowers that were white in color on an individual plant as our explanatory variable, as some individuals had both white and non-white flowers. For all analyses, residuals were normally-distributed and homoscedastic.

Characterizing Environmental Correlates of Floral Color Frequencies

To evaluate if abiotic environmental factors correlate with flower color frequency, we measured soil pH (Kelway Soil pH Meter, Kel Instruments Co., NJ) and soil moisture (HydroSense Soil Moisture Meter, Campbell Scientific Australia, AU) in five locations at each plot. We performed logistic regression with a binomial distribution and a logit link function to test whether environmental conditions significantly influence the probability

of individuals within a plot with entirely white flowers (Proc Glimmix), including plot nested within site as a random effect. This analysis was done at the plot-level.

RESULTS:

Quantifying Differences in Fitness Traits Between Morphs

Plant height at the first census varied significantly with flower color ($F_{1,640}=8.04$, p-value 0.0047) (**Table 2, Figure 2**), in which white flowered plants were shorter than purple flowered plants. There was also significant site-to-site variation in the proportion of white flowers in each plot ($\chi^2=168.3$, p-value < 0.0001). Flower color did not significantly influence the number of siliques or silique length (**Table 2**).

Characterizing Environmental Correlates of Floral Color Frequencies

The probability that a plot consisted of entirely white flowered plants varied significantly with soil water content ($F_{1,17}=11.2$, p-value= 0.0038): for every 1% increase in soil water content, there was a 10.9% increase in the probability that a plot was composed only of white flowers (**Table 3, Figure 3**). The probability that a plot consisted of entirely white flowered plants also varied significantly with soil pH ($F_{1,17}=8.04$, p-value 0.0047): for every unit increase in soil pH, there was a 98% decrease in the probability that a plot was composed only of white flowers (**Table 3, Figure 4**). There was a trend with elevation, in which for every 1 foot increase in elevation, there was a 0.3% increase in the probability that a plot was composed only of white flowers (**Table 3, Figure 5**). We also found significant site-to-site variation in the probability that a plot consisted entirely of white flowered individuals ($\chi^2=105.63$, p-value < 0.0001).

DISCUSSION

The purpose of this observational study was to assess the differences in fitness-related traits between flower color morphs and to quantify the environmental correlates of variation in floral color frequencies among populations. We found that (1) purple flowered plants had a taller initial height than white flowered plants; (2) fruit production and length did not vary as a function of flower color; and (3) the probability of white

flowered plants increased significantly with more soil moisture, soil acidity (lower soil pH), and marginally with higher elevation.

Because there was no difference in silique production and length between flower morphs, the purple flowered plants were not at a selective disadvantage this year, as we used silique production as the metric of fitness. However, purple flowered plants were taller at the initial census, which suggests that either purple flowered plants emerged earlier, or they emerged at the same time as white-flowered plants but had greater initial growth rates. Plant height is a central component of a plant's ecological strategy because it is crucial to a plant's carbon gain (Mole et al. 2009), and height is a major factor in a plant's ability to compete for light (Falster & Westoby, 2003). Plant height is strongly correlated with life span and time to reproduction (Moles & Leishman, 2008), and Enquist et al. (1998) found that plant size is also correlated with metabolic rate and maximum population density. However, in our study, final plant height did not significantly vary as a function of flower color, which suggests that if the purple morph does have a fitness advantage over white flowered plants, it is in early season growth. Several seasons of field data need to be collected in order to (1) assess the temporal variability of fitness related traits of the flower morphs; (2) calculate the frequency of purple flowered morphs in *B. stricta* populations around RMBL, and (3) decouple the influence of environment and genotype on morphological traits.

We also found that the probability of white flowered plants is greater in soils with higher soil moisture, which suggests that darker pigmentation may be associated with drought-related issues. This is in accordance with existing experimental evidence, which found that in some plant species, periods of drought favor pink/purple flowered individuals over white-flowered ones because increased anthocyanins levels enhance stress tolerance (Strauss and Whittall 2006). This is true for *Linanthus parryae* blue-flower morphs, which have greater fitness than white flower morphs during years of drought because the increased anthocyanin pigmentation enhances its water use efficiency (Schemske and Bierzychudek, 2001). Dick et al. (2011) observed a similar pattern in the purple/white flower polymorphism in the arctic mustard *Parrya nudicaulis*. They found that the frequency of white flowered *P. nudicaulis* increased with favorable environmental factors such as warming temperatures and increased water availability,

which is consistent with the role of anthocyanin pigments in stress tolerance. Manipulative laboratory studies need to be conducted in order to establish causality between water stress and higher frequencies of purple flowered *B. stricta*.

We also concluded that white flowered plants are more likely in plots with lower soil pH. Anthocyanins, the most common flower pigments in angiosperms, are highly unstable molecules that are susceptible to degradation from extreme light conditions, soil pH and temperature (Wesche-Ebeling & Argaiz-Jamet 2002). The ionic nature of anthocyanin facilitates changes in the molecule structure according to pH, which can yield distinct colors at different pH values (Brouillard 1982). Wesche-Ebeling & Argaiz-Jamet (2002) and Rein (2005) have shown that anthocyanins in several commercial fruits are more stable in acidic soils than in alkaline soils, which is inconsistent with our findings. However, this mechanism may be different in flowers, as the flowers of hydrangea cultivars change from red to blue in soils with low pH, which increases Al^{3+} availability (Schreiber et al. 2010). In this system, Al^{3+} removes H^+ ions from delphinidin, the anthocyanin responsible for the blue pigmentation, which accentuates the blue color of the flowers. However, further experiments are needed to assess the relationship between soil pH and flower color in *B. stricta*.

Finally, our results also show a trend between flower color and elevation, in which white flowered plants are more frequent at higher elevations. Plants at higher altitudes experience environmental stresses such as temperature extremes and decreased water availability, which can affect flower color (Arnold et al 2009). This result is inconsistent with the prediction that if anthocyanin production increases stress tolerance, then purple flowered individuals should be more frequent in stressful environments. Therefore more seasons of field data are needed to evaluate the correlation between elevation and flower color frequency.

If natural selection favors phenotypes with the greatest fitness, then understanding why there is so much phenotypic variation within species is critical for our knowledge of evolutionary ecology. Our preliminary results suggest that variation flower color in *B. stricta* could be a highly plastic response to edaphic conditions including soil moisture and soil pH. We currently do not know whether flower color also has a genetic component. However, over the course of one field season, we found that white flowered

individuals do not have a fitness advantage or disadvantage relative to non-white flowered individuals. The results of this observational study will provide essential data for further experiments to investigate the ecological and evolutionary causes and consequences of purple flower morphs of *B. stricta* and determine if purple morphs experience selective disadvantage integrated across sites and seasons. Furthermore, because the flower morphs fluctuate in frequency in differing environments, with further analysis and field studies these results may provide data that will allow scientists to predict the consequences of shifting global environments on the phenotypic variation of *B. stricta* and the impacts it may have on the larger ecological community.

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Figure 1: Flower guide used to categorize flower color variation in *Boechera stricta*.

Table 1: Site habitat and environment				
Site	Elevation (m)	Mean Soil Moisture (%) $\pm$ SE	Mean Soil pH $\pm$ SE	Habitat description
1	3009	5.91 $\pm$ 0.33	6.75 $\pm$ 0.03	Dry meadow, rocky, little vegetation
2	3002	8.35 $\pm$ 0.40	6.54 $\pm$ 0.10	Dry meadow, rocky, little vegetation
3	3011	11.97 $\pm$ 0.90	6.33 $\pm$ 0.03	Wet meadow, heavily vegetated
4	3018	16.5 $\pm$ 1.94	6.43 $\pm$ 0.08	Rocky, inclined habitat at edge of aspen forest, little vegetation
5	3006	8.27 $\pm$ 1.14	6.67 $\pm$ 0.03	Aspen forest edge, moderately vegetated
6	2975	7.23 $\pm$ 0.88	6.70 $\pm$ 0.01	Dry meadow, moderately vegetated
7	3223	6.5 $\pm$ 0.40	6.57 $\pm$ 0.07	Conifer forest edge, dry, rocky, little vegetation
8	3024	8.6 $\pm$ 0.6	6.75 $\pm$ 0.05	Wet meadow, exposed, moderately vegetated
9	3083	17.2 $\pm$ 1.8	6.45 $\pm$ 0.05	Wet meadow, moderately vegetated, slightly rocky
10	2947	12.73 $\pm$ 0.67	6.47 $\pm$ 0.03	Exposed wet meadow, heavily vegetated

Table 2: ANOVA results comparing phenotypic traits among flower morphs. Five separate ANOVAs were performed to examine the effect of the proportion of white flowers on an individual on key fitness-related traits. Random effect of site was significant ($\chi^2=168.3$, p-value < 0.0001).				
Trait	Numerator df	Denominator df	F-value	p-value
Foliar damage	1	670	1.1963	0.2745
Initial height	1	669	8.04	0.0047*
Final height	1	640	3.1261	0.0775
Silique production (final census)	1	633	1.5533	0.2131
Length of longest silique (final census)	1	632	0.6528	0.4194

***significant p-value**

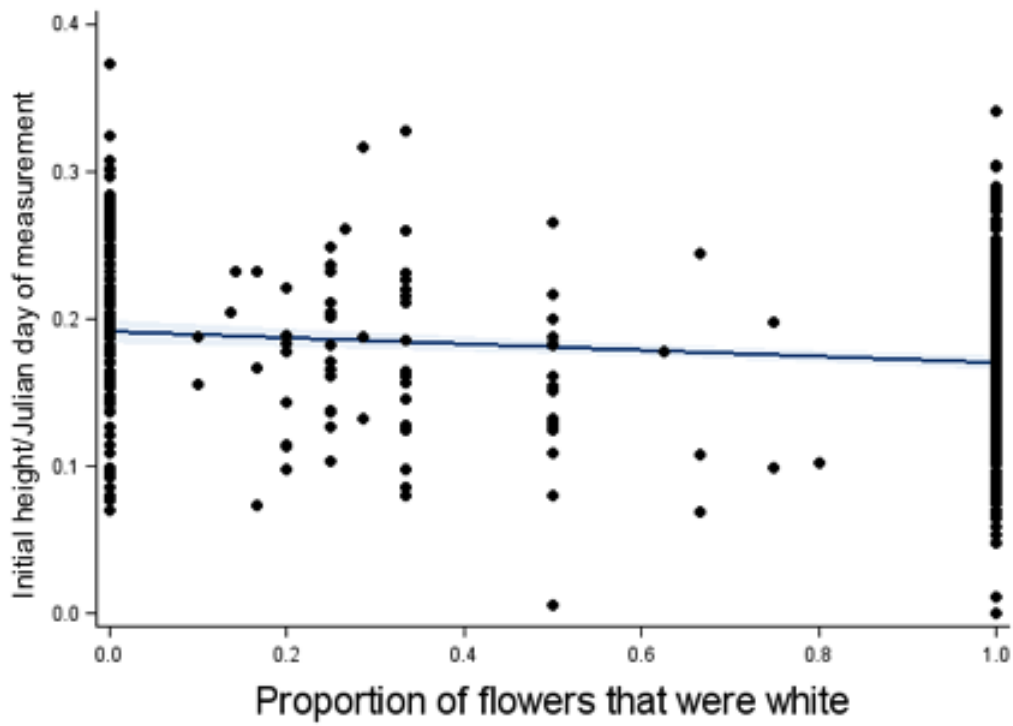


Figure 2: Initial plant height as a function of proportion of white flowers on an individual. Initial plant height varies significantly with the proportion of white flowers ($F_{1, 640}=8.04$, $p\text{-value}=0.0047$). Each data point represents an individual plant.

Table 3: Environmental correlations with flower color frequencies: logistic regression results. We performed logistic regression with a binomial distribution and a logit link function to test whether environmental conditions significantly influence the probability of individuals within a plot with entirely white flowers. Random effect of site was significant ($\chi^2=105.63$, p-value < 0.0001).						
<i>Factor</i>	<i>Numerator df</i>	<i>Denominator df</i>	<i>f-value</i>	<i>p-value</i>	<i>e^B (odds ratio)</i>	<i>95% confidence interval of odds ratio</i>
Elevation	1	17	3.25	0.0894	1.003	(1.000, 1.006)
Soil moisture	1	17	11.20	0.0038*	1.109	(1.039, 1.184)
Soil pH	1	17	8.76	0.0088*	0.02	(0.001, 0.327)

***significant p-value**

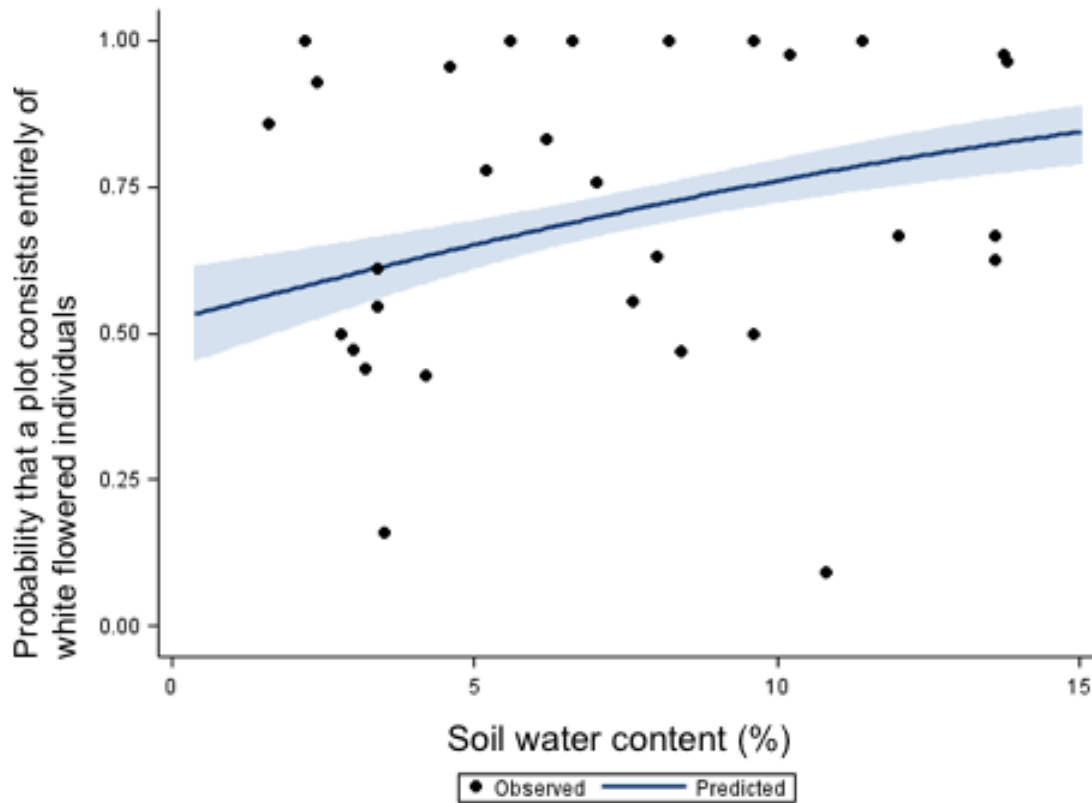


Figure 3: Probability of white flowers as a function of soil water content. The probability that a plot consisted of entirely white flowered plants varied significantly with soil water content ($F_{1, 17} = 11.2$, $p\text{-value} = 0.0038$). Each data point represents plot-level data, the line is the prediction, and the blue shaded area is the confidence interval of the prediction.

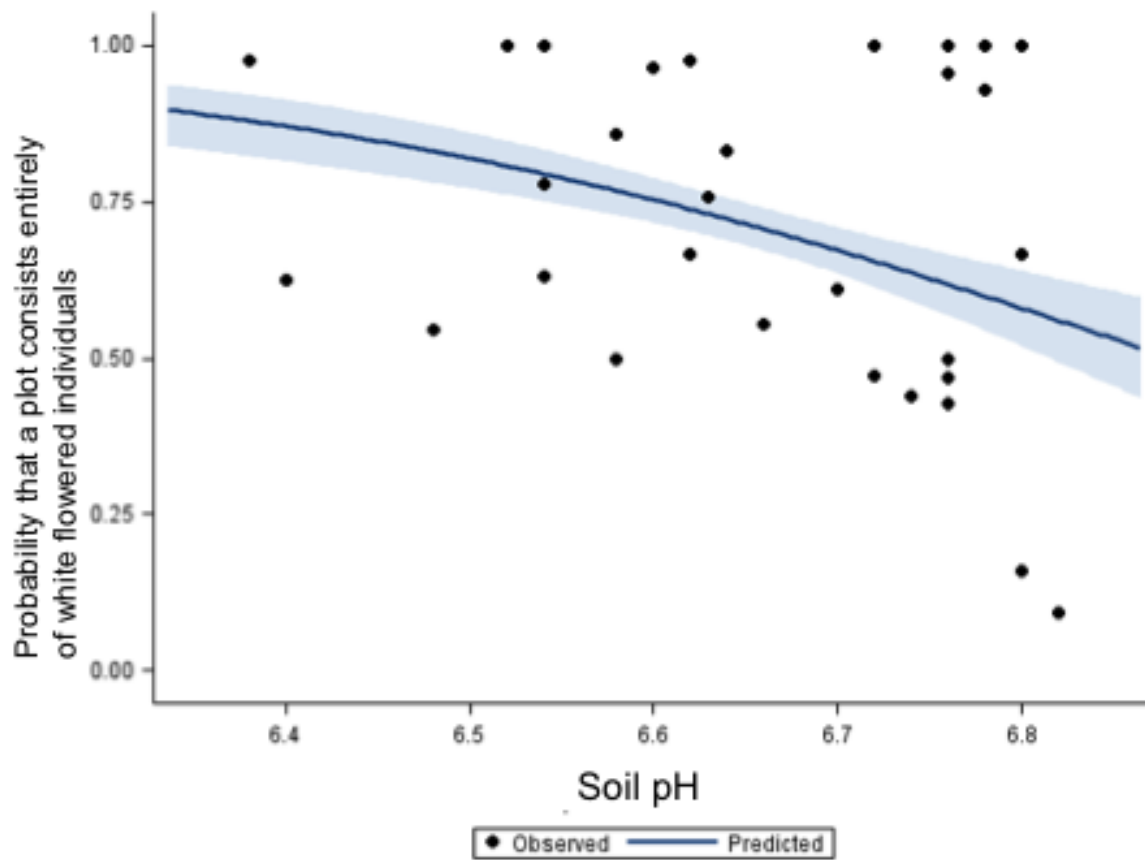


Figure 4: Probability of white flowers as a function of soil pH. The probability that a plot consisted of entirely white flowered plants varied significantly with soil pH ($F_{1,17} = 8.76$, $p\text{-value} = 0.0088$). Each data point represents plot-level data, the line is the prediction, and the blue shaded area is the confidence interval of the prediction.

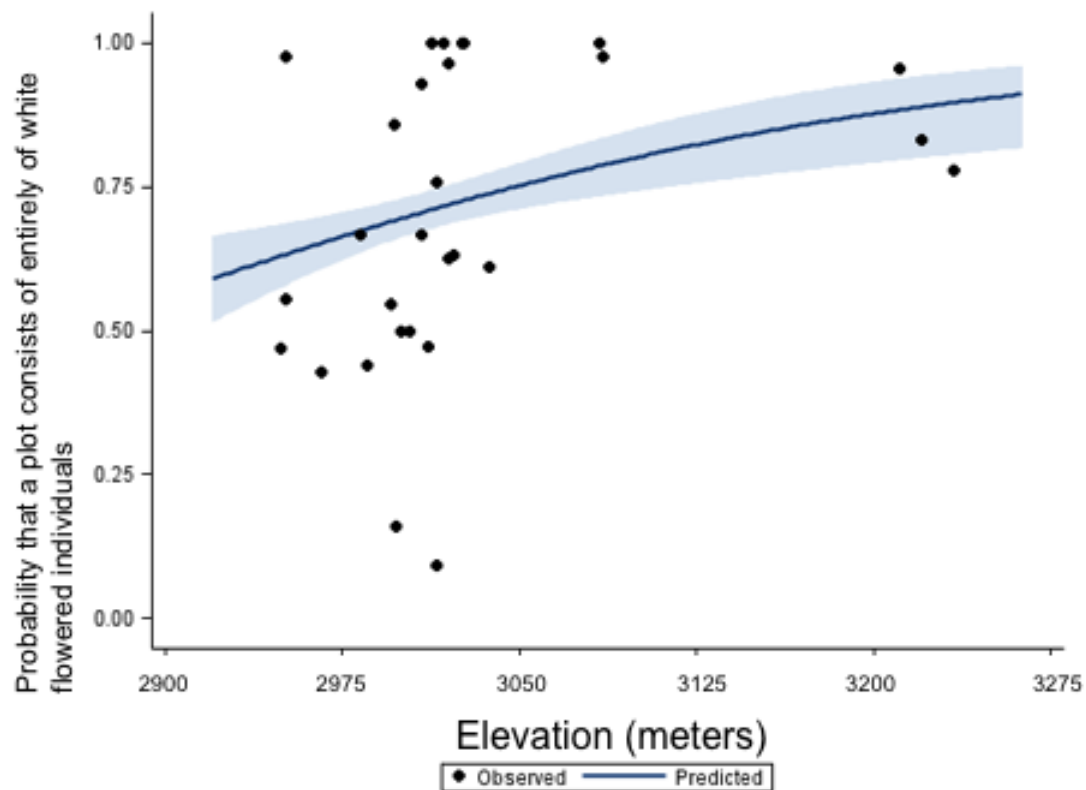


Figure 5: Probability of white flowers as a function of elevation. There was a trend in which the probability that a plot consisted of entirely white flowered plants increased at higher elevations ($F_{1, 17} = 3.25$, $p\text{-value} = 0.0894$). Each data point represents plot-level data, the line is the prediction, and the blue shaded area is the confidence interval of the prediction.