EVOLUTIONARY MAINTENANCE OF GEOGRAPHIC VARIATION FOR FLOWER COLOR IN *LEAVENWORTHIA STYLOSA*

by

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ABSTRACT

Flower color polymorphism is a striking feature of some angiosperm species, especially when it is geographically structured. Geographic patterns of flower color can be maintained by either non-adaptive processes, like genetic drift, or by natural selection, acting directly or indirectly. *Leavenworthia stylosa*, an endemic to the cedar glades of middle Tennessee, occurs mostly in monomorphic populations of yellow or white flowered morphs. The overall objective of my study was to understand why most of the *L. stylosa* populations are monomorphic and what maintains the geographic pattern of flower color variation of the species. I studied the pollinator assemblages, flower color preferences, and their constancy in foraging and seed predation across the species range. To check the differences in abiotic factors in different sites, I studied the soil chemistry, water holding capacity and water loss in white and yellow sites. Additionally, I conducted a series of reciprocal transplant experiments at different life history stages and evaluated the performance of the early life history stages of the two morphs across a water-related stress gradient. In both white and yellow sites, pollinators favored the white morph over the yellow morph across the species range. Seed predation was lower on the white morph compared to the yellow morph. *Bombylius* showed exclusive visits to the white morph in white sites favoring the white morph and preventing the yellow morph from increasing in frequency. In yellow sites, the fitness-enhancing pollinators preferred yellow morph and fitness-reducing seed predators showed no preference. There was no difference between white and yellow sites in soil chemistry. But yellow sites had

higher water holding capacity and higher water loss rate than white sites. During the early life history stages the white morph was more successful over yellow morph while during late life history stages yellow morph was more successful over white morph. The yellow morph showed limited evidence for local adaptation in flower number and fruit number survival through reproductive stage. Stress experiments showed conflicting results. Thus, it is likely that the maintenance of geographic variation for flower colors of *L. stylosa* is influenced by multiple selective agents including pollinators, seed predators and abiotic conditions.

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CHAPTER 1

GENERAL INTRODUCTION

Understanding the mechanisms that maintain variation among populations is a main focus of evolutionary biology. Many plant species exhibit conspicuous variation in flower color among individuals as well as among sites in different parts of their ranges. This variation is often attributed to pollinators as they can exert selection on floral traits by various mechanisms, such as geographic variation of the pollinator assemblages or pollinator preferences for particular flower colors (Waser, 1986; Gómez and Zamora, 2000; Aldridge and Campbell, 2007). The morph that a pollinator favors will gain a selective advantage over the discriminated morph, and thus, maintain the polymorphism of flower color.

In addition to their interactions with pollinators, plants experience antagonistic interactions with pre-dispersal seed predators, which can exert selective pressures for or against particular flower color morphs depending on seed predator color preferences. Different color morphs of the same plant species can also experience different effects of seed predators depending on their ability to produce plant defense compounds, many of which are pleiotropically associated with plant pigment biosynthesis pathways (Strauss and Irwin, 2004; Strauss and Whittall, 2006). If both pollinators and seed predators prefer the same flower color morph, this conflicting selection can result in multiple morphs being maintained within populations, since the morph which is less preferred by pollinators also experiences lower costs of seed predation. In contrast, in cases where the

preferences of the two agents are opposite (the morph less preferred by pollinators is preferred by seed predators), the net effect of selection should be a monomorphic population (Strauss and Whittall, 2006).

Indirect selection by abiotic factors may also play a role in maintaining among population variation in flower color (Armbruster, 2002). Spatial variation in abiotic conditions may result in local adaptation of different genotypes in various parts of the species range (Kawecki and Ebert, 2004). This can produce geographic variation in flower color if flower color morphs also differ in their abiotic tolerances. This adaptation may not be a product of a single selection event at a particular life history stage, but rather a multiplicative function of the selection episodes that take place at each stage of the life cycle (Sobral et al., 2015).

In addition to the direct or indirect selection exerted by biotic and abiotic agents, geographic variation can arise and be maintained by non-adaptive processes, such as genetic drift within populations in combination with restricted gene flow among populations (Epling and Dobzhansky, 1942). This can lead to fixation of alternate flower color alleles in different populations.

My study species*, Leavenworthia stylosa* A. Gray (Brassiceae), is a winter annual endemic to cedar glades that co-occurs with three other congeners in the Central Basin of Tennessee: *L. uniflora* (Michx.) Britton, *L. exigua* Rollins, and *L. torulosa* A. Gray (Tennessee Flora Committee, 2015). Among the four species in Tennessee, *L. stylosa* has the most restricted distribution, being documented in only seven counties (Rollins, 1963; Chester et al., 1997). Flower color is polymorphic within the genus *Leavenworthia*: Three species (*L. alabamica, L. torulosa,* and *L. uniflora*) produce white flowers, while

two species (*L. aurea* and *L. texana*) produce yellow flowers, and two other species (*L. crassa* and *L. exigua*) have yellow- and white-flowered populations classified as separate varieties or subspecies (Lloyd, 1969). Individuals of *L. stylosa* produce flowers that are either white, yellow, or rarely lavender. Individual populations may contain one, two, or all of these flower color morphs, but typically white- or yellow-flowered individuals are found in monomorphic or nearly monomorphic populations that are geographically structured in Middle Tennessee. In *L. stylosa*, there is considerable gene flow among populations and little genetic structure between populations, which does not match the flower color distribution pattern (Dixon et al., 2013), suggesting that the geographic pattern of flower color variation may be the result of differences in direct or indirect selection for flower color in different parts of the species range.

The overall objective of my dissertation was to investigate the role of selection by biotic and abiotic factors in the maintenance of flower color polymorphism and its geographic distribution in *L. stylosa*. My dissertation has two main chapters – Chapter 2 (biotic factors) and Chapter 3 (abiotic factors) - and an overall conclusion (Chapter 4).

In Chapter 2, I focus on biotic factors that may influence flower color. I present the results of a pollinator study done in white- and yellow-flowered sites by establishing reciprocal transplant arrays, and I test three possible mechanisms that pollinators use to exert selection on flower color leading to spatial variation of flower colors. In addition, I present data on pre-dispersal seed predation. Overall, I discuss how pollinators interact with seed predators to maintain the geographic pattern of flower color distribution in *L. stylosa*.

In Chapter 3, I focus on abiotic factors that may influence flower color. I present the results of (1) soil analyses performed in white- and yellow-flowered populations; (2) a series of reciprocal transplant experiments done on seed after-ripening, seed germination, juvenile and adult rosettes, and reproductive stages of the life cycle; and (3) laboratory experiments testing the performances of white and yellow morphs under water-related stress at the seed after-ripening, seed germination, and seedling growth stages. In the same chapter, I discuss the differences in fitness components between the two color morphs and evidence for local adaptation of color morphs at each life history stage.

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CHAPTER 2

ROLE OF BIOTIC FACTORS IN THE MAINTENANCE OF GEOGRAPHIC VARIATION FOR FLOWER COLOR IN *LEAVENWORTHIA STYLOSA*

ABSTRACT

When a polymorphism in a floral trait arises in a population, it may be maintained through adaptive processes like pollinator-mediated selection or through non-adaptive processes such as pleiotropic effects or genetic drift. The white and yellow flower color morphs occur in my study species, *Leavenworthia stylosa*, that are geographically structured in middle Tennessee (USA). I conducted a reciprocal transplant experiment (among four sites with two morphs) to determine whether pollinator assemblages, preferences, constancy, and seed predation differ between morphs and across the geographic range, and whether they exert concordant or conflicting selection on flower color. Pollinator assemblages at white sites were significantly different from those in yellow sites, but pollinators as a whole did not show different visitation rates to a particular morph at a site. Pollinators as a whole showed a color constancy. Seed predation was higher in white than in yellow sites, with the white morph having higher predation than the yellow morph. In white sites, exclusive visits by *Bombylius* contributed to maintaining the white morph and preventing the yellow morph from increasing in frequency within those sites. On the other hand, yellow morphs were less preferred by the pollinators and in yellow sites, *Bombylius* showed equal preferences and equal number of constant transitions between white to white as well as yellow to yellow without increasing the fitness of one morph over the other.

INTRODUCTION

Flowering plants show extraordinary diversity, especially in their reproductive organ – the flower (Clegg and Durbin, 2000; Conner, 2006; Rieseberg and Willis, 2007). Among floral traits, flower color is especially variable both among and within many species (Lloyd, 1969; Kay, 1978). Hence, understanding the processes that generate and maintain floral diversity in natural populations is a major goal in ecology and evolutionary biology (Mojonnier and Rausher, 1997; Subramaniam and Rausher, 2000). In species with flower color polymorphism, cases of geographic patterns of flower color variation are especially interesting (Mascó et al., 2004; Schemske and Bierzychudek, 2007; Hopkins et al., 2012; Arista et al., 2013). Variation in flower color across a species range could emerge due to several different reasons (Herrera et al., 2006). In species with a genetic basis of petal color, a geographic pattern of color variation could arise and be maintained through purely neutral processes such as genetic drift within populations in combination with restricted gene flow among populations, leading to the fixation of different flower color alleles in different portions of the geographic range (Epling and Dobzhansky, 1942). The resulting pattern of "isolation by distance" could prevent exchange of flower color alleles and maintain genetic differentiation of populations (Wright, 1943). Geographic variation in flower color could also result from natural selection exerted by local biotic or abiotic factors favoring one color over the other in a particular habitat or portion of the range leading to local adaptation of flower color morphs (Rausher, 2008). Selection could be acting on flower color directly, or indirectly as a response to selection acting on traits genetically correlated to flower color (Armbruster, 2002; Rausher, 2008).

Flower color is likely to have a strong influence on the reproductive success of individuals, as it provides visual cues for pollinators, and can influence their movement patterns (Galen, 1999; Raguso and Willis, 2002). Therefore, in many cases, selection by pollinators is thought to be a more likely mechanism to maintain geographic variation of flower color than are selectively neutral processes (Fenster et al., 2004; Rausher, 2008; but see Wang et al., 2016). Hence, in any study considering geographic variation of floral characters, one critical step is to investigate the relationship between flower color and the differences in the local selective pressures exerted by pollinators (Herrera et al., 2006; Anderson and Johnson, 2008; Niet et al., 2014). Pollinators are considered to be the primary selective agents on floral traits, and the existence of "pollination syndromes" is often cited as evidence for this (Knudsen and Tollsten, 1993; Fenster et al., 2004; Pauw, 2006; Rausher, 2008).

Pollinators may drive geographically structured divergence of floral characters by several mechanisms. One way is by creating a geographical pollinator mosaic or a "pollinator climate" (Grant and Grant, 1965). Pollinator assemblages likely vary geographically in terms of species identity and abundance, especially in plant species with generalist pollinators (Moeller, 2005). Differences among pollinators in their foraging behaviors (visitation frequency and efficiency) and preferences for floral traits can result in spatial variation in selection pressures on different floral traits (Gómez and Zamora, 2000). Alternatively, the color preference of pollinator species may vary spatially (Aldridge and Campbell, 2007), mainly due to the spatial variation of floral rewards (pollen and nectar) that attract pollinators (Heinrich and Raven, 1972; Waser, 1983).

Additionally, pollinators can exert selection on floral traits by means of floral constancy (Waser, 1986), which is the tendency of an individual pollinator upon leaving a flower of a particular color to visit another flower of the same color, even when alternative colors are present (Bateman, 1951). This type of behavior results in assortative mating among the members of the same color morph (Jones, 1997). As a result, the favored morph will gain a selective advantage, and the discriminated morph will get a selective disadvantage by means of differential seed set and paternity. Hence, the selection generated by preferential visitation and assortative movement of pollinators can preserve the monomorphic nature of populations by preventing the establishment of new morphs in the population (Waser and Price, 1981; Stanton et al., 1986; Stanton, 1987).

In addition to their mutualistic interactions with pollinators, plants experience antagonistic interactions with other animal visitors that can exert different types of selective pressures on floral traits (Strauss and Irwin, 2004). Herbivores are the major antagonistic animals encountered by plants throughout their life. Among them, pre-dispersal seed predators are especially important because, unlike other herbivores who generally damage only a part of the plant, seed predators destroy entire individuals (seeds), and can therefore have a large influence on plant fitness (Kolb et al., 2007). A growing body of knowledge supports the idea that pre-dispersal seed predators can exert selective pressure on floral traits via selection on pleiotropically-related plant traits associated with protection against herbivores (Linhart, 1991; Strauss et al., 1996; Galen, 1999; Strauss and Irwin, 2004). For example, flower color has been shown to be associated with both attracting pollinators and deterring seed predators (Carlson and Holsinger, 2010; Pérez-Barrales et al., 2013; Sobral et al., 2015). This occurs because

some floral pigments (e.g. anthocyanins) are produced via the same biosynthetic pathway as plant defense compounds that protect against predators (Strauss and Whittall, 2006; Caruso et al., 2010). Consequently, plants producing more floral pigments may also produce more defensive compounds (Strauss and Irwin, 2004). Therefore, through color preference, pre-dispersal seed predators can differentially reduce the fitness of different flower color morphs in a population with variable flower color (Kolb et al., 2007; Sobral et al., 2015). In addition to the color preference of seed predators, the overall seed predation rate in a particular habitat is likely to also be influenced by seed abundance, seed predator distribution pattern, and the presence of other interacting species (reviewed in Kolb et al. 2007).

Under a scenario where both mutualistic and antagonistic selective agents act on a particular floral trait (in this case, flower color), the net effect on the morph composition of the population will be determined by the balance of these conflicting pressures. If the preferences of mutualists and antagonists for a particular trait are opposed to each other (e.g. if one flower color morph is favored by pollinators and avoided by seed predators), this should result in a monomorphic population (Strauss and Whittall, 2006). Alternately, if mutualists and antagonists have similar preferences (the flower color morph preferred by pollinators also suffers greater seed predation), polymorphic populations could result (Strauss and Whittall, 2006). The variation in composition of floral visitors (both mutualistic and antagonistic) across the landscape forms a geographic mosaic of phenotypic selection with populations experiencing different evolutionary trajectories, depending on relative strengths of selection exerted by different floral visitors (Thompson, 1999). The variation in floral visitor assemblages can generate spatial

structure in floral traits (Jager and Ellis, 2014), and when there are several selective agents acting on a particular trait it is important to consider the role of all agents simultaneously in order to understand the mechanisms that maintain the geographic variation seen in floral traits (Caruso et al., 2010).

Leavenworthia stylosa A. Gray (Brassicaceae) has a genetically-based flower color polymorphism in which individuals produce either yellow or white flowers (Rollins, 1963; Norton et al., 2015). A survey of flower color variation across the species range found that among 77 populations, 66 of them were totally or nearly monomorphic in flower color (30 yellow, 36 white), while the remaining 11 populations were polymorphic for yellow and white flowers (Fig. 1) (Norton et al., 2015). Geographically, yellow-flowered populations occur predominantly in the northwestern part of the species range and white-flowered populations in the southeastern part.

This strong geographic pattern of flower color is puzzling given that Dixon et al. (2013) found low genetic differentiation among *L. stylosa* populations, and the patterns of differentiation did not correspond to the pattern of flower color variation. Moreover, the average pairwise F_{ST} value between populations of different flower colors was not significantly different from the average value between populations of the same flower color, suggesting significant gene flow among populations with different flower colors. The high prevalence of monomorphic populations suggests that there is strong natural selection occurring in the face of this significant gene flow to favor different flower colors in different parts of the range. This selection could be driven by biotic factors such as pollinators and seed predators, or by local adaptation to abiotic environmental conditions.

In this chapter, I investigated the role of biotic factors in shaping the geographic pattern of flower color in *L. stylosa* by a reciprocal transplant experiment at four study sites across the range of the species examining pollinators and seed predators. The self-incompatible flowers are pollinated by a diverse community of pollinators (Lloyd, 1969; Norton et al., 2015), and upon fertilization, *L. stylosa* seeds are produced in siliques. However, seeds are predated by the larvae of the cabbage seedpod weevil (*Ceutorhyncus obrstrictus*), which is a specialist seed predator of the Brassicaceae (Nielsen, 1989). Female weevils oviposit into immature fruits, and the resulting larvae consume seeds as the fruit develops. I addressed the following questions: (1) Do pollinator assemblages, preferences, constancy, and seed predation differ between floral color morphs of *L. stylosa*? (2) Is there geographic variation among *L. stylosa* populations in local selective pressures exerted by pollinators and seed predators? and (3) Do pollinators and seed predators exert a concordant or conflicting selection on flower color?

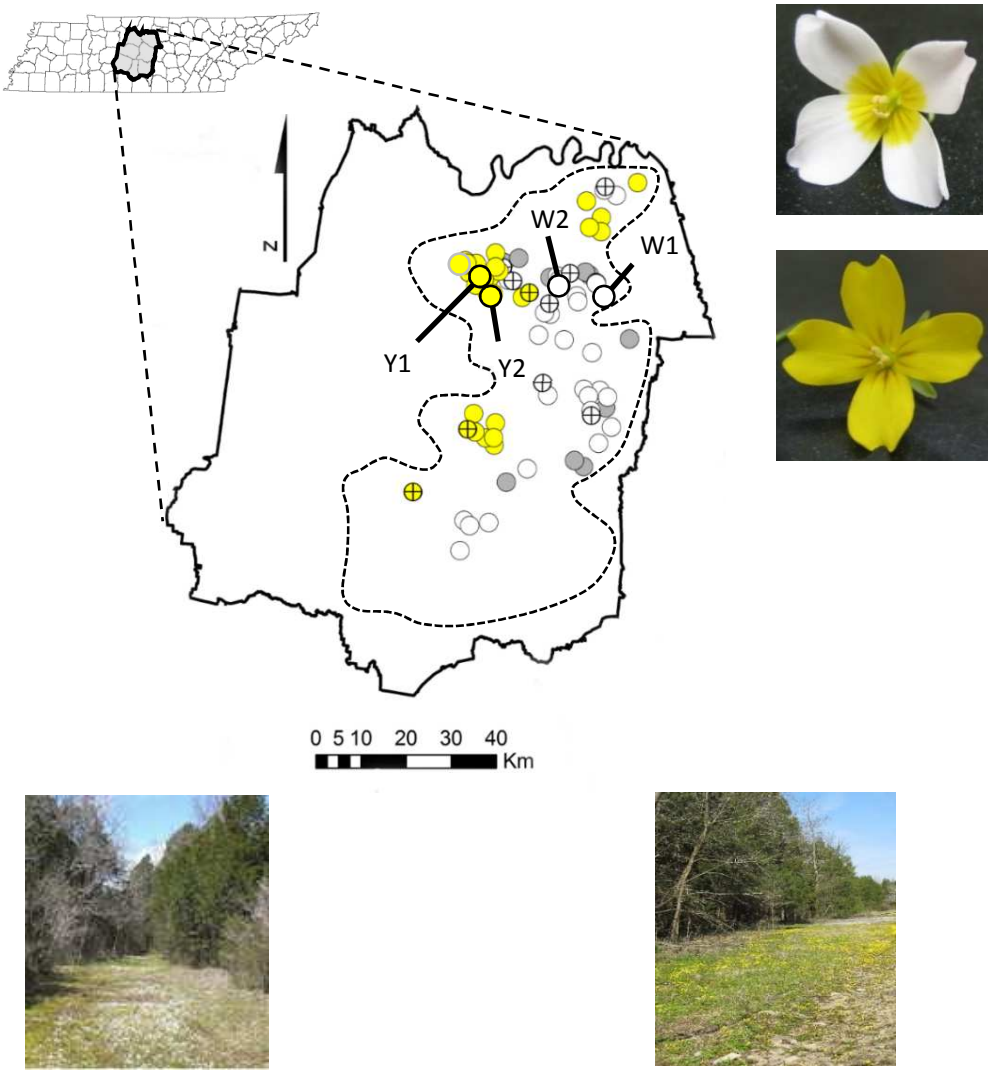

Figure 1. Distribution of *Leavenworthia stylosa* populations. *L. stylosa* is restricted to seven counties (shaded in the map) within the Central Basin of Tennessee (dashed line) and shows a petal color polymorphism of white or yellow flowers (pictured to the right of map). Circles represent the locations of the populations: open white and yellow circles for populations containing exclusively white or yellow morphs (pictured at the bottom of the map) and hatched white or yellow circles representing >99% monomorphic populations. Gray circles represent the populations with intermediate color frequencies. Thick-outlined, labeled circles represent the populations used in this study.

MATERIALS AND METHODS

Study species

Leavenworthia stylosa is endemic to the limestone cedar glades of the Central Basin of Tennessee, USA (Fig. 1). It is a winter annual in which seed dispersal occurs in late spring, and germination takes place in autumn. The plant overwinters as rosettes and flowers in early spring.

Experimental design

I collected similar-sized *L. stylosa* rosettes that were entering the early stages of flowering in March 2013 from two, white-flowered source populations [named W1 (Sue Warren) and W2 (Shooting Range) sites] and two yellow-flowered source populations [named Y1 (Smith Springs) and Y2 (Quarterman) sites] (Fig. 1). In each of these four sites, I established an array of 3 m x 3 m by planting 25 rosettes from each source population in a fully reciprocal design (alternating the color and source population, Fig. 2). Thus, each plant in an array originated from the home site or from an away site (e.g. at the W1 site, W1 plants were planted at their home site and W2, Y1, and Y2 plants from away sites). Plants were spaced a distance of approximately 0.3 m apart from each other. All of the arrays were established within populations of *L. stylosa* that served as sources. Prior to transplanting into the arrays, existing *L. stylosa* rosettes from the natural population were removed within the area of the array. The transplanted rosettes were watered for 2 weeks to ensure they became established, and after two weeks were allowed to grow under natural conditions. I revisited the arrays one week after

establishment, and I replaced dead rosettes with equally sized living rosettes; no additional rosettes died after this one-week period.

Pollinator preference and visitation

During the flowering season in April 2013, I conducted a series of pollinator observations on the plants in the arrays at each of the four sites. At the beginning of each observation session, I recorded the number of open flowers on each individual. All observations were done between 10:00 am and 3:00 pm when pollinators showed the highest activity; each observation session lasted 30 min. The observations were completed on days experiencing nearly full sun and no rain. During observations, each pollinator that entered into the array was followed until it left the array; the landing of a pollinator on petals of a flower was considered as a floral visit. The flower visitation pattern of each potential pollinator was recorded as it moved among the flowers on different individual plants (and rarely between flowers on the same individual plant, which accounted for 0.02% of total visits) inside the arrays. I quantified visitation rates as the number of pollinator visits per flower per hour of observation. To assess color constancy, I recorded the number of pollinator movements (transitions) between flowers of the same color morph and between flowers of different color morphs. Insects were not captured but were identified into five groups: Lepidoptera, non-*Bombylius* fly (hereafter, referred to as fly), bee fly (*Bombylius*), solitary bees (Andrenidae and Halictidae), and others. Twenty-three pollinator observation sessions were conducted over 12 days.

Seed predation

After flowering, flowers were allowed to develop into fruits in the field. In late May 2013, the plants in the arrays were harvested and the number of successful fruits (i.e. those that contained seeds) and failed fruits (i.e., those that did not contain seeds) were counted on each plant. I was able to identify failed fruits because in *L. stylosa,* flowers are borne singly, and the pedicels remain on the plant even when a flower fails to produce a fruit. Two mature fruits were randomly selected from each individual plant in the array, and the number of weevil predated and non-predated (intact) seeds were counted for each fruit. The total number of predated and non-predated seeds per plant was estimated by multiplying the numbers of predated and non-predated seeds per fruit by the total number of fruits produced by each plant.

Statistical analyses

Pollinator assemblages, preferences and constancy

Spatial variation of flower color, due to selection by pollinators, could result from three possible mechanisms (see Introduction in this Chapter). The first mechanism is that the abundance of different pollinator groups varies spatially, and the pollinator group that prefers a particular color morph is most common in the populations in which that particular morph predominates. I tested this mechanism by performing two heterogeneity G-tests (Sokal and Rohlf, 1995). The first G-test was done to identify a pollinator group that was more likely to occur at a particular site than the pollinator community as a whole. The number of individuals in a pollinator group observed at a particular site was compared to neutral expectations (i.e., the pollinator group visited the white and yellow

sites at the same frequency as all pollinator groups across all sites). The second G-test was performed to identify the pollinator group that was more likely to visit a particular color morph. For that test, the observed number of visits of a particular pollinator category to a particular color morph was compared to neutral expectations (i.e., that pollinator category had visited that color morph at the same frequency as all pollinator categories across all sites). For the first G-test, pollinators in the W1 site were not analyzed since a considerable part of the array was flooded for several days during the observation period, and very few pollinators visited our array during that period. For the second G-test, the "other" pollinator group was excluded from analyses since it was assumed to include multiple pollinator types with diverse preferences.

The second mechanism by which spatial variation of flower color could be determined by pollinator preference is if there is a spatial variation of color preference by pollinators such that the local color morph is visited more frequently. I tested this mechanism by analyzing the pooled visitation rates of all pollinators using an analysis of variance (ANOVA) with the factors site and morph and the interaction site x morph. A significant main effect of morph would reflect a pollinator preference (as a total pollinator biota) of a particular morph, while a significant site x morph interaction would reflect spatial variation in morph preference. A Tukey's post hoc comparison of means was done to determine the preferred color morph in each site. The visitation rates were square-root transformed to satisfy the normal distribution assumption. The color preferences of the individual pollinator group in white and yellow sites was compared, but an overall statistical analysis was not possible because some pollinator groups had very small sample sizes in one site type.

A third mechanism by which pollinators could influence the geographic pattern of flower color is through color constancy. If pollinators exhibit a significantly higher proportion of movements between flowers of the home color morph in the arrays, a reproductive advantage to the individuals of the home color morph over the away color morph would occur. To test this mechanism, two Chi-square tests (one for white sites and one for yellow sites) were used to compare the observed number of transitions with the expected numbers, which were calculated by multiplying the independent probabilities that pollinators originated on a particular morph and moved to a particular morph. If the result was significant, I then performed separate exact binomial tests with Bonferroni correction to determine if the constant and inconstant transition within or between morphs were significantly different from their null expectations (McDonald, 2008). In addition, to test the overall color constancy of the pollinator biota of *L. stylosa*, I pooled the count transitions shown by all pollinator groups across the four arrays and calculated Bateman's Constancy Index (BCI) (Bateman, 1951) using the following formula (Waser, 1986):

$$BCI = [(AD)^{1/2}-(BC)^{1/2}]/ [(AD)^{1/2} + (BC)^{1/2}],$$

where A and D represent constant transitions between the same color morph (A: white to white, D: yellow to yellow) and B and C indicate the number of inconstant flights between the morphs (B: white to yellow, C: yellow to white). BCI values range from -1 indicating complete inconstancy (pollinators always moving to the alternate morph) through 0 (random movements between morphs) to +1 indicating complete constancy (pollinators always moving between flowers of the same morph). BCI values were compared to 0 by a Chi-square test.

Seed predation

Seed predators could influence the evolutionary maintenance of flower color in three ways; by one morph always suffering higher seed predation (main effect of morph), by higher seed predation at certain sites (main effect of site) and by the cost of seed predation on specific morphs varying spatially (morph x site interaction). The proportion of predated seeds from each source population in white and yellow sites was analyzed using generalized linear model (binary logistic model) with morph and site (W1, W2, Y1, Y2) as factors and with a morph x site interaction, followed by least significant difference (LSD) test.

W1	Y1	W2	Y2	W1	Y1	W2	Y2	W1	Y1
Y1	W2	Y2	W1	Y1	W2	Y2	W1	Y1	W2
W2	Y2	W1	Y1	W2	Y2	W1	Y1	W2	Y2
Y2	W1	Y1	W2	Y2	W1	Y1	W2	Y2	W1
W1	Y1	W2	Y2	W1	Y1	W2	Y2	W1	W2
Y1	W2	Y2	W1	Y1	W2	Y2	W1	Y1	W2
W2	Y2	W1	Y1	W2	Y2	W1	Y1	W2	Y2
Y2	W1	Y1	W2	Y2	W1	Y1	W2	Y2	W1
W1	Y1	W2	Y2	W1	Y1	W2	Y2	W1	Y1
Y1	W2	Y2	W1	Y1	W2	Y2	W1	Y1	W2

Figure 2. Experimental arrays used to observe pollinator preference and visitation and seed predation. The yellow cells represent yellow-flowered *Leavenworthia stylosa* individuals and the white cells represent white-flowered individuals. W1, W2, Y1, and Y2 represent the source populations (see Fig. 1) from which individuals were collected and planted in the array.

RESULTS

Pollinator assemblages, preferences and constancy

In total, I observed the four arrays for 11.5 h, recording 300 flower visits by 165 individual pollinators. Solitary bees were the most common visitor (n = 77) and *Bombylius* showed the longest foraging bouts (mean = 2.4 flower visits per individual) within the arrays (Table 1).

To address the first mechanism, that abundance of different pollinator groups varies spatially and they prefer the predominant morph in their abundant site, the first heterogeneity G-test revealed that the pollinator assemblages at white sites were significantly different from those in yellow sites (Total $G = 31.55$, $P < 0.0001$). The number of Lepidoptera was significantly greater in yellow than in white sites ($G = 14.07$, $P < 0.001$), whereas that of solitary bees was significantly greater in white than in yellow sites ($G = 16.45$, $P < 0.0001$) (Table 1). The second heterogeneity G-test revealed that the five pollinator groups showed a significant difference in their color preferences (Total G = 43.029, P < 0.0001). Three of the four pollinator groups exhibited color preference, with Lepidoptera, fly, and *Bombylius* all visiting white morphs significantly more frequently than yellow morphs ($P < 0.001$, $P < 0.01$, $P < 0.01$, respectively) (Fig. 3). The visitation pattern of solitary bees did not statistically differ from equal visitation to both color morphs. In the G-test done on preference of different pollinator groups for different morphs, both total G and pooled G values were highly significant ($G = 43.07$, $P < 0.0001$ and $G = 24.53$, $P < 0.0001$, respectively) indicating an overall trend in favor of visitation to the white morph. However, the heterogeneity G in the same analysis was also

significant ($G = 18.54$, $P < 0.001$) indicating that the preference for visiting the white morph was not uniform in magnitude.

For the second mechanism, that pollinator preference varies spatially such that the local color morph is visited more frequently, pollinators as a whole showed slightly, but not significantly, higher visitation rates to the white morph at each site (Fig. 4). Pooled across all sites, white morphs experienced significantly higher visitation rates than yellow morphs (white: 0.58 ± 0.11 visits/flower/h; yellow: 0.40 ± 0.08 visits/flower/h; morph main effect, $F = 10.046$, $P < 0.05$). Visitation rates among sites were not significantly different (site main effect, $F = 1.415$, $P = 0.252$), they were similar among sites and morphs (site x morph interaction, $F = 1.012$, $P = 0.397$). A statistical test was not performed on the data concerning number of visitations of specific pollinator groups due to small sample sizes. However, color preference of pollinator groups varied spatially depending on the site (Fig. 5). *Bombylius* and flies visited the white morph more frequently in white sites but visited both morphs equally in yellow sites. Solitary bees and Lepidoptera visited both morphs equally in white sites but visited the white morph more frequently in yellow sites.

For the third mechanism, by which pollinators could influence the geographic pattern of flower color is through color constancy, I observed pollinators make 123 transitions between *L. stylosa* flowers (Table 2). The observed number of transitions was significantly different from that expected under random pollinator movements for both white sites ($\chi^2 = 8.73$, $P < 0.01$) and yellow sites ($\chi^2 = 6.28$, $P < 0.05$). The subsequent binomial tests showed a significantly higher white to white transition of pollinators than other transitions in white sites as well as yellow sites ($P = 0.0005$ and $P = 0.0031$,

respectively). Considering the pollinator biota of *L. stylosa* for all sites, all transitions yielded a BCI value of 0.352, which was significantly greater than zero ($\chi^2 = 14.49$, $P < 0.001$) suggesting that pollinators, as a community, showed a color constancy.

Seed predation

Seed predation differed among sites (site main effect: Wald $\chi^2 = 754.708$, $P < 0.001$) and seeds from the white morph had significantly higher predation than those from the yellow morph (morph main effect: Wald $\chi^2 = 625.205$, $P < 0.0001$). However, the morph x site interaction was not significant (Wald $\chi^2 = 4.478$, $P = 0.107$). Seeds from the white morph were heavily predated as compared to those from the yellow morph ($P < 0.01$) in both white sites. No seed predation occurred in the Y1 site, and very low predation occurred in the Y2 site with the proportion of predation on white and yellow morphs equal ($P = 0.596$).

Table 1. Number of individuals and the average number of visits for each pollinator group in the experimental arrays at each *Leavenworthia stylosa* study site.

	Pollinator groups									
	Lepidoptera***		Fly		*Bombylius*		*Solitary bees****		Other	
Site	Number of individuals	Mean visits/ individual	Number of individuals	Mean visits/ individual	Number of individuals	Mean visits/ individual	Number of individuals	Mean visits/ individual	Number of individuals	Mean visits/ individual
W1	0	n/a	1	1.0	3	0.7	8	1.1	0	n/a
W2	3	1.0	11	1.4	14	2.1	51	1.7	5	1
	3	**1.0**	**12**	**1.4**	**17**	**1.8**	**59**	**1.7**	**5**	**1**
Total										
Y1	19	2.3	2	1.0	12	2.8	8	2.0	0	n/a
Y2	1	1.0	5	1.0	4	4.3	10	1.4	8	1
	20	**2.2**	**7**	**1.0**	**16**	**3.0**	**18**	**1.7**	**8**	**1**
Total										

Asterisks indicate pollinator group was more likely to be observed at one site than at the other sites (*** $P < 0.001$, **** $P < 0.0001$). n/a is not applicable since no individuals visited. Bold values indicate totals within each group.

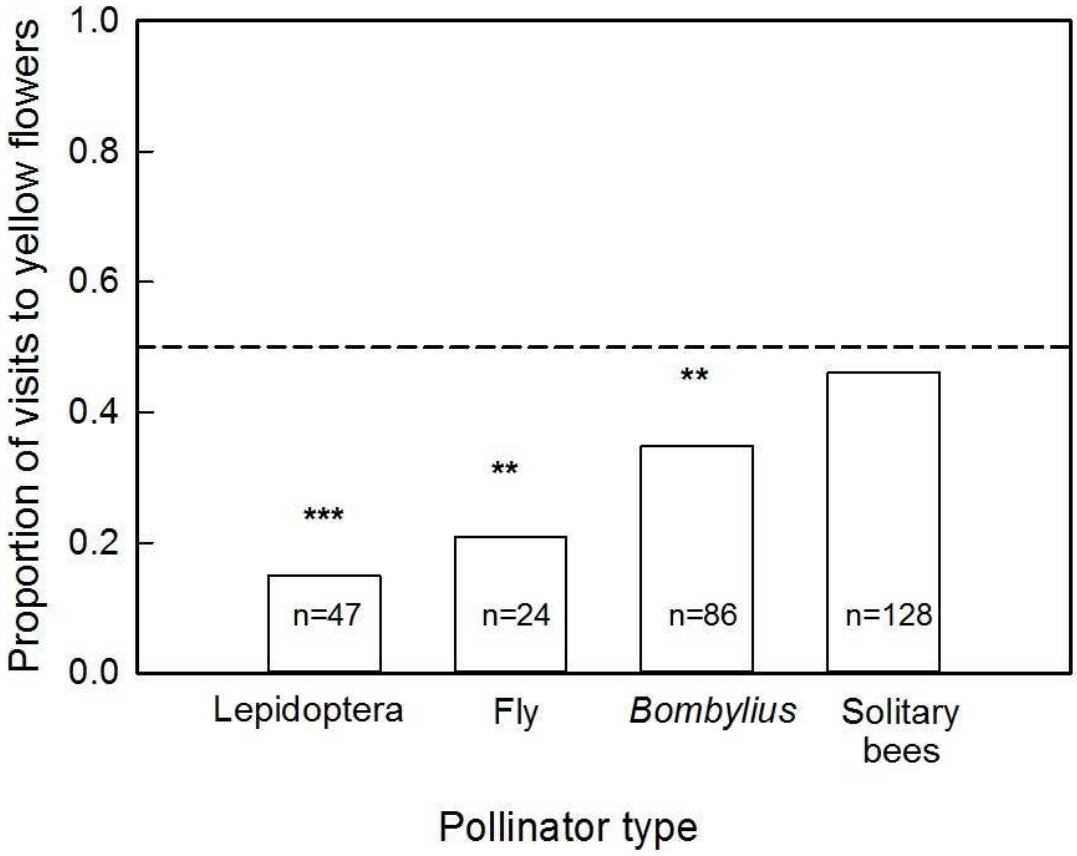

Figure 3. Preference of each pollinator group for yellow *Leavenworthia stylosa* morphs across all sites. The dashed line indicates no preference. Asterisks indicate pollinator groups with significant preference for a particular color morph of *L. stylosa* (G-test, ** $P < 0.01$, *** $P < 0.001$). The total number of visits made by each pollinator group is denoted by n.

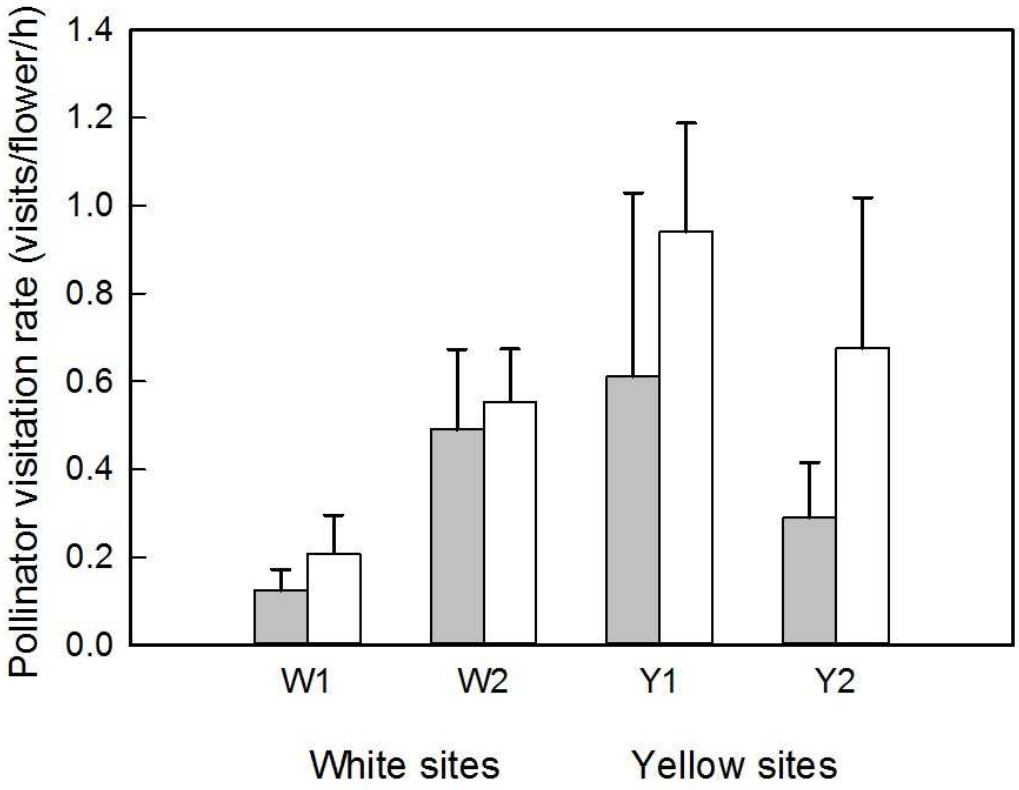

Figure 4. Visitation rates of the pollinators as a community to yellow morph (gray bars) and white morph (white bars) *Leavenworthia stylosa* flowers in the experimental arrays established in two white (W1, W2) and two yellow (Y1, Y2) sites. Pollinators did not show a significant difference in visitation rate for a particular morph at any site. Error bars represent SE.

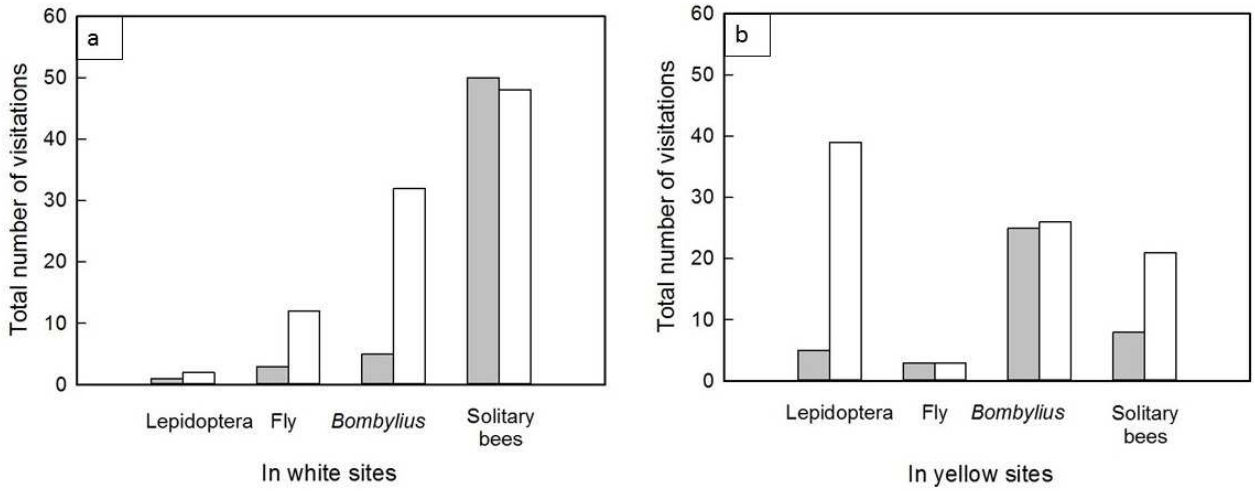

Figure 5. Preference of pollinator groups for yellow (gray bars) or white (white bars) *Leavenworthia stylosa* morphs in (a) white sites and (b) yellow sites.

Table 2. Counts of pollinator transitions made by each pollinator group between white (W) and yellow (Y) flower color morphs in experimental arrays at the study sites for *Leavenworthia stylosa*.

	Transitions between color morphs							
	In white sites				In yellow sites			
Pollinator	W to W	Y to Y	W to Y	Y to W	W to W	Y to Y	W to Y	Y to W
Lepidoptera	0	0	0	0	17	0	4	3
Fly	1	0	0	0	0	0	0	0
Bombylius	21	0	1	1	12	12	5	3
Solitary bees	8	11	7	6	5	0	4	2
Total (Observed)	**30**	**11**	**8**	**7**	**34**	**12**	**13**	**8**
Total (Expected)	**(25.11)**	**(6.11)**	**(12.89)**	**(11.89)**	**(29.46)**	**(7.64)**	**(17.54)**	**(12.54)**

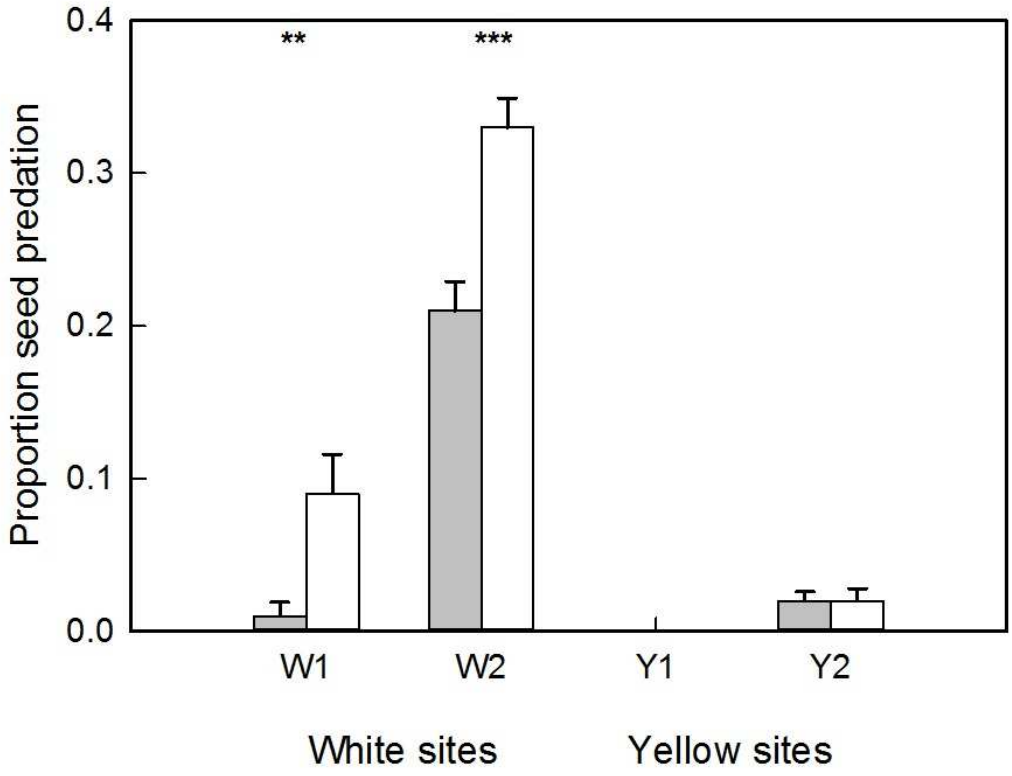

Figure 6. Proportion of seed predation of yellow morph (gray bars) and white morphs (white bars) of *Leavenworthia stylosa* in experimental arrays established in white and yellow sites. Significant differences in seed predation within sites are indicated with asterisks (** $P < 0.01$, *** $P < 0.001$; LSD test). Error bars represent SE.

DISCUSSION

The main goal of this study was to evaluate the role of biotic factors in the maintenance of the spatial variation of flower color in *L. stylosa*. A population genetics study by Dixon et al. (2013) found little genetic differentiation among populations and significant gene flow between white- and yellow-flowered populations. These findings suggest that "isolation by distance" is not a likely mechanism maintaining the geographic variation of flower color in *L. stylosa*, and implies that current or recurrent natural selection may be acting locally to maintain flower color variation among populations.

An increasing number of studies suggests the importance of considering both mutualistic and antagonistic relationships generated by pollinators and herbivores in shaping geographically structured floral trait variation (Galen, 1996; Strauss and Armbruster, 1997). Some of them have found evidence for sculpting various floral traits by the selection exerted by both pollinators and seed predators. In *Protea aurea*, white morph flowers were more attractive for avian pollinators as well as seed predators (Carlson and Holsinger, 2013). Selection pressure exerted by pollinators favors floral exertion of the corolla beyond the bract, while seed predators exert selection to reduce the exertion of the corolla in *Pedicularis rex* (Sun et al., 2016). In *Dalechampia scandens*, blossoms with larger bracts were heavily visited by both bee pollinators and seed predators (Pérez-Barrales et al., 2013). To investigate the role of pollinators and pre-dispersal seed predators in maintaining geographic variation of flower color in *L. stylosa*, I performed pollinator and seed predation studies in four experimental arrays containing yellow and white morphs.

Geographic variation of flower color within a species can arise as a response to adaptive as well as non-adaptive processes (Armbruster, 2002), and a simple demonstration of pollinator discrimination among different morphs is not sufficient to conclude the existing variation is a result of pollinator-mediated selection on floral traits (Rausher, 2008). Though studies on the role of pollinators in maintaining flower color polymorphism are abundant in the literature, very few of them [e.g. *Mimulus aurantiacus* (Streisfeld and Kohn, 2007); *Disa ferruginea* (Newman et al., 2012)] have provided conclusive evidence for pollinator-mediated selection in shaping the geographic variation of flower color. A reciprocal transplant experiment can provide direct evidence for pollinators as a selective agent and can test whether the overall floral phenotype is adapted to the local pollinator environment (Niet et al., 2014).

My experimental arrays were visited by a diverse group of pollinators whose abundance differed between white and yellow sites. There are several possible reasons for the geographic variation in pollinator assemblages. The difference may be due to pollinators having geographic distributions limited to a subset of *L. stylosa*'s range. However, given the very small geographic range of *L. stylosa*, this seems unlikely. A second possibility is that certain pollinators are not foraging on *L. stylosa* in all parts of the range (Eckhart, 1992). I observed all pollinator groups at all four transplant sites (with the exception of Lepidoptera at site W1). However, there was substantial variation in pollinator abundance among the sites. To confirm the role of pollinators' foraging only in a part of the species range, additional studies on the distribution of the pollinators and their abundance are needed. In addition, my results came from only four populations during a single flowering season. By expanding the number of populations across the

species range and the number of flowering seasons, variation in pollinator assemblages in space and time could be documented.

The pollinators observed in my experimental arrays did not show an over riding preference towards a particular morph across all sites, suggesting that their preferences are labile and vary on the availability of floral rewards in space and time. Though Lepidopterans were the most abundant pollinators in yellow sites, they preferred the away morph (white) of *L. stylosa* over the home color morph in their home color sites. Solitary bees also preferred white morph over yellow in yellow sites. In addition, *Bombylius* showed an equal preference for both white and yellow morphs in yellow sites. In general, the pollinators that had been foraging exclusively on the yellow morph in yellow sites changed their preference to the white morph or equally preferred the white morph when it was available. However, none of the pollinator groups preferred yellow over white morph even in the yellow sites. The abundant pollinators' preference towards the away morph has been documented in *Mimulus aurantiacus* (Streisfeld and Kohn, 2007), a well-studied species with a red-yellow flower color system. Hummingbirds in the natural inland populations of the yellow-color race shifted their preference to red flowers in experimental arrays in the same habitat. A correlation between flower color and other floral traits such as flower size, shape, and nectar volume has been identified in the *M. aurantiacus* floral races, with red flowers usually producing higher nectar volume than yellow flowers (Streisfeld and Kohn, 2005). Thus, hummingbirds preferred the red morph wherever it was available.

On the other hand, in my study, pollinators exhibited constancy on the white morph in both white and yellow sites. In white sites, it was largely driven by the constant

transitions shown by *Bombylius* that preferred white over yellow, whereas in yellow sites by both *Bombylius* and Lepidopterans. I observed a very high constancy of *Bombylius* when they foraged in natural populations of *L. stylosa* with both color morphs present (data not shown) with over a hundred consecutive white to white transitions. Solitary bees, on the other hand, showed equal preferences for both morphs and visited the morphs randomly in both white and yellow sites. Lepidopterans are known for shifting their color preferences when the color of the most rewarding flower changes (Goulson and Cory, 1993; Kandori and Ohsaki, 1996; Weiss, 1997). *Bombylius* adults are also nectar feeders (Boesi et al., 2009), whereas many solitary bees depend on flowers for pollen and oil but rarely for nectar (Wcisio and Cane, 1996). The shift of Lepidopteran preference to the white morph, their highly constant white-to-white transitions in yellow sites, and *Bombylius*' preference to the white morph and constant transition pattern suggest that both pollinators are attracted to a floral reward available in the white morph.

In several polymorphic species, it has been reported that white flowers are larger in size compared to colored flowers [e.g. *Protea aurea* (Carlson and Holsinger, 2013); *Lobularia maritima* (Gomez, 2000); *Claytonia virginica* (Frey, 2004)]. In addition, several studies on species in the Brassicaceae [*Raphanus sativus* (Stanton and Preston, 1988); *Erysimum mediohispanicum* (Gomez et al., 2008)] as well as members of other angiosperm families [e.g. *Eichhornia paniculata* (Pontederiaceae, Worley and Barrett, 2000); *Nicotiana* spp. (Solanaceae, Kaczorowski et al., 2005); *Silene virginica* (Caryophyllaceae, Fenster et al., 2006)] have shown a positive association between corolla size and nectar volume. Though a morph-specific nectar volume has not been studied in *L. stylosa*, its white flowers are slightly larger than the yellow (pigmented)

flowers (M.T.R. Fernando, unpubl. data), and these larger white flowers may have the capacity to produce higher nectar volumes than the yellow morph. If the slightly larger flowers of the white morph of *L. stylosa* are in fact more rewarding in terms of nectar and pollen than the yellow morph, this may explain the higher preference for the white morph and higher constant transitions between the white morph flowers across the species range.

A pollinator's contribution to the fitness of a plant it visits can be divided into two components: the number of flowers visited by the pollinator (quantity of visits) and the effectiveness of pollen transfer (quality of visits) during foraging (Herrera, 1987). The most abundant pollinator group may not be the most effective one and differences in pollinator efficiency among pollinator groups have been found in other systems (Mayfield et al., 2001; Madjidian et al., 2008) and visitation does not imply successful pollination (Olsen, 1997). As an example, in *Alstroemeria aurea,* the native bee *Bombus dahlbomii* was more efficient in terms of quantity and quality of pollen deposition compared to the invasive *Bombus ruderatus*, which was the more frequent visitor (Madjidian et al., 2008). Thus, there is a possibility that the fitness of *L. stylosa* individuals depends more on the number of visits by the effective pollinator groups, rather than the abundance of particular pollinator groups. I focused only on frequency of pollinator visitation and not the efficiency of the pollinators. A pollinator study on *L. alabamica* has shown that solitary bees and *Bombylius* are effective pollinators, as single visits from them resulted in high fruit and seed production, while flies other than *Bombylius* were ineffective pollinators (Layman et al., 2017).

Unfortunately, Layman et al. (2017) did not assess the effectiveness of Lepidopteran pollinators in *L. alabamica* due to low visitation rates. The Lepidopteran

pollinator of *L. stylosa* was most commonly *Anthocharis cardamines* (orange tip butterfly), which mainly feeds on cruciferous species (Wiklund and Åhrberg, 1978; Courtney, 1980) by landing on flowers and feeding on nectar with their long mouthparts. As their bodies do not appear to consistently come into contact with the anthers and stigmas of *L. stylosa* flowers, they may not be very effective pollinators (C.R. Herlihy, pers. comm.). On the other hand, solitary bees and *Bombylius* were the most abundant pollinators in my arrays, accounting for 47% and 20% of all observed pollinators, respectively. If, as in *L. alabamica*, these are also the most effective pollinators, their visitation patterns are likely to have the strongest influence on pollinator-mediated selection on flower color. Considering the white sites, *Bombylius* visited almost exclusively white flowers and was very constant. This behavior may help maintain the frequency of the white morph within these sites while preventing the yellow morph from increasing in frequency. Whereas in yellow sites, *Bombylius* visited both morphs equally, but they also moved in a constant way (yellow to yellow and white to white) which would not increase the frequency of one morph relative to the other.

Regardless of the flower color morph in *L. stylosa*, seed predation was higher on plants in white sites compared to yellow sites and white morph suffered higher seed predation than the yellow morph. This result was somewhat consistent with earlier observations in natural populations of *L. stylosa,* which showed that the weevil (*C. obstrictus*) did not discriminate between white and yellow morphs when ovipositing (Kaysar, 1985). White-flowered individuals more commonly lost seeds to seed predation and weevils ate more seeds from the fruits of white- than yellow-flowered plants (C.R. Herlihy, unpubl. data). In addition, the non-significant site x morph interaction in the

seed predation analysis suggested that predation on yellow and white morphs was similar among sites. Several lines of evidence show that members of the Brassicaceae have different susceptibilities to *C. obstrictus* depending on the various physical and chemical properties of the host plant, such as flower color (Buechi, 1990), levels of glucosinolate production (Ulmer and Dosdall, 2006), or nutrients in seeds (Slansky and Panizzi, 1987).

However, in some species with anthocyanin-based flower color polymorphism, white morphs are more susceptible to seed predation than the colored morph (e.g. *Protea* spp., Carlson and Holsinger, 2010). This is due to increased production of secondary metabolites in colored morphs resulting from the shared biosynthetic pathway of pigments and defense compounds such as glucosinolate. As no study has been done on morph-specific secondary metabolite production in *L. stylosa*, I cannot conclude that the yellow morph can tolerate seed predation due to glucosinolate produced by the flavonoid biosynthetic pathway. On the other hand, the rarity of seed predation in yellow sites may be due to a spatiotemporal fluctuation of *C. obstrictus* density. My study was done only in one year and considered only two yellow and two white populations of *L. stylosa*. A broader sampling of natural populations and replication of the study for several years would be needed to confirm that seed predation is indeed higher in white populations. Nevertheless, considering the selection exerted by pollinators and seed predators together, the white morph was preferred over the yellow morph by both pollinators and seed predators. Yellow flowers were less preferred by pollinators throughout the range, especially in their home sites, but they had a low cost of seed predation. When the fitness-enhancing pollinators and fitness-reducing seed predators have opposite

preference in the yellow site, the coincident selection can favor a monomorphic population (Strauss and Whittall, 2006).

Considering the selection exerted by pollinators and seed predators together, the white morph was preferred over the yellow morph. Yellow flowers were less preferred by pollinators throughout the range, especially in their home sites, but they had a low cost of seed predation. When the fitness-enhancing pollinators and fitness-reducing seed predators have opposite preference in the yellow site, the coincident selection can favor monomorphic populations (Strauss and Whittall, 2006). Thus, I did find some evidence of selection by pollinators and seed predators, but other factors are likely involved.

In addition to pollinators and seed predators, abiotic factors play an important role in shaping flower color by pleiotropically related functions of floral pigments leading to local adaptation (Strauss and Armbruster, 1997; Armbruster, 2002; Strauss and Whittall, 2006; Rausher, 2008). Unlike pollinators and seed predators, these abiotic factors interact with all stages of a plant's life cycle and the magnitude and direction of the selection exerted at one stage could be different from the other. Therefore, it is essential to consider all the life history stages of the plant when studying the role of abiotic factors in maintaining the geographic variation of flower color; which is the main focus of Chapter 3.

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CHAPTER 3

ROLE OF ABIOTIC FACTORS IN THE MAINTENANCE OF GEOGRAPHIC VARIATION FOR FLOWER COLOR IN *LEAVENWORTHIA STYLOSA*

ABSTRACT

Geographic variation of flower color can be maintained through selection resulting from abiotic as well as biotic conditions. In *Leavenworthia stylosa,* two main flower color morphs occur that are geographically structured in Middle Tennessee: yellow and white, with most populations containing only one flower color morph. Although considerable gene flow occurs among color morph populations, pollinators and seed predators influence color morph variation. The goal of this study was to determine if local adaptation to abiotic factors and differential responses to water-related stress during different life history stages can explain the geographic pattern of flower color distribution. I conducted soil analyses, multiple reciprocal experiments, and water-related stress experiments focusing on early life history stages as well as on reproduction. The white morph performed better in seed germination and early seedling survival, while the yellow morph performed better in winter survival, reproductive stage survival, and flower and fruit production. Seed production was equal between the morphs. The water-related stress experiments yielded conflicting results. Thus, I found limited evidence for local adaptation of yellow and white morphs of *L. stylosa* in distinct life history stages, but they are not strong enough to maintain the geographic pattern of flower color variation found in this species.

INTRODUCTION

Pollinators have frequently been credited as the main selective agents responsible for generating and maintaining floral diversity in angiosperms (Fenster et al., 2004). Trait variation among populations, such as that seen in flower color, was long believed to be maintained by selection by pollinators, either through spatial variation in their distributions or through their differential preferences for different morphs among populations (Grant, 1949; Proctor, 1996). However, many studies on pollinator-mediated selection have failed to demonstrate that the agent causing selection on flower color was pollinators (Clegg and Durbin, 2000; Rausher, 2008). Thus, other causal mechanisms have been proposed over time. There is a growing body of evidence suggesting that an alternative mechanism might be indirect selection by abiotic factors (Strauss and Armbruster, 1997; Armbruster, 2002; Rausher, 2008). A long-running debate focused on the roles of genetic drift versus natural selection as the mechanism maintaining the spatial pattern of flower color in *Linanthus parryae* (Epling and Dobzhansky, 1942; Wright, 1943a; Endler, 1986; Coyne et al., 1997). Although initially attributed to genetic drift (Epling and Dobzhansky, 1942; Wright, 1943b), the pattern of flower color variation was ultimately found to be due to the spatio-temporal variation of selection exerted by spring rainfall and the differential water use efficiencies of the color morphs (Schemske and Bierzychudek, 2007).

Anthocyanins and carotenoids are the main pigment groups responsible for flower color in angiosperms and other products of their biosynthetic pathways play a vital role in physiological functions (Delgado-Vargas et al., 2000; Grotewold, 2006). Products from

the anthocyanin (flavonoid) biosynthetic pathway are among the best characterized plant secondary metabolites (Tanaka et al., 2008). Some of these compounds are involved in UV protection (anthocyanins and flavonols), structural support (lignins), microbial interactions (isoflavonoids), pollen viability (flavonols), and stress tolerance (flavonols and flavones) (Durbin et al., 2003). Products of the carotenoid biosynthetic pathway serve in UV protection (xanthophylls), stress tolerance, and seed germination (abscisic acid) (Hirschberg, 2001). Thus, the flavonoid and carotenoid pigments may have pleiotropic effects on plant phenotype, which is the effect of a locus (or several loci) on multiple phenotypic traits (Armbruster, 2002). Importantly, mutations in genes in the biosynthetic pathways of anthocyanins and carotenoids can result in the joint effect of altering flower color (Sobel and Streisfeld, 2013; Zhang et al., 2015), as well as altering the functions of pleiotropically-related characters (Rausher, 2008), and consequently, selection on one trait will cause a correlated response in the other.

Most of the physiological functions mentioned above can strongly influence plant fitness, and both biotic and abiotic agents can serve as selective pressures on them. Selective forces with spatial variation may provide an advantage to different genotypes in local populations leading to local adaptation (Kawecki and Ebert, 2004). These locally adapted genotypes have higher fitness in their home sites than in other environments (McKay et al., 2005). If local selective pressures are strong enough, and flower color covaries with the trait under selection, local adaptation can generate and maintain a geographic pattern of flower color variation, even in the face of considerable gene flow (Waser and Price, 1985; Linhart and Grant, 1996; Arista et al., 2013; Blanquart et al., 2013).

To demonstrate that a particular flower color is adapted to local conditions, it is important to show the relationship between variation of flower color among the populations and the covarying differences in local selective pressures (Sobral et al., 2015). A reciprocal transplant experiment is a powerful approach to detect this pattern of local adaptation in the wild (Kawecki and Ebert, 2004; Herrera et al., 2006). While these field studies can demonstrate that particular genotypes have higher fitness in certain environments, they are not always able to identify the local selective agent responsible for adaptation (Stratton, 1994). Experiments under controlled conditions that manipulate the environment are helpful in dissecting the mechanisms of natural selection (Schmid, 1992; Latta and McCain, 2009). Various abiotic factors have been identified as selective agents driving flower color variation, including heat (Coberly and Rausher, 2003), rainfall (Schemske and Bierzychudek, 2001), and drought (Warren and Mackenzie, 2001), and local differences in these selective forces can lead to geographic patterns of color variation (Arista et al., 2013).

Studies on floral adaptation have sometimes failed to identify local adaptation because they have focused only on one part of the plant's life cycle, particularly the flowering stage (Volis et al., 2002; Sobral et al., 2015). However, plants experience changes in environmental factors in their habitats throughout the life cycle (Linhart and Grant, 1996). Therefore, on top of the spatial variation of selective forces, these numerous environmental factors can exert selection during different stages of the life cycle from seed maturation to flowering (Ernst, 1987; Veiga et al., 2015).

While many studies of local adaptation of floral traits have been done at the flowering stage (Streisfeld and Kohn, 2005; Sorbral et al., 2015; Veiga et al., 2015),

plants experience considerable selection by abiotic factors at early life history stages. The seed and seedling stages are highly susceptible to unfavorable environmental conditions that change the degree of seed dormancy, delay germination, and/or kill the seedling (Ratcliffe, 1961; Karssen, 1980). Seed dormancy release, seed germination, and seedling growth have different combinations of environmental requirements (Jorritsma-Wienk et al., 2006), while later life history stages can often tolerate stresses better than the early stages (Lee et al., 2003). Therefore, the effect of a particular selective agent could be different from one life history stage to another (Jorritsma-Wienk et al., 2006). As a whole, the net selection experienced by an individual is a multiplicative function of the selection episodes that take place at each stage of the life cycle (Volis et al., 2002). To understand the relative contribution of each selection episode, reciprocal transplant studies covering different life history stages are essential (Niet et al., 2014).

My study species, *Leavenworthia stylosa* A. Gray (Brassiceae), produces flowers that are yellow, white, or rarely lavender (Rollins, 1963), with the yellow and white individuals usually making monomorphic or nearly monomorphic populations (Lloyd, 1969). The distribution of flower color is geographically structured in Middle Tennessee (Fig. 1). The distribution of flower color and morph frequencies appears to have remained stable over the time period for which historical records have been compiled (Norton et al., 2015).

A population genetic study on *L. stylosa* suggested that little genetic structure existed between populations, and the observed structure did not match flower color distribution pattern. Moreover, there is considerable gene flow between white and yellow populations (Dixon et al., 2013). This suggests that strong local selective forces may be

operating to maintain the monomorphic nature of populations in the face of this significant gene flow. For Chapter 2, I performed a reciprocal transplant study with two yellow-flowered and two white-flowered populations to examine the role of pollinators and pre-dispersal seed predators in the maintenance of geographic variation of flower color at the reproductive stage. The results of these studies suggested that exclusive visits by the pollinator *Bombylius* contributed to maintenance of the white morph, and seed predators and pollinators contributed to maintenance of the yellow morph.

Leavenworthia stylosa occurs in limestone cedar glades. These glades are rocky and have shallow soils. As such, water may stand for extended periods during winter and early spring, whereas the same areas may become excessively dry in late summer and autumn (Quarterman, 1989). This species is a winter annual, germinating in the fall, overwintering as a rosette, and flowering in the early spring. Consequently, across the life cycle, individual plants experience a dramatic range of hydrological conditions. During summer, while seeds are undergoing after-ripening and are breaking dormancy, soils vary in their moisture levels – from being completely saturated to being completely dry. After-ripening is particularly sensitive to moisture, with the process occurring optimally at intermediate levels of moisture (Walck et al., 2011). Thus, for seeds, stress may occur at both low and high moisture levels. Seedlings and early rosettes of *L. stylosa* are susceptible to drought but tolerant of flooding. Although adaptation to saturated soils (flooding) has not been studied in *L. stylosa*, rosettes of *L. uniflora* were shown to be metabolically adapted to growing with their roots under anaerobic conditions (Baskin and Baskin, 1976). Thus, all stages of the life history of *L. stylosa* experience water-related stresses, though the nature of the stress may differ between stages of the life cycle.

In the present chapter, I examined whether local adaptation to abiotic conditions may help explain the geographic pattern of flower color variation in *L. stylosa.* First, I examined differences in soil parameters among populations of the two color morphs. Second, I used multiple reciprocal transplant experiments at different life history stages (seed after-ripening, seed germination, early seedling growth, juvenile and adult rosettes). Third, I tested the roles of water-related stress on seed after-ripening, seed germination, and seedling growth. Together, these three parts allowed me to address the following questions: (1) Are there differences in soil parameters between white- and yellow-flowered sites? (2) Is there evidence of local adaptation, and does it vary across stages of the life cycle? (3) Do white- and yellow-flowered individuals show different responses to water-related stresses during different stages of their life cycle?

MATERIALS AND METHODS

Study species

The flowers of *L. stylosa* are self-incompatible and are pollinated by a diverse group of pollinators, including bees, bee-flies, butterflies, and flies (Chapter 2; Norton et al., 2015). Upon fertilization, *L. stylosa* seeds are produced in siliques, and seed dispersal occurs from mid-May to early June. Seeds are primarily dispersed by gravity, and water may also act as a dispersal agent (Rollins, 1963) as water flows over the surface of cedar glades during rain events (Morris et al., 2002). The plant is a winter annual. Its seeds are dormant at the time of dispersal, and they after-ripen during summer and germinate during late September and October (Baskin and Baskin, 1971). Seedlings overwinter as rosettes and flowering occurs from March until early May (Rollins, 1963).

Differences in soil properties among populations

The chemistry of soils from two, monomorphic white (W1, W2) and two, monomorphic yellow (Y1, Y2) sites, used for reciprocal transplant experiments in Chapter 2 (Appendix A), were determined. Five soil samples (up to 1-2 cm deep) were collected from random locations within each array in mid-January 2015. The samples were sent to A & L Eastern Laboratories (Richmond, VA) and analyzed for calcium (ppm), magnesium (ppm), phosphorus (ppm), potassium (ppm), cation exchange capacity (CEC) (milli-equivalent per 100 g of soil), organic matter (%), and pH.

Water holding capacity of the soils was calculated from five, 50 cm^3 sub-samples taken from a composite soil sample from each site in May 2015. The composite sample was made by combining >10 discrete soil samples collected randomly from a site. Water

holding capacity was determined using a modified version of the procedure in Harding and Ross (1964). Each composite soil sample was sieved through 1 mm openings and put in a 50 ml plastic centrifuge tube. Water was added until the soil was saturated, and the tube was kept vertical, plugged with wool, in a water bath overnight with a 1:2 soil (in tube) to water (in bath) ratio. The soil sample was then allowed to drain for 3 h in a funnel plugged with wool, and the water holding capacity of each sub-sample was determined by the weight of the water held in the sample vs. the sample dry mass (dried at 105°C for 24 h).

Volumetric water content (VWC) was recorded from mid-October to mid-November 2015, the time when seed germination occurs in the field, using soil moisture smart sensors (model: S-SMx-M005; Decagon Devices Inc., Pullman, WA) and HOBO data loggers (Onset Computer Corporation, Bourne, MA). The sensors were buried (up to 5 cm deep) immediately adjacent to each array (Chapter 2) at each site. The data logger recorded the VWC at 1 h intervals.

Reciprocal seed sowing experiments

I conducted three reciprocal transplant experiments using seeds that had been collected from >100 individuals in each of the four sites (W1, W2, Y1, Y2) used in Chapter 2; the populations at these sites served as seed sources for the experiments. Two reciprocal experiments were conducted in the field during consecutive seed germination seasons: one in 2014 starting with after-ripened (non-dormant) seeds and the other one in 2015 starting with fresh (dormant) seeds. The third reciprocal experiment was conducted in an incubator in 2015, using soils collected from each of the sites.

Reciprocal seed sowing experiment with non-dormant seeds

Seeds collected in May 2014 were after-ripened in dry paper envelopes under laboratory conditions (21°C, relative humidity 50-60%) for 4 months. Fifty after-ripened seeds each were placed into polyester mesh fabric bags (10 cm x 6 cm), and bags were then sewn shut and color-coded to identify the source population of the seeds. In mid-October 2014, during the normal germination period in natural populations, 40 bags (10 from each source population) were placed in each site in an array (3 m x 3 m), alternating the flower color and the source population of seeds (Fig. 2a). Bags were placed 30 cm apart from each other and were secured and kept in contact with the soil with nails. Seed germination took place under natural environmental conditions and after three weeks (in mid-November 2014), the bags were carefully collected with the underlying soil layer and brought to the laboratory at Middle Tennessee State University. Each bag was cut open, and the soil was washed away. Seedlings, non-germinated seeds, and empty seed coats of germinated seeds [identified by a characteristic opening (split) in the seed coat] were counted.

A parallel seed germination experiment was conducted in the laboratory with seeds from the same collection used in the 2014 reciprocal sowing experiment to compare germination under field conditions to germination under controlled laboratory conditions. Fifty seeds from each site were placed on moist Whatman #1 filter papers in each of five, 5-cm diameter petri dishes; the petri dishes were wrapped with a transparent polythene film to reduce evaporation. Seeds were incubated at 20/10^{o}C alternating day/night temperatures and a 12 h photoperiod, which are conditions that approximated natural temperatures and photoperiod during the germination period in the field. The

number of germinated seeds was counted every week until no additional seeds germinated. Filter papers were kept moist throughout the experiment by adding distilled water as needed. All non-germinated seeds were dissected to check for viability. Firm, light green embryos were considered viable, while soft, brown embryos were considered non-viable.

Reciprocal seed sowing experiment with dormant seeds

Fresh (dormant) seeds collected in May 2015 were used in this experiment. The same procedure was followed as with the non-dormant seeds (see above), with the exception that 25 seeds per bag were used instead of 50 seeds per bag. Bags were placed in each array (Fig. 2a) in early May 2015 and retrieved in early November 2015, after the germination period in natural populations. Seedlings, non-germinated seeds, and empty seed coats of germinated seeds were counted, and viability of non-germinated seeds was assessed, as above.

Reciprocal seed sowing experiment under controlled conditions

Soil was collected from each of the four sites, air-dried and sieved through 1 mm openings to remove large soil particles and *L. stylosa* seeds from the seedbank. Twenty transparent plastic containers [10.5 (diameter) × 4.0 cm (depth)] per site were filled with 200 g of soil. Seeds collected in May 2015 were reciprocally sown in the containers of each other's soils (n = 25 seeds per container on top of the soil), such that seeds from each population were sown onto all four soils. Seeds were moved through a series of temperature regimes that simulated conditions they experience in natural habitats from

May to November (Appendix B). At the start of the experiment, soil was moistened with distilled water. During the summer months (May to August 2015), the lid of each container was kept closed for 1 day after watering and kept open for the next 13 days (to allow the soil to dry) before the next watering event. From September to the end of the experiment in November, the lids remained on the containers to allow the soil to stay moist. The position of containers within the incubator was randomized once a week. Germination was checked weekly, by counting the number of germinated seeds and removing seedlings. All non-germinated seeds were dissected to check embryo viability. Firm, light green embryos were considered viable, while soft, brown embryos were considered non-viable.

Reciprocal rosette transplant experiments

I conducted two reciprocal transplant experiments with *L. stylosa* rosettes: one starting with juvenile rosettes transplanted in mid-December 2013 and followed into May 2014 and another, starting with adult rosettes transplanted in mid-March 2013 and followed into May 2013. In each of the four sites, I established an array by planting 25 rosettes from each source population in a reciprocal design (alternating the color and source population, Fig. 2b). See Chapter 2 for additional details on the arrays. For the experiment started in the winter with juvenile rosettes, data on overwinter survival of rosettes were collected in February 2014, and data on final survival and reproductive success were collected in May 2014.For the experiment started in the spring with adult rosettes, data on survival and reproductive success were collected in May 2013. Reproductive success was quantified as the total number of flowers produced, total

number of fruits produced, and average number of seeds per fruit from two randomly selected siliques per individual. Total seed production per individual was estimated by multiplying total fruit number by the average number of seeds per fruit.

Performance under water-related stress conditions

I did a series of experiments focusing on three early stages of the life cycle (seed after-ripening, seed germination, early seedling growth) under various moisture conditions. The conditions were created by utilizing a gradient of relative humidities or water potentials, or by a series of different watering regimes.

Seed after-ripening stage

Two experiments were conducted to examine water-related stress at the seed after-ripening stage. The first experiment used different watering regimes with seeds collected in May 2012 from three white populations (W3, W4, W5) and three yellow populations (Y1, Y2, Y3) (Figure 1; Appendix A). After-ripening of seeds from the six populations was tested under four watering regimes: constant wet (T1), watering once per week (T2), watering once every 2 weeks, and watering once every 4 weeks (T4). Over the course of the experiment the dishes were moved through a series of temperature regimes in incubators, simulating temperatures in the field (Appendix B). Each treatment was conducted on three replicates per population, each having 25 seeds on white quartz sand in 6 cm diameter plastic petri dishes. The experiment started within 2 weeks of collection in May 2012. On the first day of the experiment, all petri dishes, except those in T1, were saturated with water and dishes were kept closed with lids for 24 h. After 24

h, lids were removed and the dishes were kept open for the rest of the days in the watering regime. In T1, the sand was moistened and then kept constantly moist with the dishes kept closed until the end of the experiment. The watering regimes in all treatments were followed for 16 weeks to allow for after-ripening. Starting on the 17th week and continuing for 2 weeks, petri dishes in all treatments were watered and the substrate was kept moist by keeping the lids closed, wrapped with transparent polythene film, and adding water when needed, to assess germination. The position of the petri dishes inside the incubator was randomized once a week. Seed germination was recorded daily, with root emergence as the criterion used to identify germination. After two weeks, all non-germinated seeds were dissected to check embryo viability. Firm, light green colored embryos were considered viable, while soft, brown embryos were considered non-viable.

In the second experiment, fresh seeds collected in May 2015, from two yellow (Y1, Y2) and two white (W1, W2) populations, were placed in envelopes into tightly sealed polycarbonate boxes (28 cm width x 28 cm length x 13 cm depth; Fibox Inc., Glen Burnie, MD) over saturated solutions of LiCl (Sigma-Aldrich, St. Louis, MO). These solutions created a relative humidity gradient (11, 25, 50, 75, or 95%) to allow for after-ripening (Appendix C). All boxes were kept inside a light- and temperature-controlled incubator at 30°C, which simulated summer temperatures. After 16 weeks of after-ripening, the seeds were incubated to test for germination on moist filter papers in petri dishes, wrapped by transparent polythene films to reduce evaporation. Incubation temperature was alternating (12/12 h) 20/10°C, simulating autumn temperatures, with a 12 h daily photoperiod during the high temperature. The positions of the petri dishes inside the incubator were randomized once a week and petri dishes were checked weekly

for germinated seeds for 2 weeks. After these 2 weeks, all non-germinated seeds were dissected to check the viability. Firm, green embryos were considered as viable, whereas soft, brown embryos were non-viable.

Seed germination stage

Water potential of soil solution varies with changes in the moisture content of the soil (Hayward and Wadleigh, 1949), and depending on the extent of soil water evaporation, plants and their seeds may experience different water potentials of the substrate. I studied the germinability of seeds from the two color morphs under a water potential gradient (0, -0.25, -0.5, -0.75, -1.0 MPa) created by polyethylene glycol (PEG)-8000 (Sigma-Aldrich, St. Louis, MO) (Appendix D). Non-dormant seeds (after-ripened in the laboratory for 16 weeks) from two yellow- (Y1, Y2) and two white-flowered (W1, W2) populations were incubated inside petri dishes on filter papers moistened with distilled water (control) or PEG solution. Each treatment was conducted on three replicates per population, with 25 seeds per replicate. Filter papers were kept moist with water or PEG solution, and the petri dishes were wrapped with polythene films and incubated at 23°C constant temperature (approximately the average high October temperature in Middle Tennessee; NOAA, 2016) for three weeks followed by 15°C constant temperature (approximately the average high November temperature in Middle Tennessee for an additional three weeks. The daily photoperiod was 12 h. These temperatures were selected since water potential of the solutions vary with temperature, and these temperatures approximated natural temperatures during germination in the field for which information was available for water potential (Michel, 1983). Seed germination

was recorded each week, and filter papers were replaced every four days to maintain constant water potential. After six weeks from the start of the experiment, the filter papers in each petri dish were replaced by filter papers moistened with deionized water and incubated at 15°C constant temperature for an additional three weeks to check the total germination. The position of the petri dishes inside the incubator was randomized every four days throughout the study period. At the end of the experiment (nine weeks), non-germinated seeds were dissected to check viability. Firm, light green embryos were considered viable and soft, brown ones were non-viable.

Initial seedling stage

I studied the growth of seedlings under a water potential gradient (-0.25, -0.5, -1.2 MPa) created by infusing PEG solutions with different water potential into agar substrate, following the protocol of Verslues et al. (2006). After-ripened seeds collected from two yellow (Y1, Y2) and two white (W3, W4) populations were incubated at 10°C constant temperature on filter papers moistened with 100 ppm gibberellic acid solution in petri dishes. After seeds germinated, 10-day old seedlings of approximately equal size were selected for the experiment, and pictures of the seedlings were taken using a digital camera before planting. Each seedling was transplanted into a separate, plastic centrifuge tube (50 ml volume) with agar medium, and the caps were closed loosely on the tubes to allow for air exchange. Each treatment was applied to 25 seedlings per population. All seedlings were grown under 20/10°C alternating temperatures with a 12 h photoperiod. The position of tubes within the incubator was randomized every three days. After four weeks, seedlings were removed from the agar and re-photographed. The area of the shoot

was measured using ImageJ2 software (Schindelin et al., 2015), and the change in two-dimensional shoot surface area between the initial and final photographs was calculated for each seedling.

Statistical analyses

Differences in soil properties among populations

Differences in soil properties among the four sites were analyzed using multivariate analysis of variance (MANOVA) in SPSS (Chicago, IL) and a canonical centroid plot was created in JMP® 13.1.0 (SAS Institute Inc, Cary, NC). Prior to the analysis, data were checked for normality and Ca and CEC were transformed using Box–Cox procedures to fit the normal distribution using the Box–Cox normality plot (Wessa, 2015). As the levels of Ca were highly correlated with CEC ($r = 0.996$), CEC was removed from the model to reduce collinearity (Scheiner, 1993). Post-hoc least significance difference (LSD) tests were conducted for each parameter to check differences among sites. A separate MANOVA was conducted to determine whether the parameters in white sites differed from those in yellow sites. The change in volumetric water content was calculated by determining the difference between maximum and minimum content over time following two rain events that occurred at all sites.

Reciprocal seed sowing experiments

I performed separate analyses on the three reciprocal seed sowing experiments and the parallel laboratory experiment using SPSS. In each analysis, the proportion of seed germination and proportion of seedlings that survived were analyzed as dependent

variables in generalized linear models (with logit link function), which included site (or soil type) and flower color morph as factors along with their interaction. LSD tests were conducted to determine whether morphs significantly differed in seed germination and seedling survival within a site (or soil type).

Reciprocal rosette transplant experiments

I analyzed the survival data with generalized linear model (with logit link function), with site and morph as factors including their interaction, using SPSS. The reproductive success data were tested with an analysis of variance that included site and morph as factors as well as their interaction. The parameters with non-normal distributions were Box-Cox transformed to satisfy the normal distribution assumption.

Performances under water-related stress conditions

For experiments on after-ripening and germination, I analyzed the proportion of germinated seeds as dependent variables in generalized linear model (with logit link function), which included morph and treatment as factors and their interaction, using SPSS. For seedling growth data, Box-Cox transformation was used to satisfy the normal distribution assumption. Seedling growth (area) was tested with an analysis of variance with morph and treatment as factors and an interaction term. LSD tests were conducted to determine whether morphs had significant differences in seed germination or seedling growth across treatments.

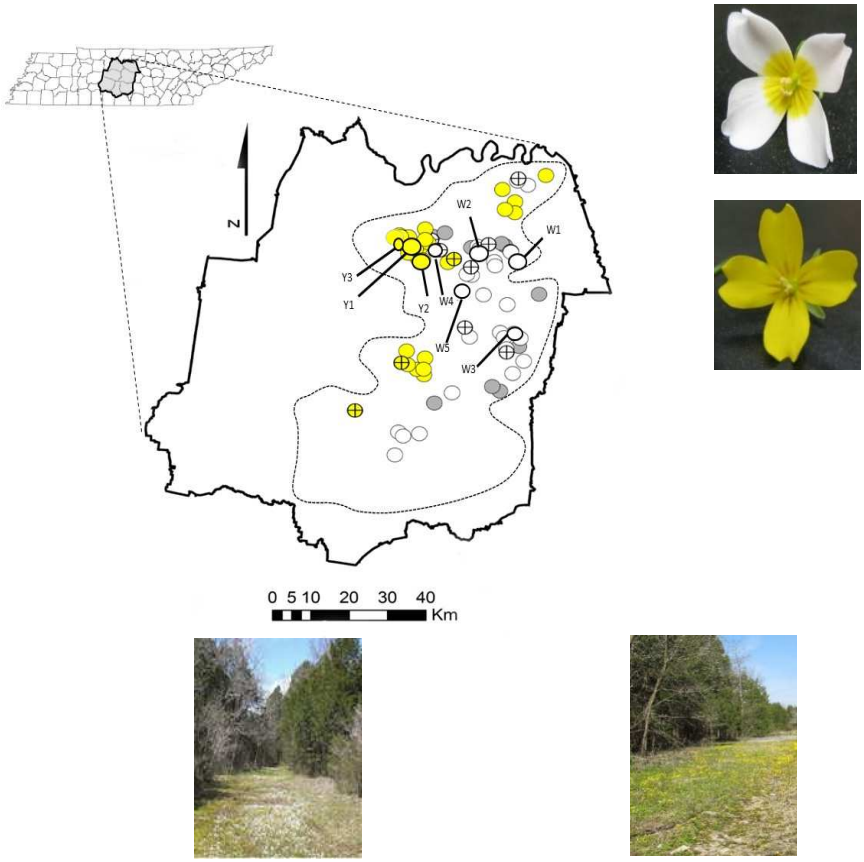

Figure 1. *Leavenworthia stylosa* is restricted to seven counties (shaded in the map) in the Central Basin of Tennessee (dashed lines). This species exhibits a petal color polymorphism where individuals produce either white or yellow flowers (pictured to the right of map). Circles represent the locations of the populations: open white and yellow circles indicate exclusively white- or yellow-flowered populations (pictured below the map), and hatched white or yellow circles indicate populations with >99% of a single color morph. Gray circles represent polymorphic populations with intermediate color morph frequencies. Thick-outlined, labeled circles represent the populations used in this study. Reciprocal sowing or transplant experiments were conducted in W1, W2, Y1 and Y2 populations while other populations (W3, W4, W5, Y3) were used in the water-related stress experiments.

Array (a)

W1	Y1	W2	Y2	W1	Y1	W2	Y2
Y1	W2	Y2	W1	Y1	W2	Y2	W1
W2	Y2	W1	Y1	W2	Y2	W1	Y1
Y2	W1	Y1	W2	Y2	W1	Y1	W2
W1	Y1	W2	Y2	W1	Y1	W2	Y2

Array (b)

W1	Y1	W2	Y2	W1	Y1	W2	Y2	W1	Y1
Y1	W2	Y2	W1	Y1	W2	Y2	W1	Y1	W2
W2	Y2	W1	Y1	W2	Y2	W1	Y1	W2	Y2
Y2	W1	Y1	W2	Y2	W1	Y1	W2	Y2	W1
W1	Y1	W2	Y2	W1	Y1	W2	Y2	W1	W2
Y1	W2	Y2	W1	Y1	W2	Y2	W1	Y1	W2
W2	Y2	W1	Y1	W2	Y2	W1	Y1	W2	Y2
Y2	W1	Y1	W2	Y2	W1	Y1	W2	Y2	W1
W1	Y1	W2	Y2	W1	Y1	W2	Y2	W1	Y1
Y1	W2	Y2	W1	Y1	W2	Y2	W1	Y1	W2

Figure 2. Experimental arrays used to study local adaptation in different life history stages. (a) The array used in reciprocal seed germination experiments started with non-dormant seeds and dormant seeds. (b) The array used in reciprocal transplant experiments started with juvenile rosettes and adult rosettes. The yellow cells represent yellow-flowered *Leavenworthia stylosa* individuals and the white cells represent white-flowered individuals. The letters (W1, W2, Y1, Y2) inside the cell represent the source population of the seeds and rosettes.

RESULTS

Differences in soil properties among populations

Soil parameters as a whole differed significantly among the four sites (Y1, Y2, W1, W2) (Wilk's lambda = 0.007, $F = 6.316$, $P < 0.0001$). None of the soil parameters clustered either white or yellow sites together, suggesting high heterogeneity among all sites (Fig. 3). Calcium, magnesium, potassium, cation-exchange capacity, organic matter, and water holding capacity differed among sites, but phosphorus and pH did not (Table 1). In addition, soil parameters as a whole did not differ between white and yellow sites (Wilk's lambda = 0.428, $F = 2.292$, $P = 0.099$). However, water-holding capacity of yellow sites was significantly higher than that of white sites ($P < 0.05$). In addition, the greatest change in volumetric water content following two rainfall events that occurred at all sites occurred in yellow sites (Table 2)

Reciprocal seed sowing experiments

In the reciprocal seed sowing experiment started with non-dormant seeds, the overall germination proportions were similar across the four sites (44-49%). However, overall seed germination of white morphs was significantly higher (morph effect, Wald $\chi^2 = 36.659$, $P < 0.001$) than that of yellow morphs, which was similar across sites (site x morph, $P = 0.764$). Seed germination proportions did not differ among sites (site effect, Wald $\chi^2 = 7.213$, $P < 0.05$). The white morph had higher seed germination proportions ($P < 0.05$) than the yellow morph in each site (Fig. 4a). The incubator experiment done in parallel to the field experiment starting with non-dormant seeds (using the same seed batch) showed no consistent differences between white and yellow morph germination

proportions (white: 0.73 ± 0.021 and yellow: 0.72 ± 0.023; Wald $\chi^2 = 0.141$, $P = 0.707$) (Fig. 4b).

In the reciprocal seed sowing experiment started with dormant seeds, which after-ripened under field conditions, the overall germination of white morph seeds was higher than that of yellow morph seeds (morph effect, Wald $\chi^2 = 56.678$, $P < 0.001$) and the seed germination proportions differed among sites (site effect, Wald $\chi^2 = 222.009$, $P < 0.001$). The site x morph interaction also was significant (Wald $\chi^2 = 9.289$, $P < 0.05$). White morphs had higher germination than yellow morphs in all sites except in Y2 (Fig. 5a), and this difference was more pronounced in white sites ($P < 0.001$).

The proportion of seedlings surviving was significantly different among sites (site effect, Wald $\chi^2 = 67.489$, $P < 0.001$) and white morph seedlings survived better than yellow morphs (morph effect, Wald $\chi^2 = 8.094$, $P = 0.004$); the site x morph interaction was also significant (Wald $\chi^2 = 17.186$, $P = 0.001$). Seedlings from white morph seeds had higher survivorship than those from the yellow morph at both white sites, and seedlings from yellow morph seeds had higher survivorship than those from the white morph at one yellow site (Y1) (Fig. 5b). However, seedlings from white morph seeds had higher survivorship than those from the yellow morph at the Y2 site.

The seed germination experiment that tested after-ripening and germination on soil from each of the four sites showed a significant soil effect (Wald $\chi^2 = 64.235$, $P < 0.001$). Overall, the germination proportions of white morph seeds were significantly higher than those of yellow morph seeds on all soil types (morph effect, Wald $\chi^2 = 26.049$, $P < 0.001$), but the soil x morph interaction was not significant (Wald $\chi^2 = 1.222$,

$P = 0.748$). Germination proportions of white morph seeds were significantly higher than those of yellow morph seeds on soil from one white site and both yellow sites (Fig. 5c).

Reciprocal rosette transplant experiments

In the experiment started with juvenile rosettes, the overall overwinter survival ranged from 64-98% across sites. Survival in the Y2 site was low (64%) compared to other sites because part of the array was destroyed by human activities (Fig. 6a). The survival proportions differed significantly among sites (site effect, Wald $\chi^2 = 38.271$, $P < 0.001$). Considering all sites together, significantly more yellow morph plants survived than did white morph plants (morph effect, Wald $\chi^2 = 4.578$, $P < 0.05$), and the site x morph interaction was also significant (Wald $\chi^2 = 9.204$, $P < 0.05$). The yellow morph had higher survival than the white morph in both white sites ($P < 0.05$), but the two morphs survived equally well in both yellow sites, indicating no evidence for local adaptation for overwinter survival. The subsequent survival up to the reproductive stage was not significantly different among sites (site effect, Wald $\chi^2 = 2.971$, $P = 0.396$), but the yellow morph had an overall higher survival than the white morph across all sites (morph effect, Wald $\chi^2 = 8.971$, $P < 0.001$). The site x morph interaction was not significant (Wald $\chi^2 = 5.505$, $P = 0.138$). Survival to reproductive age was significantly higher for the yellow morph than the white morph in one yellow site (Y1) and one white site (W2) (Fig. 6b). In the other sites (Y2, W1), there were no differences in survival to reproductive stage between morphs.

In the reciprocal transplant experiment done with adult rosettes, survival differed between sites (site effect, Wald $\chi^2 = 5.949$, $P < 0.001$) but did not differ between morphs

(morph effect, Wald $\chi^2 = 1.187$, $P = 0.276$) and no significant site x morph interaction was found (Wald $\chi^2 = 4.884$, $P = 0.180$). The W1 site had high mortality of individuals due to a flood (Fig. 6c). Though the survival of white and yellow morph plants was similar in most sites, the yellow morph plants had higher survival in one yellow site (Y1) (Fig. 6c).

Considering reproductive success of the plants in the reciprocal transplant experiment, site effects were significant for all parameters (flower number, fruit number, seeds per fruit, and estimated total seed number) for both juvenile and adult rosettes (Table 3). In the experiment started with juvenile rosettes, the yellow morph produced significantly higher numbers of flowers (white: 5.18 ± 0.481; yellow: 7.41 ± 0.749; $P < 0.01$) and fruits (white: 2.65 ± 0.188; yellow: 3.45 ± 0.231; $P < 0.05$). However, both morphs produced similar numbers of seeds per fruit (white: 9.10 ± 0.97; yellow: 9.74 ± 0.839; $P = 0.259$) and similar total seed number (white: 9.10 ± 0.970; yellow: 9.74 ± 0.839; $P = 0.310$) (Fig. 7). In the experiment started with adult rosettes, the yellow morph produced significantly more flowers (white: 5.91 ± 0.33; yellow: 7.06 ± 0.37; $P < 0.05$) and fruits (white: 3.29 ± 0.201; yellow: 4.18 ± 0.204; $P < 0.01$). However, there was no significant difference between morphs in seeds per fruit ($P = 0.284$) or total seed number ($P = 0.084$). No significant site x morph interactions were found for any of the reproductive parameters examined in either transplant experiment (Table 3).

Performance under water-related stress conditions

Seed after-ripening stage

The proportion of seeds germinating differed significantly among treatments (treatment effect, Wald $\chi^2 = 518.983$, $P < 0.001$), and yellow morph seeds had significantly higher germination than white morph seeds across all treatments (morph effect, Wald $\chi^2 = 29.548$, $P < 0.001$). The morph x treatment interaction was not significant (Wald $\chi^2 = 3.672$, $P = 0.299$). The yellow morph had significantly higher seed germination than did the white morph in the T1, T2 and T4 treatments ($P < 0.01$), but germination did not differ between morphs in the T3 treatment (Fig. 8).

In the relative humidity experiment, all of the seeds at 75% and 95% relative humidity rotted due to fungal activity during after-ripening and were therefore excluded from the analysis. Seed germination proportions were significantly different among treatments (treatment effect, Wald $\chi^2 = 32.134$, $P < 0.001$) and overall, white morph seeds germinated at a significantly higher frequency than did yellow morph seeds (morph effect, Wald $\chi^2 = 20.249$, $P < 0.001$). The treatment x morph interaction was not significant (Wald $\chi^2 = 1.634$, $P = 0.442$). A greater proportion of white morph seeds after-ripened at 11% and 25% relative humidities, germinated than yellow morph seeds ($P < 0.01$), but germination did not differ between morphs when after-ripened at 50% (Fig. 9).

Seed germination stage

Seed germination significantly decreased with an increase in water potential (treatment effect, Wald $\chi^2 = 115.331$, $P < 0.001$), and this decrease was similar between

the morphs (morph effect, Wald $\chi^2 = 0.299$, $P = 0.585$; treatment x morph interaction, Wald $\chi^2 = 1.497$, $P = 0.827$). (Fig. 10).

Initial seedling stage

As all seedlings at a water potential of -1.0 MPa died before the end of the experiment, this treatment was excluded from data analysis. Both white and yellow morphs had similar seedling growth at a water potential of -0.25 and -0.5 MPa (morph effect, $F = 2.019$, $P = 0.159$), and growth of both morphs decreased similarly from -0.25 to -0.5 MPa (treatment effect, $F = 28.216$, $P < 0.001$; morph x treatment interaction, $F = 3.714$, $P = 0.058$). Growth at -0.25 MPa was similar between morphs, but growth at -0.5 MPa was significantly higher for the white morph compared to the yellow morph ($P < 0.01$) (Fig. 11).

Table 1. Soil parameters at yellow (Y1, Y2) and white (W1, W2) sites of *Leavenworthia stylosa*.

Parameter	Site	Mean ± SE
Calcium (ppm)	Y1	7198.6 ± 121.09 b
	Y2	4460.4 ± 136.55 a
	W1	6789.4 ± 549.86 b
	W2	5072.6 ± 109.42 a
Magnesium (ppm)	Y1	85.00 ± 3.36 bc
	Y2	114.40 ± 4.81 a
	W1	77.20 ± 8.36 c
	W2	94.00 ± 4.1 b
Phosphorus (ppm)	Y1	2.80 ± 0.73
	Y2	4.60 ± 0.75
	W1	3.00 ± 0.63
	W2	3.20 ± 0.66
Potassium (ppm)	Y1	101.60 ± 2.83 ab
	Y2	107.40± 5.31 a
	W1	88.20 ± 4.2 b
	W2	107.40 ± 5.31 a
Cation exchange capacity (mili-equivalents/100g of soil)	Y1	36.94 ± 0.61 b
	Y2	23.52 ± 0.70 a
	W1	34.82 ± 2.80 b
	W2	26.40 ± 0.56 a
Organic matter (%)	Y1	7.54 ± 0.22 b
	Y2	5.20 ± 0.35 ac
	W1	4.42 ± 0.46 c
	W2	6.00 ± 0.46 a
pH	Y1	7.82 ± 0.07
	Y2	7.56 ± 0.10
	W1	7.82 ± 0.11
	W2	7.56 ± 0.68
Water holding capacity*	Y1	66.96 ± 1.53 a
	Y2	50.66 ± 8.04 b
	W1	35.35 ± 0.72 c
	W2	53.35 ± 0.55 d

Means ± SE followed by different letters indicate significant differences (LSD test, $P < 0.05$).

* [(wet soil mass – dry soil mass)/dry soil mass] x 100

Table 2. Change in volumetric water content after two rain events in two yellow (Y1, Y2) and two white (W1, W2) sites of *Leavenworthia stylosa.*

Site	Change in volumetric water content in soil (m^3/m^3)	
	Rain event 1 (within 160 h)	Rain event 2 (within 40 h)
Y1	-0.000159	-0.000490
Y2	-0.000227	-0.000200
Average Yellow	**-0.000193**	**-0.000345**
W1	-0.000199	-0.000255
W2	-0.000132	-0.000128
Average White	**-0.000166**	**-0.000192**

Table 3. Summary of analysis of variance results on the effects of site and flower color morph and their interaction on reproductive parameters of *Leavenworthia stylosa* in two reciprocal transplant experiments.

Effect		Flower number		Fruit number		Seeds per fruit		Est. total seed number	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Started with juvenile rosettes									
Site	3	27.583	***	14.675	***	25.334	***	25.288	***
Morph	1	10.483	***	4.218	*	1.281	ns	1.036	ns
Site x morph	3	1.043	ns	0.908	ns	0.561	ns	0.164	ns
Started with adult rosettes									
Site	3	4.276	**	14.357	***	6.319	***	15.287	***
Morph	1	5.639	*	7.865	**	1.152	ns	2.995	ns
Site x morph	3	1.691	ns	1.822	ns	1.403	ns	1.325	ns

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant

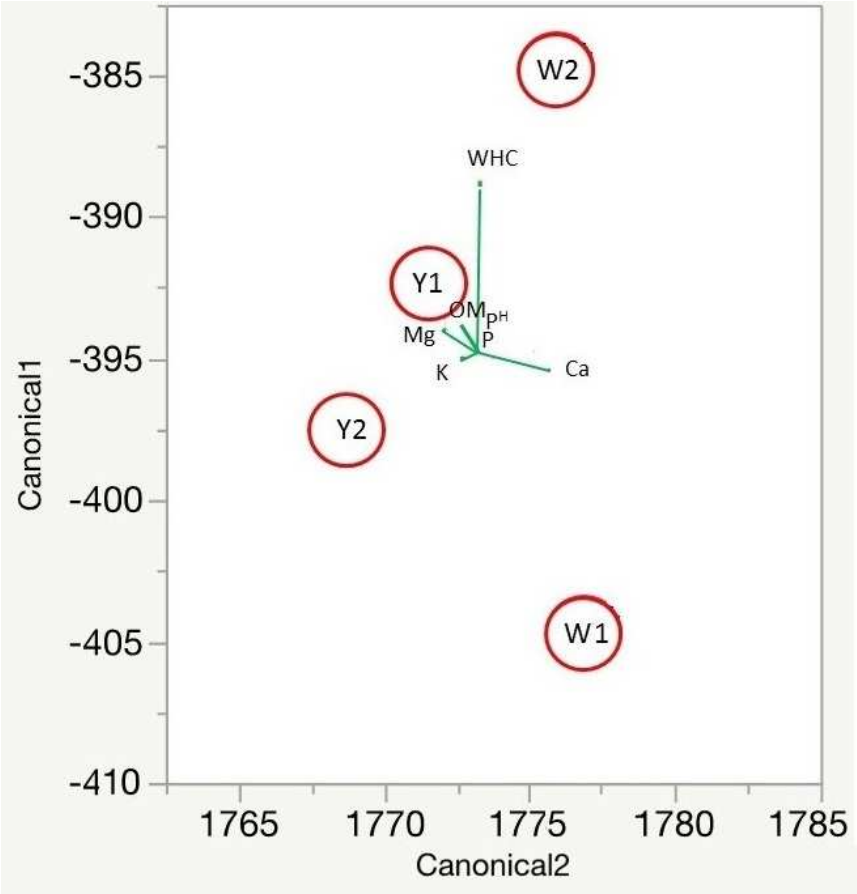

Figure 3. Canonical centroid plot of the multivariate effects of two yellow (Y1, Y2) and two white (W1, W2) sites on the soil parameters Ca, Mg, K, organic matter (OM) and water holding capacity (WHC). Circles represent the 95% confidence region and biplot vectors show the directions of the response variable, e.g. WHC increased when moving from W1 to W2 and Ca increased when moving from Y2 to W1.

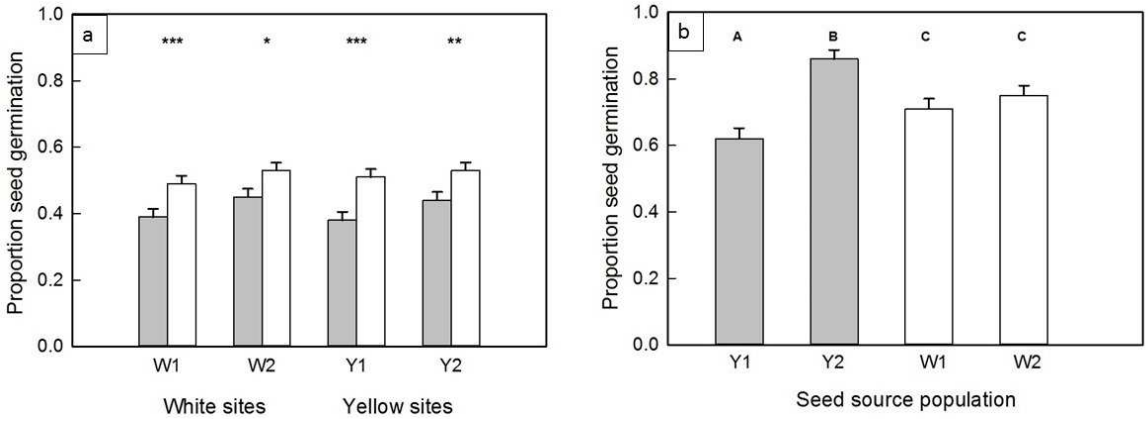

Figure 4. Seed germination of yellow (gray bars) and white (white bars) *Leavenworthia stylosa* morphs in experiments started with (a) non-dormant seeds in a reciprocal seed sowing experiment or (b) under controlled laboratory conditions. Significant differences in seed germination proportions between yellow and white morphs within sites are indicated with asterisks in graph a (LSD test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) and those among sites are indicated by different letters above bars in graph b (LSD test; $P < 0.05$). Error bars represent SE.

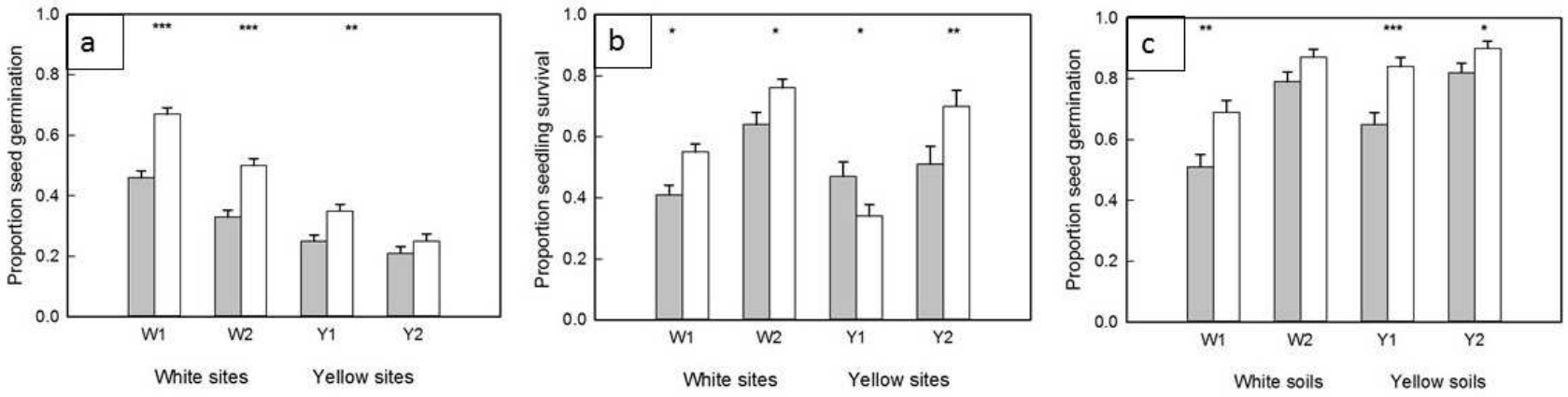

Figure 5. Seed germination and seedling survival of yellow (gray bars) and white (white bars) *Leavenworthia stylosa* morphs. (a) Seed germination or (b) seedling survival proportions in a reciprocal seed sowing experiment started with dormant seeds. (c) Germination proportions of seeds after-ripened and germinated on soil from each of the four sites. Significant differences in germination or seedling survival proportions between yellow and white morphs within sites/soils are indicated with asterisks (LSD test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Error bars represent SE.

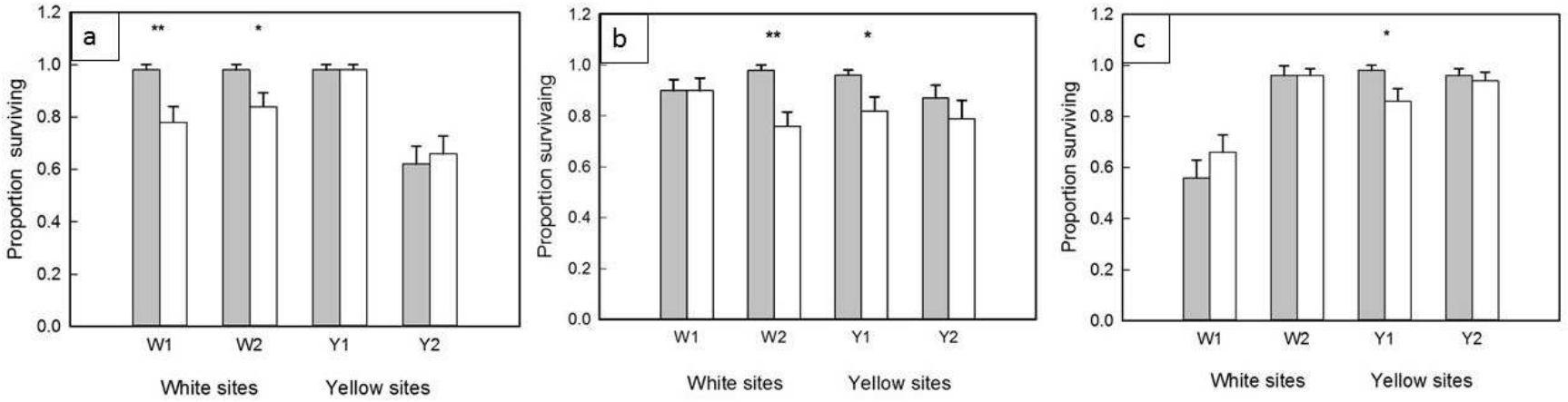

Figure 6. Proportion of plants surviving in reciprocal transplant experiments: (a) overwinter survival of transplanted juvenile rosettes, (b) post-winter survival through the end of the flowering period of juvenile rosettes, and (c) survival to the end of the flowering season of transplanted adult rosettes. Significant differences in survival proportions of yellow and white morphs within sites are indicated with asterisks (LSD test; * $P < 0.05$, ** $P < 0.01$). Error bars represent SE.

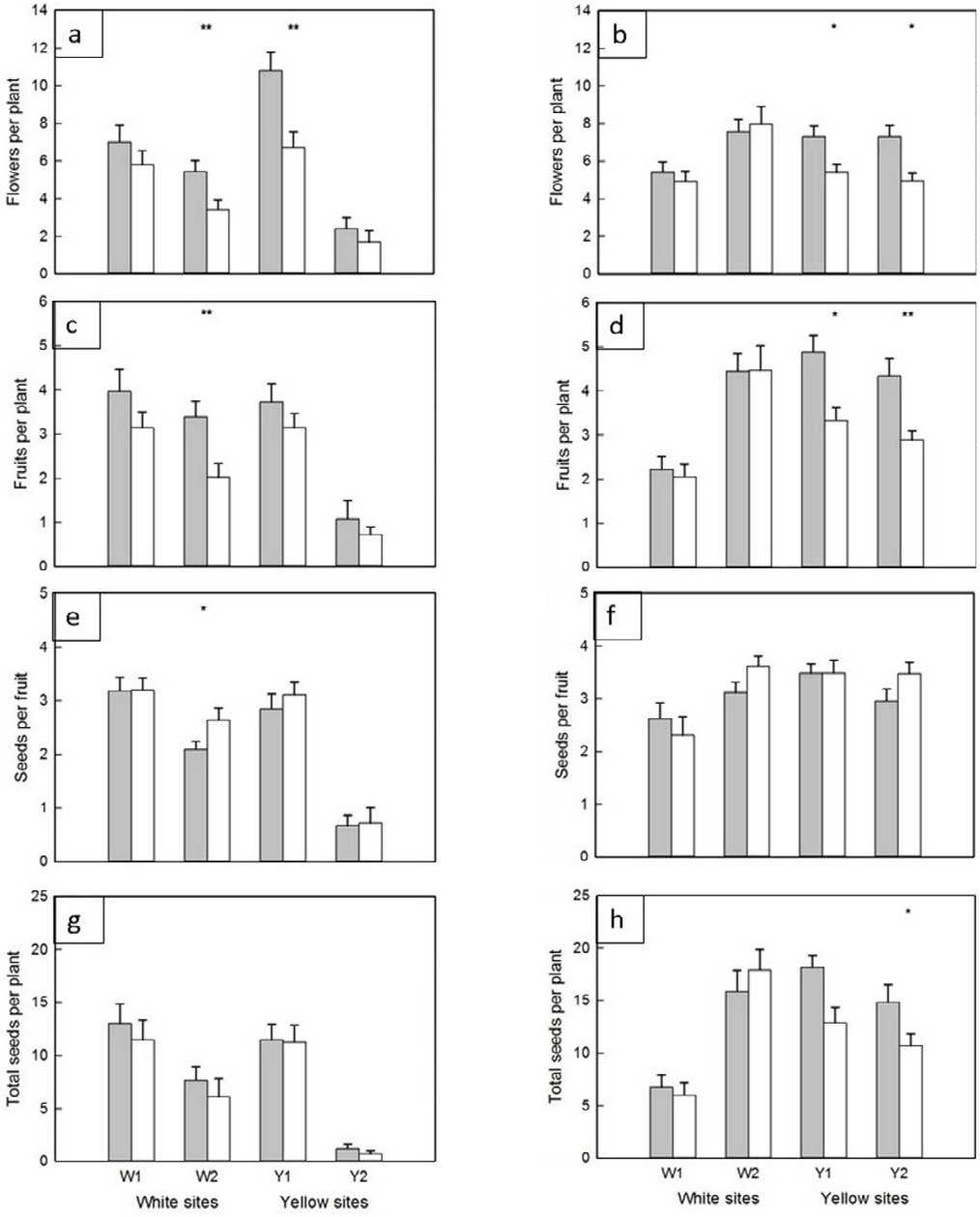

Figure 7. Reproductive success of yellow (gray bars) and white (white bars) *Leavenworthia stylosa* morphs in a reciprocal transplant experiment started with juvenile rosettes (graphs a, c, e, g) and adult rosettes (graphs b, d, f, h). Significant differences in performance between morphs within sites is indicated with asterisks (LSD test; * $P < 0.05$, ** $P < 0.01$). Error bars represent SE.

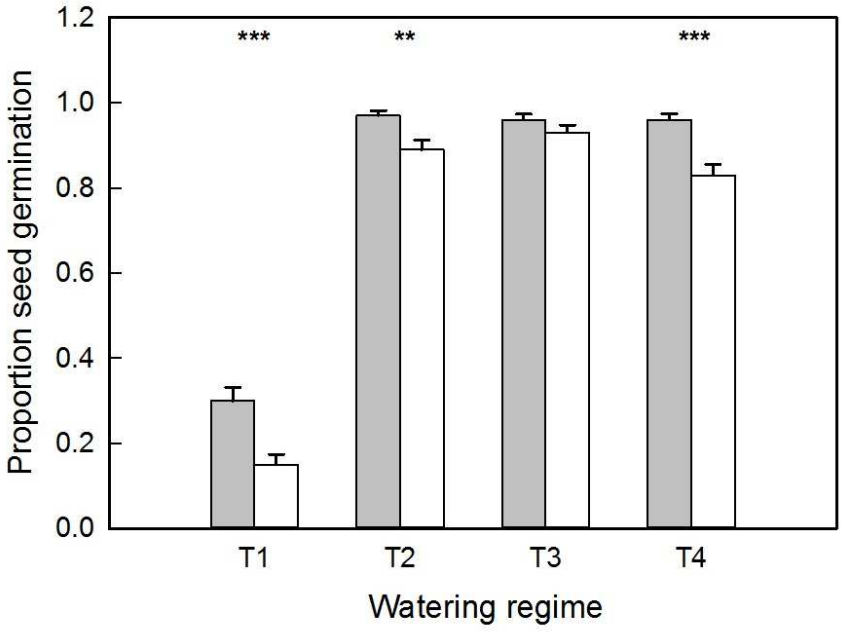

Figure 8. Proportion of seed dormancy loss (as indicated by germination) for yellow (gray bars) and white (white bars) *Leavenworthia stylosa* morphs under different watering treatments: constant wet (T1), watering once per week (T2), watering once every 2 weeks (T3), and watering once every 4 weeks (T4). Significant differences in germination proportions between morphs within each treatment is indicated with asterisks (LSD test; ** $P < 0.01$, *** $P < 0.001$). Error bars represent SE.

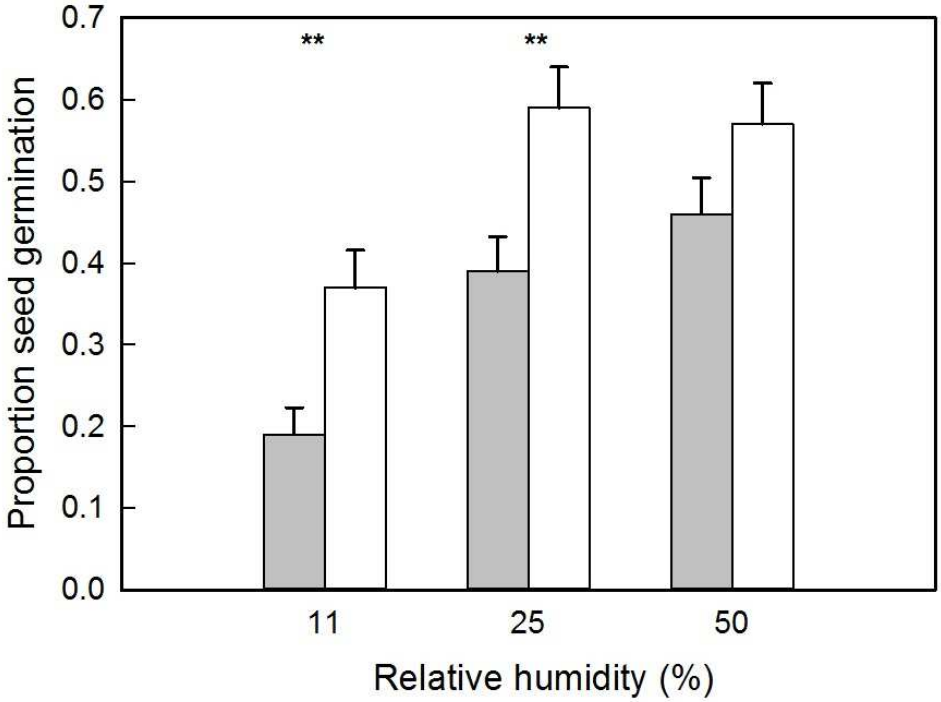

Figure 9. Proportion of seed dormancy loss (as indicated by germination) for yellow (gray bars) and white (white bars) *Leavenworthia stylosa* morphs under a relative humidity gradient at 30ºC. Significant differences in germination proportions between morphs within each treatment is indicated with asterisks (LSD test; ** $P < 0.01$). Error bars represent SE.

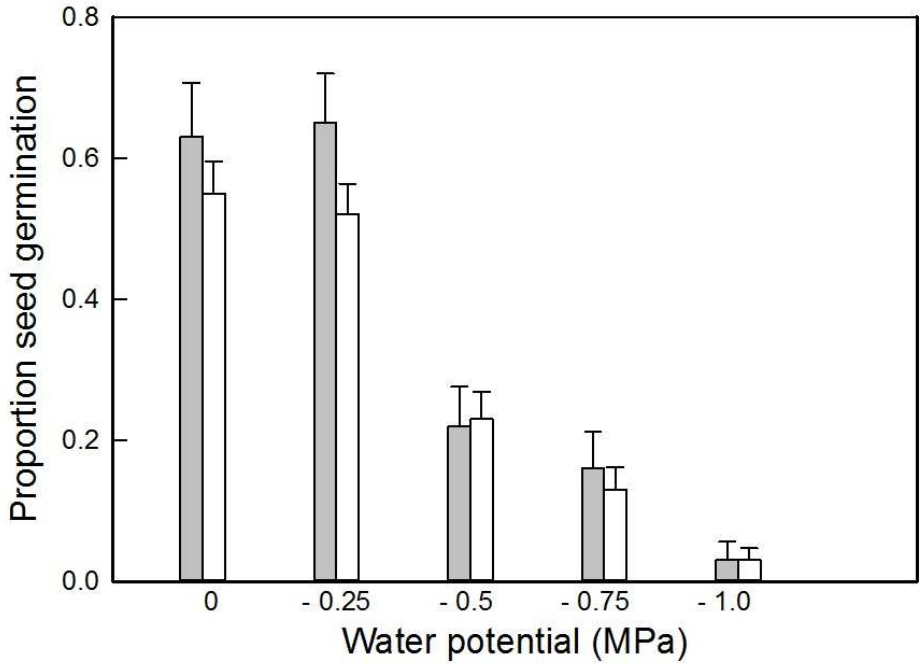

Figure 10. Proportion of seed germination for yellow (gray bars) and white (white bars) *Leavenworthia stylosa* morphs under a water potential gradient. Error bars represent SE.

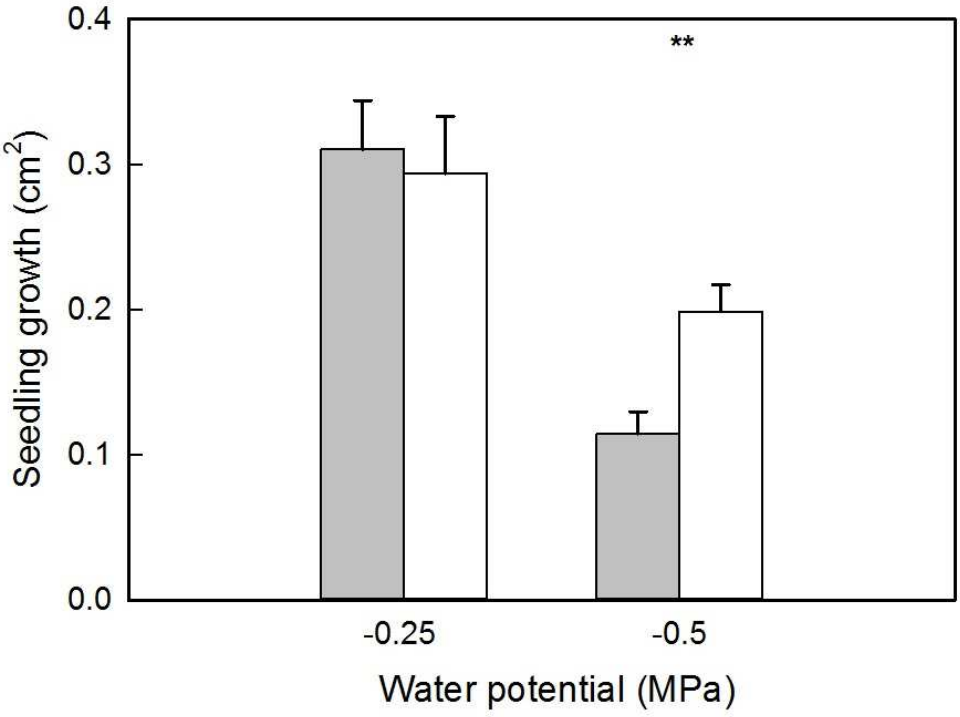

Figure 11. Average seedling growth (area) for yellow (gray bars) and white (white bars) *Leavenworthia stylosa* morphs under a water potential gradient. Significant differences in seedling growth between morphs within treatments is indicated with asterisks (LSD test; ** $P < 0.01$). Error bars represent SE.

DISCUSSION

To evaluate the role of local adaptation to abiotic factors in maintaining the geographic pattern of flower color variation in *L. stylosa*, I conducted three sets of complimentary experiments. First, soil properties were compared between sites containing different flower color morphs. Second, reciprocal transplant experiments were conducted at multiple early life history stages. Third, a series of studies was done on the effects of water-related stress at early life history stages. Taken together, these experiments allowed me to evaluate how selection, operating at different life history stages in relation to soil properties and water-related stress, affects the performance of the two color morphs and their distributions in geographic space.

I found strong evidence for morph-specific variation in different fitness-related components and limited evidence of local adaptation of white and yellow morphs of *L. stylosa* at different life history stages. Seed germination is one of the earliest expressed traits of a plant that can be influenced by selection (Donohue et al., 2010). In both field reciprocal sowing experiments, germination was higher for the white morph than for the yellow morph across most sites. In the laboratory reciprocal sowing experiment, germination was also higher for the white morph than for the yellow morph regardless of soil type. In contrast, germination did not differ between morphs when they were incubated on filter paper moistened with distilled water in the laboratory. Germination also did not differ between morphs when seeds were incubated over a water potential gradient. Thus, these sets of observations suggest that the soil substrate plays a key role for influencing germination.

The higher germination of white morph seeds over yellow morph seeds observed at most sites in the reciprocal sowing experiments started with non-dormant or dormant seeds suggests that the white morph has wider germination success across the species range compared to the yellow morph. Differences in germination between laboratory and field experiments is usually attributed to environmental conditions such as temperature, humidity, soil chemical properties, and/or photoperiod (Travest, 1998). A study done on fruit color polymorphism of *Rubus spectabilis* found a higher seed germination of orange morph seeds than red morph seeds in the soil collected from orange-morph dominated sites Travest and Willson, 1998), and it has been suggested that the action of some edaphic factors can inhibit seed germination or modify the osmotic pressure of the soil, which can affect the metabolic processes related to pigmentation. For *L. stylosa*, the reason behind the higher germination of white morph seeds compared to yellow morph seeds in the field is unclear, and detailed studies are needed to determine which edaphic factors can impact seed germination of the yellow morph. But differences in germination between the white and yellow morphs might be related to the action of abscisic acid.

Abscisic acid is a phytohormone that plays a major role in seed germination and dormancy. This compound is derived from the carotenoid biosynthetic pathway (Frey et al., 2012). Preliminary investigations suggest that carotenoid pigments are responsible for the yellow flower color of *L. stylosa* (Norton et al., 2015). The white morph of *L. stylosa* produces yellow pigment in the center of the flower, while the yellow morph produces yellow pigment throughout the entire petal. If the yellow morph is producing more carotenoid pigment, it is possible that it is also producing greater amounts of chemical signaling compounds, which are products of the carotenoid biosynthetic pathway. As

such, it is possible that seeds of the yellow morph contain greater amounts of abscisic acid. Other studies have shown that plants producing greater amounts of carotenoids also produce greater amounts of abscisic acid (Lindgren et al., 2017). A transgenic study done on *Arabidopsis* by overexpressing the seed-specific phytone synthase gene has shown a significant increase in carotenoids in seeds as well as in the plants derived from seeds, and these transgenic seeds had shown a delayed germination. Given the importance of germination timing on survival and seed production in *L. stylosa* (Baskin and Baskin, 1972b), minor variation in moisture and temperature regimes in the habitats may exert local selection on the expression of these important signaling compounds and may result in differences in germination between morphs and among sites.

There are some other possibilities that could explain differences in germination of the white vs. yellow morphs in the reciprocal sowing experiments in the field and laboratory. In all experiments, I used field-collected seeds that had been matured on their mother plants. The maternal environment in which seed maturation took place has a strong influence on seed dormancy and seed germination characteristics as they are primarily controlled by tissues surrounding the embryo, which have a maternal origin. (Roach and Wulff, 1987; Donohue et al., 2010). Moreover, maternal nutrient supply and hormone levels can also affect dormancy and seed germination (Gutterman, 1980). Thus, when the field-collected seeds were sown in other sites, this maternal effect could have carried over to them. However, the germination of white and yellow morph seeds did not differ in the parallel laboratory experiment, which was done with the same batches of seeds as the reciprocal field study (in 2014). If the maternal effect on seed dormancy break and germination is responsible for the difference between white and yellow morph

seed germination, the same difference should be seen in the laboratory experiments. Thus, the involvement of maternal effects is unlikely as germination proportions of white seed and yellow seed were equal in the laboratory experiment, which tested non-dormant seed germination on filter papers.

Other factors which could have influenced germination are seed size and seed mass. In general, larger or heavier seeds might be expected to have a higher probability of germination, and differences in seed size have been found among flower color morphs of other species. A positive relationship between seed mass and germination probability was found in *Protea* spp. to be associated with flower color polymorphism, in which white morph seeds were 10% heavier than the pink morph seeds and 3.5 times more likely to germinate than the pink morph (Carlson and Holsinger, 2010). However, in *L. stylosa,* neither seed mass nor seed surface area differed between color morphs (M.T.R. Fernando, unpubl. data). Since the reciprocal seed sowing experiments in the field yielded the same results using non-dormant and dormant seeds, I suggest that it is the germination stage (and not the after-ripening stage) that is causing the differences in seed germination proportions between morphs.

The initial establishment phase of a plant occurs in two steps: Seed germination and subsequent seedling establishment and survival (e.g. Galen and Stanton, 1999; Molofsky et al., 2000). In my study, I found significantly higher survival after germination in white than in yellow morph plants in both white sites and in one yellow site (Y2). Although seed germination of the white morph was significantly higher than the yellow morph in the Y1 site, subsequent seedling survival of the white morph in Y1 was significantly lower than the yellow morph, negating the germination advantage

shown by the white morph. An association between flower color and seedling survival has been shown in multiple species in the literature. In *Echium plantagineum,* the blue-purple flower morph survived better than the white morph due to its competitive ability at the seedling stage (Burdon, 1983). Survival of seedlings of the red flower morph of *Lysimachia arvensis* was lower than that of the blue morph in dry conditions (Arista et al., 2013). In *Digitalis purpurea,* the "red parent" seedlings survived better than the "white parent" seedlings (Ernst, 1987), and in *Clarkia unguiculata* an association between flower color genes and seedling pigmentation has been identified (Bowman, 1987). Photoprotective function of anthocyanin pigments has been identified in many systems as they can neutralize free radicals generated in the plant bodies under adverse environmental conditions (Løvdal et al., 2010). Since there is no study done on morph-specific anthocyanin production in *L. stylosa* seedlings, we cannot suggest that the higher survival is due to anthocyanin pigments in white seedlings. However, the environment experienced by seeds at the germination stage are most likely the environment that will be experienced by seedlings in their establishment stage, such that germination cues can be considered a mechanism of habitat selection (Donohue, 2003). This may be the reason for higher seedling survival of white morph I observed in most of the sites.

Being a winter annual species in cedar glade ecosystems, *L. stylosa* seeds cope with extreme wet and dry conditions from early summer into early autumn, and seedlings tolerate frost and freeze/thaw conditions on shallow soil during the winter and extreme wet conditions during winter and spring (Quarterman et al., 1993). In my reciprocal transplant arrays, though no local adaptation was detected at the after-winter stage of the juvenile rosettes, the higher survival of yellow compared to white morph plants at some

sites may be due to the pleiotropic effects of carotenoid pigment production in the yellow morph. Compounds associated with cold stress tolerance are one of the pleiotropic characters associated with the carotenoid and anthocyanin biosynthetic pathways. Studies have shown elevated levels of carotenoids and other byproducts of this pathway (e.g. abscisic acid) in plant bodies in response to cold temperatures (e.g. Adams and Demmig-Adams, 1995; García-Plazaola et al., 1999). A transcriptome study on *Populus trichocarpa* found high expression of genes associated with carotenoid biosynthesis in winter dormant stems (Ko et al., 2011). In the arctic mustard, *Parrya nudicaulis,* the frequency of white-flowered individuals which are less resistant to cold due to lack of stress responsive flavonoid intermediates, increased with increasing growing season temperature (Dick et al., 2011). The association between floral carotenoids and vegetative carotenoid production in *L. stylosa* is unclear. However, the higher survival of the yellow morph over the white morph observed in the two reciprocal transplant experiments in winter survival and reproductive stage survival suggest that cold tolerance and higher survival are possibly due to pleiotropic effects of increased carotenoid pigment production in the yellow morph.

Considering the survival through the flowering stage and reproductive success of *L. stylosa*, the yellow morph showed a higher survival than the white morph in one reciprocal transplant experiment and yellow morphs produced higher number of flowers and fruits compared to the white morph in both experiments. I found limited evidence that the home morph performs better for some traits at some sites (but this is only one part of local adaptation). Yellow morph showed a higher reproductive stage survival of transplanted adult rosettes (in the Y1 site), flower production and fruit production (at both

the Y1 and Y2 sites), and total seed number (in the Y2 site) in at least in one reciprocal transplant experiment. However, the white morph out performed yellow only for number of seeds per fruit at the W2 site. Several studies have shown an association between flower color and survival and reproductive fitness under environmental stress conditions. In *Linanthus parrya*e, blue- and white-flowered morphs produced higher number of seeds in their home sites and these differences were attributed to the edaphic differences in the two habitats and morph specific water-use efficiencies (Schemske and Bierzychudek, 2001, 2007). A study completed on five species, each of which was polymorphic for flower color, showed that the pigmented plants performed relatively better under drought conditions while the unpigmented plants performed relatively better in the well-watered conditions (Warren and Mackenzie, 2001). All of the above species have anthocyanin-based flower color polymorphisms. Thus, the authors ascribed the fitness differences between color morphs under stressful conditions to the pleiotropically-related characters of anthocyaninin in the pigmented morph. There are very few studies on species with carotenoid-based flower color polymorphism compared to those on species with anthocyanin-based flower color, and only a few studies have compared the reproductive success of the pigmented vs. non-pigmented morphs. For example, in *Raphanus sativus* the yellow morph produced a slightly but significantly greater number of flowers than the white morph, but no difference between the morphs was seen in fruit and seed production (Stanton, 1987).

My water-related stress experiments showed conflicting results among the life history stages for *L. stylosa*. In experiments varying relative humidity, the white morph typically had greater dormancy loss than did the yellow morph, and the difference in

dormancy loss between the morphs was greatest under dry conditions. In experiments varying the watering regime, a greater proportion of seeds of the yellow morph lost their dormancy across most of the watering regimes, including both the wettest and driest treatments. In the experiment studying the seed germination under water potential gradient, seed germination decreased across the water potential gradient, but within treatments, both morphs had similar germination. Seedling growth of the white morph was highest under the lowest water potential. Thus, the laboratory experiments did not provide supportive evidence that water-related stresses at early life history stages are important selective agents that may drive maintenance of the flower color polymorphism in *L. stylosa.*

Field observations done across the geographic range of *L. exigua*, a selfing sister species of *L. stylosa*, suggested that it occupied drier cedar glade sites than other members of the genus (Rollins, 1963; Lloyd, 1965). Norton et al. (2015) found that yellow morph populations of *L. stylosa* are less geographically isolated from *L. exigua* populations than white morph populations, suggesting that the yellow morph populations occur in this drier range. However, the water-holding capacity data was not consistent with these suggestions since on average, yellow sites had higher water holding capacity (i.e. would be less dry) than did white sites. A soil's water holding capacity is influenced by its texture and organic matter content, and a positive relationship occurs between water holding capacity and the amount of moisture that a soil holds (Mahe et al., 2005). The amount of moisture in soils of cedar glades (and similar glade-like communities) is heavily influenced by soil depth, which is generally very shallow but varies greatly across sites (Kumera and Martin, 1957; Somers et al., 1986). Though an extensive survey on soil

depths and soil moisture content covering the whole geographic range in different seasons is needed to conclude that yellow sites are drier than white sites, the change in volumetric water content following rainfall suggested that the yellow sites lose water at a higher rate than white sites. Moreover, microhabitat differences in soil depth, nutrients, and soil moisture within and among other glade-like communities (Walck et al., 1999; Wolf et al., 1999) can influence reproductive performances of plants and potentially color morphs. Therefore, it is possible that plants in yellow sites experience frequent harsher (drier) conditions than those in white sites, and the yellow morph can survive and reproduce better than the white morph in drier habitats due to physiological functions pleiotropically related to the production of carotenoid pigments, such as scavenging of reactive oxygen species when plants are under stress conditions (Sachindra et al., 2007).

Though the yellow morph performed better than the white morph in flower and fruit production in both reciprocal transplant experiments across all sites, seeds per plant were not significantly different between them. I found a higher visitation rate (but not significantly higher) of pollinators to the white morph compared to the yellow morph (Chapter 2), but the white morph did not receive a fruit or seed set advantage with those higher visitation rates in most of the sites (except in W2). Seed production of an individual is limited by resource availability (water and nutrients) to the mother plant as well as the pollen supply (Haig and Westoby, 1988). In *L. stylosa*, seed set in either morph is not pollen-limited but is strongly resource-limited in their sites (Norton et al., 2015). The yellow morph showed a home site advantage in yellow sites, which appear to be drier on average (at least in terms of higher evaporation) than white sites. I suggest that the yellow morph is adapted to locally harsh conditions, enabling higher

reproduction and survival in their home sites as well as comparatively benign white sites. On the other hand, the white morph is not adapted to as harsh conditions as the yellow morph and as such has reduced survival and reproduction in yellow sites.

In my study, I found limited evidence for local adaptation at different life history stages, but this evidence was highly dependent on site. The conflicting results I observed for the experiments conducted under a drought stress gradient suggest that drought stress is unlikely to be a selective agent, at least during early life history stages. There may be different selective agent(s) in the cedar glade habitats such as a soil factor, which can exert strong differential selective pressures on the two flower color morphs at some point of their life cycles. Moreover, I did not examine drought stress during the reproductive stage of the plant's life cycle, in which the two morphs may show differential responses to drought in terms of survival and fecundity. To test all of these possibilities, laboratory experiments to check the differences in soil chemistry, as well as spatial and temporal variation of hydrological features of habitats are needed. Moreover, morph-specific variation of plant pigment (carotenoids and flavonoids) profiles in flowers and vegetative tissues and seeds, will be helpful to understand the different responses of the morphs under stress conditions. More reciprocal transplant experiments are needed using more white and yellow sites representing the breadth of geographic and physiological variation in the species range, and the experiments should be repeated for several years to capture potentially large fluctuations in magnitude and direction of the selective agents through time (Schemske and Bierzychudek 2001).

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CHAPTER 4

GENERAL CONCLUSIONS

Ecologists and evolutionary biologists have long been fascinated by the emergence and the maintenance of the flower color polymorphism (Weiss, 1995). An interesting feature of some plants with polymorphic flower color is that variation is geographically structured (Mascó et al., 2004; Schemske and Bierzychudek, 2007; Hopkins et al., 2012; Arista et al., 2013). My study species, *Leavenworthia stylosa*, is a cedar glade endemic that has a limited distribution, occurring only in the Central Basin of Tennessee (Rollins, 1963). The species has two main flower colors, yellow and white, and they occur in monomorphic populations generally with yellow and white morphs in the northwest and southeast portions of the Basin, respectively. My overarching questions were "Why are most populations of *L. stylosa* monomorphic with respect to flower color?" and "What explains the geographic distribution of flower color maintanence in *L. stylosa*?"

Several mechanisms could possibly explain the maintenance of the geographic variation for flower color in *L. stylosa*. First, if flower color is selectively neutral, genetic drift within populations in combination with restricted gene flow among populations could lead to the fixation of different flower color alleles in different portions of the geographic range (Epling and Dobzhansky, 1942). Second, pollinators may show spatial variation in their assemblages and/or preferences during foraging (Gómez and Zamora, 2000), and seed predators can exert selection on flower color depending on pollinator densities and preference (Kolb el al., 2007; Sobral et al., 2015). Third, spatial variation of

abiotic factors can exert selection on pleiotropically-related non-floral traits, and different morphs may show differential fitness across environmental gradients, leading to local adaptation (Waser and Price, 1981).

The white and yellow morph populations of *L. stylosa* are not genetically structured, but there is very little genetic structure at the levels of watersheds and populations (Dixon et al., 2013). The considerable gene flow observed among populations suggests that isolation by distance does not play a role in maintaining geographic structure of flower color in *L. stylosa*.

I found some evidence for biotic agents playing a role in maintaining spatial variation of flower color in the species. Pollinator assemblages for white morphs differ from those for yellow morphs. Pollinators preferred the white morph across the species range and showed a color constancy. Moreover, seed predators preferred the white morph over the yellow morph. In white populations, exclusive visits and constant movements by *Bombylius* contributed to maintenance of the white morph and prevented the yellow morph from increasing in frequency. Whereas in yellow sites, the yellow morph was less preferred by pollinators and had less seed predation than did white morph, which prevented the white morph from increasing in frequency.

The reciprocal transplant experiments conducted at different life history stages showed limited evidence for local adaptation. Considering both seed germination and seedling survival together, the white morph performed well in white sites at the initial life history stages of the species. Over-winter survival, adult survival, and flower and fruit production were high for the yellow morph in yellow sites, indicating local adaptation at late life history stages. Soil chemistry did not differ, but hydrological properties did, on

average, between white and yellow sites. However, experiments on water-related stress on early life history stages yielded conflicting results, suggesting that it likely does not play a major role in maintaining the geographic variation of flower color.

In addition to abiotic and biotic factors, Norton et al. (2015) found that reproductive character displacement contributed to the geographic variation of flower color of the species. The white-flowered *L. exigua* usually co-occurs with the yellow morph of *L. stylosa* in Middle Tennessee, and hybrids between these two species suffer from pollen and ovule sterility. In the experimental arrays with two species, pollinators generally showed color fidelity throughout a foraging bout, mostly visiting white flowers. Thus, pollinators tended to visit the white morph of *L. stylosa* rather than yellow morph followed by a visit to *L. exigua*. Having the yellow morph of *L. stylosa* in sympatry with *L. exigua* reduces the costly interaction between them, promoting the yellow morph of *L. stylosa* at least in the part of the geographic rage where it overlaps with *L. exigua*.

Considering all factors together, it is likely that a complex interaction occurs between pollinators, seed predators, local adaptation to abiotic factors, and character displacement with a co-occurring sister species. Hence, multiple selective agents seem to promote the maintenance of geographic variation for flower color in *L. stylosa*.

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APPENDICES

APPENDIX A

List of populations and their locations where field studies were conducted and plant materials (seeds, rosettes) or soil samples were collected in Middle Tennessee.

Abbreviation	Location name	Flower color	County	GPS coordinates	Experiments *
W1	Sue Warren (along Sue Warren Trail near Cedars of Lebanon State Forest)	White	Wilson	36.069611, -86.304937	[1], [2], [3], [4], [5], [6]
W2	Shooting Range (in Cedars of Lebanon State Forest)	White	Wilson	36.084584, -86.400900	[1], [2], [3], [4], [5], [6]
W3	Flat Rock Cedar Glades and Barrens State Natural Area	White	Rutherford	35.857710, -86.298016	[8]
W4	Bryant Grove Trail (in Long Hunter State Park)	White	Davidson	36.075737, -86.521701	[7], [8]
W5	Sunnybell Cedar Glade State Natural Area	White	Rutherford	35.967552, -86.446695	[7], [8]
Y1	Smith Springs Big Glade (along Percy Priest Lake)	Yellow	Davidson	36.078677, -86.588688	[1], [2], [3], [4], [5], [6], [7], [8]
Y2	Elsie Quarterman Cedar Glade State Natural Area	Yellow	Rutherford	36.048345, -86.558989	[1], [2], [3], [4], [5], [6], [7], [8]
Y3	Butler	Yellow	Davidson	36.089681, -86.618314	[8]

* [1] Pollinator and seed predation study, [2] Ecological differences among habitats, [3] Reciprocal seed sowing experiments, [4] Reciprocal transplant experiments with rosettes, [5] Seed after ripening under a relative humidity gradient, [6] Seed after germination under an osmotic pressure gradient, [7] Seedling growth under an osmotic pressure gradient, [8] Seed germination under different watering regimes

APPENDIX B

Conditions used for seed after-ripening and germination. The temperature regime was used in the "reciprocal seed sowing experiment under controlled conditions". The temperature and watering regimes were used in the "performance under water-related stress conditions" experiment. Watering regimes had four treatments: constant wet (T1) and watering once per week (T2), once every 2 weeks (T3), or once every 4 weeks (T4).

* Dishes were moistened for 24 h (with lids attached) and then dried by removing the lids.

Week	Month	Temp. (°C)	Treatments*			
			T1	T2	T3	T4
0			water at start	water then dry	water then dry	water then dry
1	May	25/15	always wet	water then dry	dry	dry
2	May	25/15	always wet	water then dry	water then dry	dry
3	May	25/15	always wet	water then dry	dry	dry
4	May	25/15	always wet	water then dry	water then dry	water then dry
5	June	30/15	always wet	water then dry	dry	dry
6	June	30/15	always wet	water then dry	water then dry	dry
7	June	30/15	always wet	water then dry	dry	dry
8	June	30/15	always wet	water then dry	water then dry	water then dry
9	July	35/20	always wet	water then dry	dry	dry
10	July	35/20	always wet	water then dry	water then dry	dry
11	July	35/20	always wet	water then dry	dry	dry
12	July	35/20	always wet	water then dry	water then dry	water then dry
13	Aug	35/20	always wet	water then dry	dry	dry
14	Aug	35/20	always wet	water then dry	water then dry	dry
15	Aug	35/20	always wet	water then dry	dry	dry
16	Aug	35/20	always wet	water then dry	water then dry	water then dry
17	Sep	30/15	always wet	always wet	always wet	always wet
18	Sep	30/15	always wet	always wet	always wet	always wet
19	Sep	30/15	always wet	always wet	always wet	always wet
20	Sep	30/15	always wet	always wet	always wet	always wet
21	Oct	20/10	always wet	always wet	always wet	always wet
22	Oct	20/10	always wet	always wet	always wet	always wet
23	Oct	20/10	always wet	always wet	always wet	always wet
24	Oct	20/10	always wet	always wet	always wet	always wet

APPENDIX C

Protocol for preparing lithium chloride (LiCl) solutions to equilibrate seeds to specific moisture levels over a gradient of relative humidities (Gold and Hay, 2008; Hay et al., 2008). LiCl solutions of different concentrations were made by dissolving specified weights of LiCl crystals in 200 ml of deionized water at 20ºC. The amounts are as follow:

Relative humidity (%)	Weight of LiCl (g)
11	174
25	116
50	74
75	42
95	8

Each solution was added to a separate polycarbonate box (28 cm width x 28 cm length x 13 cm depth; Fibox Inc., Glen Burnie, MD), and paper envelopes containing seeds of *L. stylosa* from each population were placed on a plastic mesh supported above the LiCl solution. Containers were closed and sealed by the transparent clear polycarbonate cover. Seeds were incubated at 30ºC simulating summer temperatures for 16 weeks; after 16 weeks, boxes were opened, and after-ripened seeds were used in the experiment.

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APPENDIX D

Protocol for preparing polyethylene glycol (PEG-8000) solutions to create a water potential gradients. To make the desired water potential solutions, the following weights of polyethylene glycol (PEG-8000) powder were measured based on Michel (1983) and dissolved in 1000 ml of deionized water, using a magnetic stirrer to mix. The solution was added to petri dishes to saturate filter papers, and then the dishes were placed at 15°C or 23°C. The amounts are as follow:

Water potential (MPa)	Grams of PEG-8000/1000 ml	
	At 15°C	At 23°C
0.00 MPa	0.0000	0.000
-0.25 MPa	128.3227	133.490
-0.50 MPa	187.6705	195.523
-0.75 MPa	233.2992	243.228
-1.00 MPa	271.7964	283.478

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