LOCAL ADAPTATION AND FITNESS TRADE-OFFS.

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Biology – Doctor of Philosophy
Ecology, Evolutionary Biology, and Behavior – Dual Major

2017

ABSTRACT

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Adaptation generates and maintains genetic and phenotypic diversity. This is thought to occur due to trade-offs, where adaptation to one environment comes at a cost in another. Although trade-offs are believed to play a prominent role in the generation and maintenance of genetic and phenotypic diversity, the mechanisms by which adaptation leads to trade-offs are not well understood.

My research explores the forces that lead to adaptive trade-offs in two systems. First, using a RIL mapping population created from natural populations of *Arabidopsis thaliana*, I studied the genetic basis of flowering time, a putatively adaptive trait and one that differs between the parental populations. I identified flowering time QTL in growth chambers that mimicked the natural temperature and photoperiod variation across the growing season in each native environment and compared the genomic locations of flowering time QTL to those of fitness (total fruit number) QTL from a previous three-year field study.

In addition, I studied two populations of *Leptosiphon parviflorus*, an annual wildflower native to California. At Jasper Ridge biological preserve, populations of *L. parviflorus* grow on and off serpentine soil in close proximity. Due to its harsh growing conditions, serpentine soil exerts strong selective pressures on plants. Despite the close proximity of study populations (<100 m) and ongoing gene flow, reciprocal transplant studies demonstrate that these populations are locally adapted to their native soil types.

To determine the selective agents operating in both habitats and the forces underlying fitness trade-offs, I performed manipulative experiments in the field and greenhouse. Results from these studies show that both soil moisture and competitive interactions are important for mediating fitness differences among the populations, and adaptation to serpentine soil might result in a cost to competitive ability.

I also addressed the causes of flowering-time differences in these populations. Field reciprocal-transplant studies and watering manipulations in the greenhouse demonstrate the contribution of both the genotype and the environment to observed flowering-time differences. The plasticity of flowering time in response to soil type appears to be driven by differences in soil moisture. In addition, selection on flowering time was measured in both soil types across four years of study using a set of F5 advanced generation hybrids and found to differ among the habitats. Therefore, both selection and plasticity contribute to flowering-time differences between these populations and thus have likely played an important role in the initiation and/or maintenance of adaptive divergence in this system.

Finally, the two populations differ in their flower color, a Mendelian trait. Pollinators do not discriminate among flower colors and are unlikely to exert selection on this trait. Instead, flower color may be related to stress tolerance if the causal gene has pleiotropic effects on other traits. Using a set of Near Isogenic Lines (NILs), I found that the flower color locus has an effect on survival in field soil and fecundity in benign conditions. Ongoing work is aimed at addressing the mechanisms underlying the relationship between flower color and soil adaptation.

ACKNOWLEDGEMENTS

Many people contributed to the work presented here. Many fabulous lab technicians greatly aided experimental logistics: Mark Hammond, Nick Batora, Jon Spoelhof, Rachel Atchison, Jessie Eilers, and Inam Jameel. Publishing the *Arabidopsis thaliana* QTL study was done with the help of co-authors Chris Oakley, Jon Ågren, and Doug Schemske. The *Leptosiphon* greenhouse work would not have been possible without the enormous help from several fantastic undergraduate assistants: David Hart, Rebecca Champney, Anna Farrell, and Carolyn Graham. Field assistance on the *Leptosiphon* project was provided by Annette Potvin, Christina Feng, Evan Patrick, and Susan Gold. I greatly appreciate the support from Jasper Ridge Biological Preserve where I performed the field-work and am particularly indebted to Nona Chiariello for help with coordination and logistics, as well as Trevor Hebert who designed the soil moisture measurement system. I also want to thank my mother Cynthia Dittmar, Kristen Nolting and Heather Stadden who visited me in CA to help with field-work. The ideas and experiments written about here were often discussed with my brilliant lab-mate Carina Baskett, who also gave valuable feedback on ideas and writing. Thanks of course to my advisor, Doug Schemske, who constantly challenged and inspired me. Finally, the Plant Biology Department and the Ecology, Evolutionary Biology, and Behavior program both provided supportive environments, many friends, and made MSU a wonderful place to be a graduate student. This work was funded in part by a Doctoral Dissertation Improvement Grant through the National Science Foundation.

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KEY TO ABBREVIATIONS

QTL Quantitative Trait Loci

RIL Recombinant Inbred Line

JRBP Jasper Ridge Biological Preserve

NIL Near Isogenic Line

INTRODUCTION

Local adaptation is a major driving force underlying the origin and maintenance of biodiversity (Schluter 2001; Via 2001) and occurs when the spatial heterogeneity of a landscape causes divergent selection pressures on populations (Hedrick 1986). Implicit in the definition of local adaptation is the existence of fitness trade-offs, which occur when a population adapted to one environment suffers a fitness cost in another environment (Blanquart et al. 2013, Kawecki and Ebert 2004). Despite its importance, the mechanisms by which adaptation leads to trade-offs are not well understood and understanding the circumstances that determine whether adaptive traits result in fitness trade-offs between environments has been a central goal in studies of local adaptation (Kawecki and Ebert 2004). My dissertation investigates how several factors contribute to adaptive trade-offs: the interaction of selection and gene flow, the role of pleiotropy, the spatial scale of local adaptation, and the genetic basis of adaptive traits. Below, I outline these concepts and their relationship to adaptive trade-offs.

Characterizing the *genetic basis of adaptive traits* may help in the development of a mechanistic understanding of trade-offs. Since the modern synthesis of evolutionary biology, researchers have been interested in the number and effect sizes of mutations involved in adaptation (Orr and Coyne 1992) and whether adaptation commonly occurs as a result of a few mutations of large effect or many mutations of small effect. Empirical data to address the effect size of adaptive mutations has lagged behind theoretical predictions, which vary widely (Orr 1998, Kimura 1983, Fisher 1930). Most recently, Orr (1998) predicted that large-effect mutations may be favored during the early stages of adaptation when a population is far from its optimum. Some empirical data supports this

hypothesis (Barrett et al. 2008, Bradshaw and Schemske 2003), but the interpretation of this data has been controversial (Dittmar et al. 2016, Rockman 2011). The question remains, do large-effect loci contribute to adaptation and trade-offs in nature?

Several key pieces are often missing in studies concerning the genetics of adaptation. First, it is rarely known whether populations under study are locally adapted to their respective habitats. Although divergent populations are often assumed to be locally adapted, supporting evidence is found in less than half of studied cases (Hereford 2009). Second, the adaptive value of traits under study is rarely known. This evidence is crucial for developing a comprehensive understanding of the genes involved in adaptation. Finally, identifying the causal genes underlying adaptive traits is difficult. While studies often identify genomic regions underlying adaptive traits, because these regions may contain hundreds of genes, whether one or many causal genes are within these regions is still not known (Mackay et al. 2009).

Chapter 1 of my dissertation takes advantage of the model system *Arabidopsis thaliana* to study the genetic basis of flowering time in natural populations. While there are a wealth of studies on the genetic basis of flowering time in *Arabidopsis*, most of them use lab strains grown in artificial conditions, therefore making it unclear whether variation in flowering time genes detected in lab settings contributes to adaptation among natural populations. The system used here is a mapping population created by Doug Schemske and colleagues from a cross between natural populations of *Arabidopsis* from Sweden and Italy that were determined to be locally adapted to their native habitats (Ågren and Schemske 2012). Flowering time is one of the many traits that differentiates these populations and therefore may be under selection. A further advantage of this

system is that the genomic regions affecting fitness in the field were identified (Ågren et al. 2013). This allowed us to determine whether the genomic regions underlying flowering time contribute to fitness trade-offs between the habitats.

When the mechanisms underlying fitness trade-offs are investigated, adaptive traits are commonly found to exhibit conditional neutrality, i.e. they are adaptive in one environment but neutral in another (Anderson et al. 2013; Lowry et al. 2008). Conditionally neutral traits appear to cause trade-offs between environments when each population has acquired a different set of adaptive traits. However, this can only occur between populations that are not experiencing gene flow since adaptive traits that have no fitness costs would otherwise spread (Kawecki and Ebert 2004). Additionally, divergent populations may display trade-offs due to the random acquisition of genes that have adverse effects in an alternate environment (Futuyma and Moreno 1988).

While *Arabidopsis thaliana* is a good study system for the genetic dissection of adaptive traits, the geographic distance among the study populations and their self-fertilizing mating system increases the likelihood that divergence among them may be due in part to random processes. Therefore, to study how adaptive traits can directly contribute to fitness trade-offs and how divergence may be initiated in adjacent (= parapatric) populations, the remaining dissertation chapters (2-4) investigate local adaptation among adjacent populations of the self-incompatible California annual, *Leptosiphon parviflorus,* that experience ongoing gene flow. This system provides insight into the *spatial scale of selection and role of gene flow in adaptive divergence.*

Chapter 2 presents results from reciprocal transplant experiments conducted in the field and greenhouse to determine whether the populations are locally adapted to their

habitats despite their close proximity and ongoing gene flow. This study provides evidence of the strength of selection needed to initiate divergence among populations experiencing gene flow, and demonstrates that adaptive divergence can occur at a short spatial scale (<100 m.) Further, manipulative field and greenhouse studies provide insight into the selective factors that contribute to fitness trade-offs in these habitats.

Pleiotropy, i.e. multiple phenotypic effects of a single allele, plays a prominent role in the theory of adaptation. Fisher (1930) assumed that large-effect mutations will often have negative pleiotropic effects and this formed the basis for his prediction that only small-effect loci are likely to contribute to adaptation. Empirical evidence appears to support the hypothesis that large-effect loci are more likely to affect multiple traits (Wang et al. 2010, Albert et al. 2007, Wagner et al. 2008). However, while pleiotropic loci are generally expected to have deleterious fitness consequences, mounting empirical examples demonstrate the role of pleiotropic loci in local adaptation, perhaps even helping to facilitate rapid adaptation (Ferris et al. 2017, Smith 2015, Baxter et al. 2010, Albert et al. 2007). Further, pleiotropy may make an important contribution to adaptive divergence among populations. Pleiotropic mutations that are beneficial in one habitat may have an increased likelihood of having negative fitness effects in another habitat, and thus may play a disproportionate role in contributing to adaptive trade-offs. In addition, adaptive traits that have pleiotropic effects on reducing gene flow (e.g. "Magic traits", Servedio et al. 2011) can also make important contributions to adaptive divergence.

Chapters 3 and 4 investigate the role of putatively pleiotropic loci in adaptive divergence among these populations. In chapter 3, the forces contributing to variation in

flowering time among the populations were investigated and the strength of selection operating on these traits in both habitats was measured. Differences in flowering time among the populations may have been instrumental in contributing to their divergence by reducing the amount of gene flow occurring among them. There are many examples of flowering time differentiation among plant populations adapted to different edaphic environments, and may be why these systems often provide the best examples of divergence at small spatial scales.

Lastly, the role of flower color is addressed. This trait is highly differentiated among the populations. However, because this trait is not under pollinator-mediated selection, it may be related to some other stress tolerance pathway through pleiotropy. In Chapter 1, I examine evidence for differential survival and/or fecundity among pink and white-flowered advanced generation hybrids. In Chapter 4, I measure differentiation in the tolerance of the parental populations to high magnesium and low calcium and investigate whether the fitness of Near Isogenic Lines (NILs) that differ only at the flower color locus are significantly different in solutions with high concentrations of magnesium.

Understanding how local adaptation can lead to fitness trade-offs between environments contributes to our knowledge of the forces that generate and maintain biodiversity. The role of trade-offs has commonly been implicated in adaptive divergence and as an explanation for divergence in the face of gene flow. However, empirical evidence for traits that cause opposite fitness effects in different environments is rare, possibly due to the fact that local adaptation commonly leads to geographic separation and thus allows the accumulation of conditionally neutral traits. I have established a

unique ecological system that allowed me to investigate the forces underlying adaptive divergence in the face of gene flow. My dissertation work provides evidence of the strength of selection required for divergence in the face of gene flow and a first step towards understanding the pleiotropic mechanisms operating on flower color.

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CHAPTER 1: FLOWERING TIME QTL IN NATURAL POPULATIONS OF *ARABIDOPSIS THALIANA* AND IMPLICATIONS FOR THEIR ADAPTIVE VALUE

Introduction

Understanding the genetic architecture of adaptive traits is a goal of many evolutionary biologists. Although progress has been made in discovering the genetic basis of many phenotypic traits (MacKay *et al.* 2009; Alonso-Blanco and Méndez-Vigo 2014), whether causative QTL and/or genes have relevance to adaptation in native environments can only be addressed through studies of locally adapted populations and a demonstration of the adaptive significance of allelic variation (Feder and Mitchell-Olds 2003; Barrett and Hoekstra 2011; Anderson *et al.* 2014). Information on the genes underlying adaptation can provide insight into how commonly adaptation is associated with fitness trade-offs due to antagonistic pleiotropy at a single locus, or due to adaptive alleles that are unique to each habitat (Anderson *et al.* 2013). Furthermore, it is only through knowledge of the genes underlying adaptive traits that we can address the long-standing question of whether adaptation is commonly due to a few mutations of large effect (Orr 1998) or to many mutations of small effect (Fisher 1930); a question that remains unresolved (Rockman 2012).

The use of a model system such as *Arabidopsis thaliana* (hereafter *Arabidopsis*) has advantages for studying the genetics of adaptive traits, as information from its sequenced and extensively annotated genome increase the likelihood of identifying causal genes. In particular, the genetics of flowering time has received much attention in *Arabidopsis* (Srikanth and Schmid 2011) due partly to the fact that flowering time is expected to be subject to strong selection (Simpson and Dean 2002). Studies on other plant systems have shown that the timing of reproduction is often crucial for fitness, as flowering too early or too late could reduce reproductive success or increase mortality

due to drought (Sherrard and Maherali 2006) or cold temperatures (Inouye 2008; Munguia-Rosas *et al.* 2011). Furthermore, there is evidence that divergent selection on flowering time can contribute to local adaptation among populations (Hall and Willis 2006). Studies on *Arabidopsis* demonstrate latitudinal clines in flowering time across accessions (Stinchcombe *et al.* 2004) and selection on flowering time in some environments (Korves *et al.* 2007; Scarcelli *et al.* 2007; Li *et al.* 2010; Fournier-Level *et al.* 2013). Genes in the flowering time pathway that perceive and respond to environmental stimuli have been identified in *Arabidopsis* (Srikanth and Schmid 2011), such as FLOWERING LOCUS C (*FLC*) and FRIGIDA (*FRI*), both of which are affected by cold temperatures (Michaels and Amasino 1999; Johanson *et al.* 2000).

Despite the numerous studies that investigate the genetic basis of flowering time in *Arabidopsis*, there is surprisingly little evidence that these genes contribute to adaptation in natural populations. One approach towards this aim has been to examine patterns of variation in candidate genes. Among *Arabidopsis* accessions, correlations between latitudinal variation and allelic variation in candidate genes such as *FLC* and *FRI* have been found (Caicedo *et al.* 2004; Mendez-Vigo *et al.* 2011). Although these results demonstrate striking correlational patterns, experimental studies are better able to show causative links between flowering time genes and fitness. For example, Korves *et al.* (2007) planted 136 European *Arabidopsis* accessions in a common garden in Rhode Island and found that functional *FRI* alleles increased winter survival in a fall cohort and decreased fecundity in a spring cohort, although these effects depended on an interaction with *FLC*.

Studies that investigate candidate genes are appealing since we ultimately hope to identify the genes important in natural variation and adaptation. However, they also assume *a priori* that these are the primary genes underlying flowering time variation in natural populations. In contrast, both genome-wide association studies and quantitative trait loci (QTL) studies use markers that are distributed across the genome, and allow the identification of genomic regions that contain the causal loci due to their linkage disequilibrium with the markers. These studies therefore, make no *a priori* assumptions about the genes important for flowering time and adaptation. While association studies have the advantage of being able to examine allelic variation across large numbers of *Arabidopsis* accessions, extensive population structure makes it difficult to distinguish adaptive allelic variation from spurious associations between markers and traits (Zhao *et al.* 2007). Atwell *et al.* (2010) performed an association study on flowering time phenotypes among 199 genotypes and found an over-representation of *a priori* candidate genes within their peaks of association. However, the authors relied heavily on the presence of these candidate genes to differentiate true associations from false, since both selection and population structure can cause linkage disequilibrium among unlinked loci. In contrast, QTL mapping studies use experimental populations such as F_2 hybrids or recombinant inbred lines (RILs) in which recombination breaks up associations among alleles. Using 117 RILs derived from five mapping populations of *Arabidopsis*, Fournier-Level *et al.* (2013) found differential selection on flowering time genomic regions across four European common gardens.

Although QTL mapping is a powerful means of detecting the genetic basis of phenotypic variation, many QTL studies of flowering time in *Arabidopsis* have used

crosses involving the laboratory strains Landsberg (*Ler*) or Columbia (*Col*), (see Grillo *et al.* 2013, for a comprehensive review). These strains have early flowering phenotypes due to mutations that impair *FRIGIDA* (*FRI*) function (Johanson *et al.* 2000) and therefore, studies using *Ler* or *Col* as a mapping parent unsurprisingly often show that *FRI* has a large effect on flowering time. While these lab strains have provided crucial information on the biochemical pathways involved in flowering time, only QTL studies that use natural populations will provide insight into the genes that are important for natural variation in flowering time. Further, it is rarely known whether the populations under study are adapted to their local habitats and this is necessary for addressing questions about adaptive trade-offs and the genetic architecture of adaptive traits.

The current study takes advantage of a large mapping population created from natural populations of *Arabidopsis* from Sweden and Italy. An extensive reciprocal transplant study conducted with these populations provided the first evidence that native *Arabidopsis* are adapted to their local habitats (Ågren and Schemske 2012, Lowry 2012), and thus presents a unique opportunity to dissect the genetic variation that is relevant to local adaptation. In addition, recent studies to map fitness QTL in RILs grown in the native environments have identified many of the genomic regions that are important for variation in fitness in the field (Ågren *et al.* 2013). Here we use a set of 528 RILs from these two locally adapted populations of *Arabidopsis* from Sweden and Italy to map QTL for flowering time in simulated environmental conditions. We then compare the genomic location of our flowering time QTL to the location of fitness QTL from three years of field studies (Ågren *et al.* 2013) to determine whether flowering time QTL affect fitness and if they contribute to fitness trade-offs among sites.

There are two reasons to suspect that flowering time may be involved in local adaptation between the parental populations used in the current study. First, the study populations are located near the northernmost and southernmost margins of the native range of *Arabidopsis* in Europe and experience large differences in temperature and photoperiod which may contribute to geographic differences in selection on flowering time. Second, there is substantial genotype by environment interaction for flowering time, with the Italy population flowering 33 – 50 days earlier in Italy but just 3 days earlier in Sweden (Ågren and Schemske 2012).

Grillo *et al.* (2013) performed a study using F_2s from these mapping parents to investigate the genetic architecture of flowering time under laboratory conditions with and without vernalization. The current study builds on those results by using a large RIL mapping population, which allows greater precision in estimating flowering time through the use of replicate genotypes and presents the opportunity to compare flowering time QTL with fitness QTL that were recently mapped using the same set of RILs in the field (Ågren *et al.* 2013). In addition, we grew plants in growth chambers programmed to mimic the natural temperature and photoperiod fluctuations found during a typical growing season in *Arabidopsis* in Sweden and Italy (Figure 1). Many studies of the genetics and fitness effects of flowering time in *Arabidopsis* do not grow plants under the environmental conditions typical of the parental populations (Grillo *et al.* 2013; but see Li *et al.* 2006), despite ample evidence that the environment has a large effect on the identity of flowering time QTL (Brachi *et al.* 2010; Li *et al.* 2006; Weinig *et al.* 2002). Measuring flowering time under relevant environmental conditions is important for elucidating the QTL that are responsible for flowering time variation in native habitats

(Zuellig *et al.* 2014). Moreover, the dynamic changes among temperature and photoperiod across a growing season may be distinct from the fixed environmental conditions that are often used in laboratory studies (Li *et al.* 2006). The use of growth chambers that mimic the range of variation in temperature and photoperiod conditions experienced in the field allows us to isolate the effects of these environmental factors believed to play a large role in flowering time variation in *Arabidopsis* without the statistical noise of microhabitat variation in soil moisture, herbivores or pathogens.

We address the following questions: 1) What are the number and effect sizes of QTL underlying flowering time under simulated environmental conditions? 2) Do these QTL co-localize with known flowering time genes? 3) Does the identity of flowering time QTL differ between plants grown in simulated Sweden and Italy environments? 4) Do flowering time QTL co-localize with genomic regions known to affect fitness in the field?

Methods

Field localities and RIL construction.

We focus on two locally adapted populations of *Arabidopsis* (Ågren and Schemske 2012); one in north-central Sweden (Rödåsen; N 62°48' E 18°12') and one in central Italy (Castelnuovo; 42°07' E 12°29'), that represent the northern and southern limits of the native range in Europe (Koorneef *et al.* 2004). Both populations exhibit a winter annual life history; seeds germinate in the autumn and overwinter as rosettes. Plants flower during March-April in Italy and May-June in Sweden (Ågren and Schemske 2012). Recombinant inbred lines (RILs, n = 528) were created by selfing F1

plants derived from a cross between an individual from the Swedish locality (♂) with an individual from Italy (♀) for nine generations. These RILs were genotyped for 348 SNPs that were evenly spaced across the five nuclear chromosomes of the Columbia physical map. For further details, see Ågren *et al.* (2013).

Experimental Setup

Approximately 40 sterilized seeds from each RIL and parents were sown on sterilized petri dishes with media consisting of Gambog's B-5© nutrient mix, Bacto© Agar, and ultrapure water. Dishes were wrapped in parafilm and cold stratified in the dark at 4°C for five days to break seed dormancy. Native populations in both Italy and Sweden experience cold periods at or below this temperature in the field during germination. Afterwards, the dishes were moved into a growth chamber with a constant temperature of 22°C, 16 hour days, and a photosynthetically active radiation (PAR) level of 125 μmol $m^{-2}s^{-1}$ using a combination of fluorescent and incandescent lights. The dishes were randomized throughout the chamber every day.

After 8-10 days in the chambers, seedlings were transplanted into 5.3 cm. long tubes filled with a 1:1:1 mixture of sure-mix, perlite, and vermiculite. Seedlings were then returned to the chamber for another 8 days before randomizing replicates from each RIL across six 75 cm x 70 cm plastic trays. We programmed two specialty chambers designed to hold sub-freezing temperatures (BioChambers Inc. Model# GC-20) to mimic the natural photoperiod and the range of temperatures of the Swedish and Italian sites (Figure 1). The programs were based on photoperiod data from the U.S. Naval Observatory and field temperatures that were recorded directly at the parental sites (see

Ågren and Schemske 2012) once each hour from Nov ember 2003 to July 2008, with a HOBO Temperature Data Logger (HOBO Pro Data Logger Series® H08-031-08). We recorded air temperatures about 30 cm above the ground and soil temperatures approximately 1 cm below the soil surface. Since *Arabidopsis* spends its early life history near the soil as a rosette, but is also exposed to air temperatures after bolting, we incorporated minimum and maximum temperatures from both the air and soil measurements to establish the chamber conditions. To simulate the pattern of variation experienced by seedlings in a typical year, temperatures in the growth chambers were varied on a 24-hour cycle and were calculated by averaging the absolute minimum and maximum temperatures between air and soil for randomly chosen days across the season. Temperature data loggers (U14 LCD) were used to record the temperature settings in the growth chambers in the Sweden experiment to verify that the chambers were holding the programmed temperatures.

The chamber regime corresponded to the growing season of *Arabidopsis*; September-June in Sweden, and October-April in Italy. This regime approximately matched the number of days of the life cycle (germination to seed production) for the Italy environment (148 days, Figure 1). However, due to space and time constraints, and because specialty chamber routinely malfunction at subzero temperatures, the Sweden environment was shortened by compressing its natural life cycle of 284 days to 142 days in the chamber, such that every two days in the field became 1 day in the chamber (Figure 1). Despite not corresponding to equal numbers of days in the field for the Sweden environment, our goal was to capture the range of variation experienced by seedlings across their life cycle in Sweden.

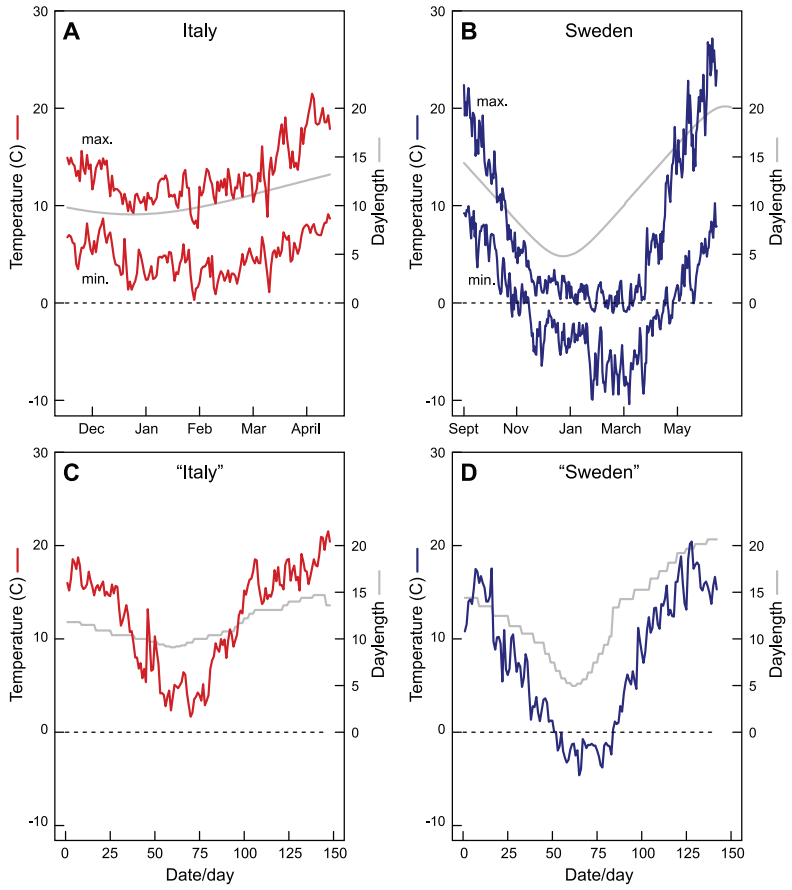

Figure 1. A comparison of field temperatures (A, B) and growth chamber temperatures (C, D). Field data were recorded from both the air and the soil over four growing seasons at the native sites in Italy (A) and Sweden (B). The colored lines represent the means of the absolute minimum and absolute maximum temperatures recorded from each day across the four growing seasons. Photoperiod is represented by the gray line and data are taken from the U.S. Naval Observatory. The bottom two panels display the temperature and photoperiod regime programmed into the chamber for each day in the Italy (C) and Sweden (D) conditions.

Six and eight seedlings from each RIL w ere used for the Italy and Sweden conditions, respectively, as well as 200 of each parent for both conditions. We used more replicates in the Sweden experiment due to the increased mortality expected from freezing damage in Sweden conditions. To compensate for having fewer plants, extra plants were used as spacers in the Italy treatment so that the density of plants remained constant between treatments.

The trays were watered with deionized water and ½ strength Hoagland's solution as needed. Every 3 days, trays were randomized both within and between the chambers until plants began flowering. To avoid damaging inflorescences, randomization was ceased when plants began to flower and during freezing in the Sweden conditions. Preliminary analyses suggested that the effect of tray explained a relatively small amount of the variation compared to the effect of line (0.3% vs. 61.5% in the Italy environment; 7.0% vs. 30.1% in the Sweden environment) and therefore was not used as a covariate in the final analysis.

In the Swedish environment, there was high mortality and tissue damage during freezing conditions. We quantified percent tissue damage using digital photographs taken before and after freezing conditions, in order to determine the extent to which variation in flowering time among genotypes was influenced by differences in tissue damage. However, analyses suggest that damage explained a relatively minimal amount of the variation in flowering time (2%) and will not be discussed further. In both environments, plants were censused every day, and date of first flowering was recorded when the first petals became visible.

QTL analysis

For each RIL in each environment we calculated the mean time to first flower. RILs that had fewer than three individuals survive to flower in the Swedish conditions were excluded from the analyses. Of the 528 lines planted in the Sweden experiment, 293 lines had three or more individuals survive to flower and were used in the analysis. We chose a minimum of three replicates per RIL as the best compromise between obtaining

RIL mean estimates averaged over multiple trays, and having a sufficient number of RILs for QTL mapping. A preliminary analysis with a minimum of two replicates per RIL surviving to flower yielded similar results to our final dataset with the exception of a loss of the small-effect QTL on chromosome 2 (not shown). It was expected that the flowering time of genotypes with high survival in Sweden would, on average, flower later in Italy than genotypes that were excluded from the Sweden analysis due to low survival, but the genotypes excluded from the Sweden analysis actually had greater average flowering times in Italy than the genotypes included in the Sweden analysis (62.1 days to 60.7 days, respectively; $p<0.0001$). In the Italy analysis, all of the 525 lines planted were used, and this included all but three of the 293 lines (1%) used in the analysis for the Sweden conditions.

QTL mapping for mean time to first flower in each environment was conducted using R/qtl (Broman *et al.* 2003) and Haley Knott regression. To calculate thresholds for incorporating additive QTL and epistatic interactions at experiment wise $\alpha = 0.05$, 10,000 permutations were performed with an automated stepwise model selection scanning for additive and epistatic QTL at each step (Manichaikul *et al.* 2009). We then fit the refined model with ANOVA to calculate the effect size and percent variance explained for each QTL. Because the automated stepwise procedure is sensitive to departures from normality, we first transformed the data by quantile normalization (Broman and Sen 2009). We then fitted this model with the non-normalized data to generate allelic effect sizes on the raw scale, which were subsequently multiplied by two to produce genotypic effect sizes for the alternate homozygotes.

Stepwise QTL analyses can sometimes result in spurious QTL that are artifacts of reduced recombination between adjacent markers. (Broman and Sen 2009). A manual inspection of our data revealed two QTL at adjacent markers on Chromosome 1 in the Italy conditions, one of which was spurious and driven by a single recombinant genotype. In this case we refitted a model with only a single QTL at this position. Between the two environmental conditions used in the current experiment, QTL were deemed to be the same if their 95% credible intervals were each less than 15.2 cM and they overlapped with each other (Ågren et al 2013).

To identify likely candidate genes within the 95% credible intervals of our flowering time QTL, we used datasets of gene annotations and genomic locations downloaded from the GO slim file (ver. 9 GFF) from TAIR (The *Arabidopsis* Information Resource). We filtered the list of genes to those containing "flowering" or "vernalization" in their "GO" terms and those for which there was experimental evidence that the gene influenced flowering (direct assay, mutant phenotypes, expression patterns, or genetic or physical interactions). Finally, we filtered this list of genes to include only those in which the start position occurred within 300 Kb (~1 cM, the average distance between markers) of the ends of the 95% credible intervals of our flowering time QTL. We did not search for candidate genes under QTL with very wide credible intervals, defined here as greater than 1/4 of the smallest chromosome (15.2 cM).

Co-localization of flowering time and fitness QTL

We compared the genomic location of flowering time QTL found in the current study to that of fitness QTL found in the field as reported in Ågren *et al.* (2013). In brief,

in three consecutive years (2009-2011), Ågren *et al.* (2013) planted seedlings of 398 RILs and the two parents into experimental gardens located at the sites of the source populations. For each site-year combination, cumulative fitness (total fruits per plant) was quantified, and QTL mapped. They identified a total of 15 distinct QTL, of which 10 were shared between sites. See Ågren *et al.* (2013) for further details.

The genomic locations of the flowering time QTL and fitness QTL were compared to determine whether they co-localize to the same genomic position. As far as we are aware, there is no standard quantitative approach for evaluating co-localization of QTL, particularly from multiple QTL models. Weinig *et al.* (2002) considered two or more QTL to co-localize if the likelihood ratio (LR) test statistic remained above the significance threshold between the two point estimates. However, it is possible for two adjacent, large effect QTL to lead to this pattern as well. Leinonen *et al.* (2013), considered QTL overlap significant if both QTL peaks overlapped with the credible intervals of one another, although in some cases credible intervals can be quite large. Huang *et al.* (2010) conducted multiple-trait composite interval mapping to evaluate the probability that more than one trait are due to a pleiotropic locus. Power for this method requires that sufficient recombination between the point estimates of adjacent QTL has occurred in the mapping population. More work is needed to establish guidelines for statistically determining whether QTL from different studies map to the same locus.

In the absence of consistent methods, we used two different criteria for evaluating co-localization of flowering time QTL and fitness QTL. The most stringent criteria for co-localization required that the point estimate of the flowering time QTL was within the range of the point estimates of unique fitness QTL identified in different years, and that

the flowering time QTL credible interval was < 15.2 cM (less than ¼ the length of the smallest chromosome) or that the range of point estimates of flowering time QTL that were shared between environments overlapped the range of point estimates of fitness QTL. The less stringent criteria required that point estimates for flowering time QTL were within the range of point estimates for fitness QTL without regard to the size of credible intervals.

Results

Flowering time phenotypes

Sweden parents flowered later than Italy parents in both environments, and flowering was delayed in the Sweden chamber relative to the Italy chamber (Figure 2). In the Sweden conditions, the average flowering time (defined as the number of days after

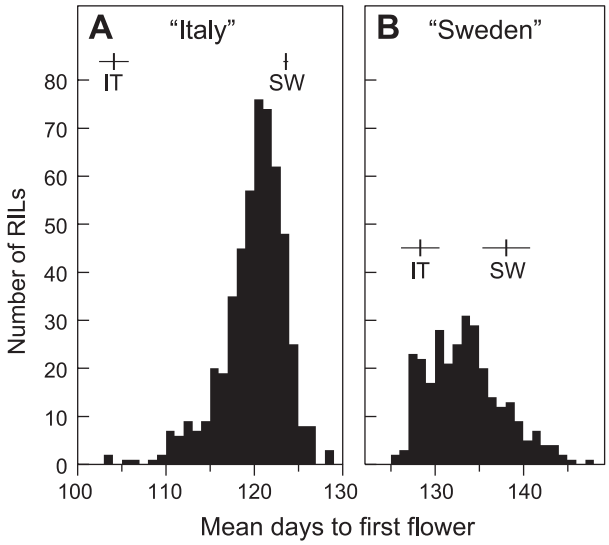

Figure 2. The distribution of RIL means for flowering time in environmental chambers simulating the temperature and photoperiod in Italy (A) and Sweden (B). 'IT' and 'SW' represent means and 95% confidence intervals.

transplanting) was 138, 128, and 132 days for the Sweden parents, Italy parents, and RILs, respectively, while in the Italy conditions the average flowering times were 124, 104, and 118 days for the Sweden parents, Italy parents, and RILs, respectively (Figure 2). There was a significant positive correlation between RIL mean flowering times between the two chamber environments ($r=0.50$, $p<0.0001$).

Genetic basis for flowering time

A total of nine QTL contributing to variation in flowering time were found in the Italy conditions and three QTL were found in the Sweden conditions. Two QTL were shared between environments (Figures 3 & 4), resulting in ten unique QTL (Table 1). The direction of the effect was the same in both environments for all QTL- the Italy genotype caused earlier flowering, while the Sweden genotype caused later flowering (Figure 5).The nine flowering time QTL found in the Italy conditions explained 61% of the difference between the parents, while the three flowering time QTL in the Sweden conditions explained 86% of the difference between the parents. The individual QTL with the largest effect on flowering time in both conditions was FlrT 5:1 (Table 1). Substitution of the Swedish genotype at this locus delayed flowering by 2.7 days in Italy and 3.8 days in the Sweden environment, which represents 14% and 39% of the parental difference in flowering times, respectively (Figure 5). Substitution of the Swedish genotype at the QTL with the next largest effect (FlrT 5:4, Table 1) delayed flowering in Italy by 2.6 days and 3.0 days in Sweden (13% and 30% of the difference between the parents, respectively). Substitution of the Swedish genotype at any of the QTL unique to the Italy environment would delay flowering by 0.7-1.3 days in Italy or 4%-7% of the

difference between the parents. A substitution of the Swedish genotype at the QTL unique to the Sweden environment delayed flowering by 1.6 days in Sweden or 17% of the difference between the parents (Figure 5; Table 1).

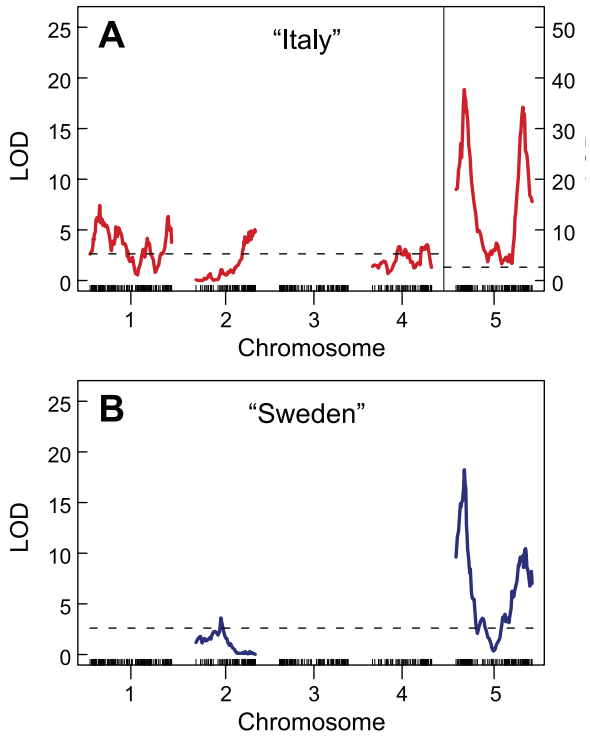

Figure 3. Stepwise LOD profiles produced from multiple QTL models (Broman and Sen 2009) for flowering time in Italy (A) and Sweden (B). Only profiles of significant QTL are shown. Note the difference in scale for chromosome five in Italy.

No epistatic interactions among flowering time QTL were detected based on the stepwise model selection procedure. Heat-maps showing strength (LOD) of pair-wise interactions among all loci do show some minor interactions, but these effects were very small when compared to the additive effects, and did not survive the model selection process.

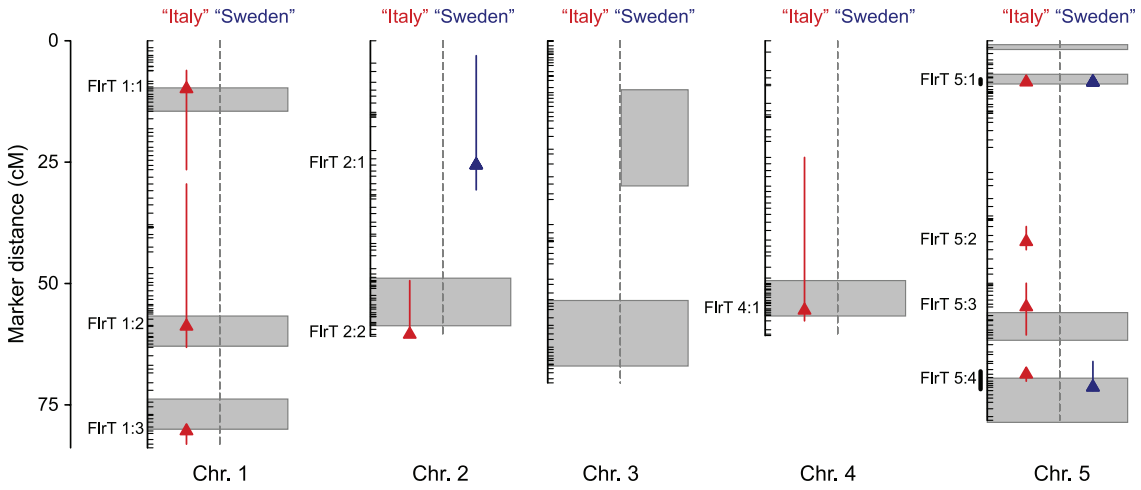

Figure 4. The genomic positions of flowering time QTL detected in growth chambers and fitness QTL detected in the field across each of the five chromosomes (vertical black lines with marker positions at tick marks). Arrows indicate flowering time QTL position and the direction of the effect of the Swedish genotype along with the 95% Bayesian credible intervals (red = Italy chamber, blue = Sweden chamber). Larger shaded boxes represent the range of point estimates from fitness QTL detected in more than one site x year combination in the field. When QTL were found in more than one environment (FlrT 5:1 and FlrT 5:4), the range of point estimates are indicated by dark lines next to the flowering time label name.

G x E interactions

Although we found one QTL unique to the Sweden environment and seven unique to the Italy environment, the two QTL with the largest effects were found in both experimental conditions. The reaction norms for the two chamber environments show that the environment causes a larger change in flowering time for the Italy parents relative to the Sweden parents (Figure 6). This is consistent with results found in field studies (Ågren and Schemske 2012). Flowering time was significantly affected by the interaction between chamber environment and genotype at the marker loci closest to four of the flowering time QTL: FlrT 1:2, FlrT 1:3, FlrT 5:2, and FlrT 5:3. In all cases, individuals with alternate alleles at these loci have larger differences in their flowering times in Italy conditions than Sweden conditions.

Table 1. Flowering time QTL and their chromosomal positions, LOD scores, and effect sizes expressed as the proportion of the difference between the parental flowering times, percent variance explained (PVE) and effect of the Swedish genotype.

Env.	QTL	Chr.	Pos.	LOD	% diff. b/w parents	PVE	Swedish genotypic effect (SE)
IT	FlrT 1:1	1	9.9	7.41	5.3	1.87	1.02 (0.19)
IT	FlrT 1:2	1	58.8	4.17	3.6	1.04	0.70 (0.21)
IT	FlrT 1:3	1	80.4	6.33	5.1	1.59	1.00 (0.21)
IT	FlrT 2:2	2	60.5	5.03	3.8	1.26	0.75 (0.19)
IT	FlrT 4:1	4	55.5	3.51	3.9	0.87	0.76 (0.20)
IT	FlrT 5:1	5	8.5	37.72	13.8	10.92	2.67 (0.21)
IT	FlrT 5:2	5	41.4	7.40	5.4	1.87	1.04 (0.24)
IT	FlrT 5:3	5	54.8	5.10	6.5	1.27	1.26 (0.28)
IT	FlrT 5:4	5	68.7	34.23	13.5	9.75	2.62 (0.24)
SW	FlrT 2:1	2	25.6	3.61	16.7	3.71	1.64 (0.41)
SW	FlrT 5:1	5	8.5	18.24	39.2	21.09	3.84 (0.41)
SW	FlrT 5:4	5	71.4	10.46	30.2	11.35	2.96 (0.41)

Candidate Genes

Candidate genes were found within several of the (<15.2 cM) flowering time QTL regions. Among the largest effect QTL, the flowering time gene Flowering Locus C (*FLC*) co-localizes with FlrT 5:1. Within the QTL region FlrT 5:4, the candidate gene *VIN3* was found within the range of point estimates and *VIP4* and *ELF5* were found within the credible interval of this QTL in the Sweden conditions.

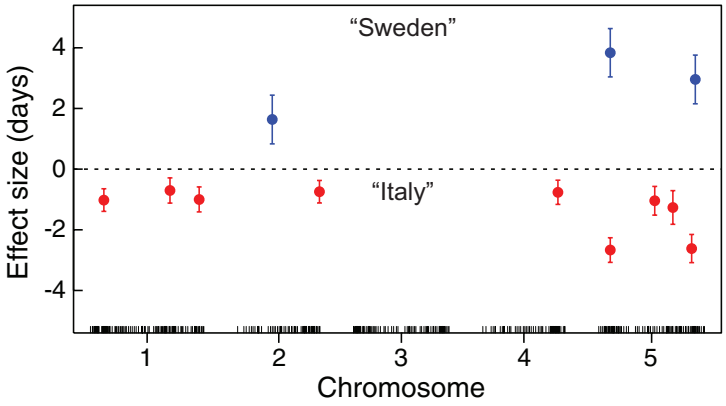

Figure 5. Effect sizes and 95% confidence intervals of the local homozygous genotypes for flowering time QTL identified in the two experimental environments. In all cases, the Italy genotype was associated with earlier flowering and the Swedish allele with later flowering.

Co-localization with fitness QTL

There was strong evidence for co-localization between fitness QTL and two flowering time QTL (Fig 4, Table 1). Both QTL FlrT 5:1 and FlrT 5:4 were found in both environments and overlapped with the point estimates of fitness QTL. Furthermore, these QTL had the largest effects on flowering time, and co-localized with the candidate genes described above. The point estimates in the two chambers for FlrT 5:1 were not only identical to each other, they were identical to the point estimate for a fitness QTL found in the field in Sweden in 2009 and within only 1 cM of a fitness QTL found in Italy in

2010 (See Figure 4; Table 1). This does not mean that we have identified the causal loci, but simply that despite recombination among markers in this genomic region, the same marker is the most closely linked with the causal loci in all of these instances. For this fitness QTL, the Italy genotype increased fitness in both Italy and Sweden.

Although the point estimates for FlrT 5:4 differed between the Italy and Sweden chambers, a likelihood ratio test comparing a two QTL model to a single QTL model (using the peak of the summed LOD profiles) indicated that the two QTL model did not offer a significant improvement over a one QTL model (χ^2=0.71, df=1, p=0.339), so we cannot reject that they are the same QTL. The range of point estimates between chambers for FlrT 5:4 also overlaps the range of point estimates for fitness QTL found in the field. For this fitness QTL, the Italy genotype increased fitness in Italy in all three years of study and the Swedish genotype increased fitness in Sweden in 2011 (Figure 4; Table 1).

Three flowering time QTL had point estimates within the range of point estimates for fitness in the field, but had confidence intervals larger than 15.2 cM: FlrT 1:1, FlrT 1:2, and FlrT 4:1 (Figure 4; Table 1). These QTL were unique to the Italy environment and co-localized with QTL for which the Italy allele increased fitness in Italy (in all three years for FlrT 1:2 and 4:1 and in 2010 for FlrT 1:1). However, due to the large credible intervals of these QTL, we are less confident about their chromosomal positions.

Discussion

Number and Effect Sizes of Flowering Time QTL

We found evidence for a relatively small number of QTL controlling flowering time in both experimental environments. The two QTL with the largest effects were also

shared between environments, and explained 13-14% and 30-39% of the difference between the parents in the Italy and Sweden environments, respectively. Using an F_2 population produced from the same parents as the mapping population in the current experiment, but grown under different experimental conditions, Grillo *et al.* (2013) also identified these QTL, which further supports their significant effects on flowering time in these populations. In addition to the two shared flowering time QTL, we found one QTL that was unique to the Sweden environment and explained 17% of the difference between the parents, while 7 QTL were unique to the Italy environment and explained between 4-7% of the difference between the parents.

The number of lines in the analysis for Italy is larger than that for Sweden due to increased mortality caused by freezing temperatures in the simulated Swedish winter. If we reanalyze the Italy data using only those lines that were included in the Sweden dataset, we lose the power to detect 2 QTL that were observed in the full Italy dataset and see a reduction in LOD scores. Although reducing the sample size by half reduced our power, we were still able to identify seven of the nine QTL from the original analysis. Even this smaller sample size (n=293) is large relative to other studies (Fournier-Level *et al.* 2013, Huang *et al.* 2010), as many previous QTL studies for flowering time have used mapping populations with <150 individuals (Grillo *et al.* 2013). Although we believe the use of a large mapping population such as ours allows adequate power to detect QTL of moderate effect, it is likely that small effect gene regions contributing to flowering time were not detected, and this may have inflated the estimation of the effects of QTL that were identified (Beavis 1998). Therefore, the ten QTL found in this experiment should be considered a minimum number. This is more than twice the number found on average in

previous studies. Among 98 QTL experiments on flowering time in *Arabidopsis*, the average number of QTLs identified for flowering time was four, with a range of 1-10 (Grillo *et al.* 2013).

Candidate Genes

Taking advantage of the well-studied flowering time pathway in *Arabidopsis* allowed the identification of several candidate genes for further investigation. The candidate gene *FLC* co-localizes with a large effect flowering time QTL found in both Italy and Sweden chamber environments (FlrT 5:1). Active *FLC* alleles repress flowering (Michaels and Amasino 1999) and vernalization reduces *FLC* expression to promote flowering (Sanchez-Bermejo *et al.* 2012). Natural variation in *FLC* has also been associated with flowering time variation in many *Arabidopsis* accessions from across its native range (Salome *et al.* 2011; Sanchez-Bermejo *et al.* 2012). *FLC* was also implicated in flowering time both with and without vernalization in the F_2 mapping population study (Grillo *et al.* 2013).

The *FLC* protein coding region was sequenced in the Sweden and Italy parents of our mapping population and no non-synonymous polymorphisms were found (Grillo *et al.* 2013). However, the cis-regulatory control of *FLC* has been supported by a number of studies. While Caicedo *et al.* (2004) identified two major *FLC* haplotypes that are differentiated by latitude among European accessions of *Arabidopsis*, no non-synonymous polymorphisms were found between these haplotypes. Instead, it appears that vernalization induces the expression of different alternatively spliced transcripts. In addition, very low non-synonymous diversity in *FLC* was found among 182 Iberian

Arabidopsis accessions, and polymorphisms were located mainly in the first intron (Mendez-Vigo *et al.* 2011). The lack of non-synonymous polymorphisms in *FLC* found across multiple studies strongly suggests that the causative allelic variation in this gene may be regulatory in nature.

Another candidate gene that co-localized with flowering time QTL in both the Italy and Sweden chamber is *VIN3*. Like *FLC*, this gene is located in the vernalization pathway, and acts to repress levels of *FLC* through recognition of the length and duration of vernalization (Sung and Amasino 2004). Allelic variation in *VIN3* may cause adaptive differences in the cold conditions that are required for sufficient *FLC* repression to allow flowering to occur. In the study by Grillo *et al.* (2013), this gene also co-localized with flowering time QTL found in the vernalization treatment. Unlike *FLC*, there is evidence for nonsynonymous polymorphisms between the two parental lines in this gene. Grillo et al. (2013) found two single base pair substitutions as well as a three base pair indel that result in different amino acids between the parents.

We did not find evidence for the importance of *FRI* in these populations, which contrasts with many studies that have identified *FRI* as a major determinant of flowering time in *Arabidopsis* (reviewed in Grillo *et al.* 2013). Many QTL studies of the genetic basis of flowering time in *Arabidopsis* have used lab strains chosen for their rapid flowering and nonfunctional *FRI* alleles (Alonso-Blanco and Méndez-Vigo 2014). Although *FRI* may be an important component of the flowering time pathway, we did not find that allelic variation in *FRI* contributes to natural variation in flowering time among the populations in our study. Ultimately, understanding the genes that contribute to natural variation in flowering time across *Arabidopsis* populations can only be evaluated

through studies that use natural populations, and these genes may not necessarily be the same genes found to be important for flowering time variation in lab strains.

G x E interactions on flowering time

Genes that regulate flowering are often involved in complex biochemical pathways that perceive environmental stimuli (e.g. vernalization and photoperiod) and initiate flowering (Simpson and Dean 2002). If different genes respond to different environmental cues, we would expect to identify unique flowering time QTL in each environment. Many QTL studies of flowering time in *Arabidopsis* have identified distinct QTL under different experimental conditions (Weinig *et al.* 2002; Li *et al.* 2006; Kover *et al.* 2009; Brachi *et al.* 2010). However, the two largest-effect QTL identified in the current study were shared between environments. Therefore, the genes underlying these QTL may be involved in multiple biochemical flowering time pathways or operate independently of the environment. Interestingly, none of the candidate genes that co-localize with flowering time QTL from the Sweden chamber are part of the photoperiod pathway. This may be due to the longer duration and stronger intensity of cold temperatures in the Sweden conditions, and therefore the signals in this treatment may override photoperiod signaling. To verify that temperature and photoperiod are more important than other, microhabitat variables in regulating flowering time, future studies will measure QTL for flowering time in the field to determine whether the same QTL are observed.

Fewer QTL were detected under Swedish conditions than Italian conditions. A re-analysis of the Italy chamber dataset using the same subset of lines used in the

Sweden chamber analysis found five of the seven QTL unique to the Italy conditions even with the reduced number of RILs. Therefore, the greater number of flowering time QTL in the Italy chamber does not appear to be solely an artifact of sample size. Instead, the greater range of phenotypic variation in flowering time observed in the Italy conditions may make it easier to detect minor effect QTL. Furthermore, the Sweden conditions may represent saturated vernalization conditions that could normalize flowering time among different genotypes and reduce or remove the contribution of some genes as a result. Strange et al. (2011) found that some QTL that had large effects on flowering time without vernalization had no effect when vernalization was saturated.

Co-localization of flowering time QTL and fitness QTL from the field

The two largest effect flowering time QTL found in both experimental conditions co-localize with fitness QTL and have tight credible intervals (Figure 4). For one of these, (FlrT 5:1), the Italy genotype is favored at both field sites (Ågren et al 2013), despite the fact that the Italy genotype decreases flowering time and the Sweden genotype increases flowering time. There are several possible explanations for why the late-flowering local genotype may be maladaptive in Sweden. First, field studies demonstrate that differences in parental fitness in Sweden are largely attributable to differential survival between the populations, not fecundity (Ågren and Schemske 2012). Therefore, early flowering may increase fecundity in Sweden as long as individuals survive the winter. In addition, recent climate warming in Sweden (Kullman 2001) may have increased the fitness of southern genotypes. In fact, winter survival of the Italian genotype in Sweden increased with higher minimum winter temperatures (Ågren and

Schemske 2012). Therefore, increased winter survival whether due to climate change or the presence of local alleles at other loci, may confer fitness advantages to early flowering in Sweden. Finally, Ågren *et al.* (2013) found that the local genotype was maladaptive in Sweden for several fitness QTL and suggest that weaker selection against non-local genotypes or genetic drift due to small effective population sizes in Sweden may have increased the chances for maladaptive alleles to become fixed.

The other flowering time QTL found in both conditions (FlrT 5:4) co-localizes with a QTL that exhibits a fitness trade-off, with the Italy genotype increasing fitness in Italy and decreasing fitness in Sweden (Figure 4). In a study of the mustard *Boechera stricta*, Anderson *et al.* (2013) also found evidence for a fitness trade-off that mapped to the same location as a known flowering time QTL detected in a growth chamber experiment. Flowering time genes may result in fitness trade-offs if there is differential selection on flowering time in different habitats or if flowering time has pleiotropic effects on other traits that affect fitness. There is evidence that selection on flowering time differs across the native range of *Arabidopsis* (Fournier-Level *et al.* 2013), and differences in climate between Sweden and Italy suggest that divergent selection on flowering time may be expected. However, studies of *Arabidopsis* and other taxa also find evidence that flowering time genes can have pleiotropic effects on traits such as water use efficiency *(Arabidopsis*; Lovell *et al.* 2013; *Brassica rapa;* Franks 2011), vegetative biomass (*Avena barbata*; Latta and Gardner 2009), and size at reproduction (*Brassica rapa*; Haselhorst *et al.* 2011). Scarcelli *et al.* (2007) found that the candidate flowering time gene *FRI* exerted a negative pleiotropic effect on fitness in *Arabidopsis* through a reduction in the number of branches. To further investigate whether flowering

time contributes to fitness trade-offs between these populations, future studies will grow Near Isogenic Lines (NILs) with flowering time QTL introgressed into the parental backgrounds in native habitats. Flowering time and fitness of these NILs will be measured relative to parental lines to determine the effects of these regions alone on both flowering time and fitness in the field and to examine evidence for fitness trade-offs caused by individual loci.

There is evidence to suggest that three of the eight QTL not shared between environments (seven unique to the Italy environment), co-localize with fitness QTL (Figure 4). In all cases, the Italy genotype increased fitness in its native environment. Between these three QTL and the two that were shared among environments, we observe a total of five instances where a flowering time QTL found in the Italy environment co-localizes with a fitness QTL in which the Italy genotype increases fitness. By comparison, we observe two instances where a flowering time QTL found in the Sweden environment co-localizes with a fitness QTL, and in only one of these does the Swedish genotype increase fitness. These results indicate that differences in flowering time may be more important for local adaptation in Italy than in Sweden. Field studies on the parental populations demonstrated that freezing tolerance likely plays a large role in local adaptation at the Swedish site and therefore, flowering time may have a relatively smaller contribution to fitness in Sweden than in Italy (Ågren and Schemske 2012). Conditional neutrality may be expected for flowering time if it is under selection in only one environment, or if, as is observed here, some genes only affect flowering time in one environment. This was observed in *Arabidopsis lyrata*, where loci that only affected

flowering time in one environment were favored in that environment, but conditionally neutral in the other (Leinonen *et al.* 2013).

Ultimately, we hope to uncover the genes underlying flowering time as well as other adaptive traits in these populations of *Arabidopsis*. Doing so will allow us to evaluate whether individual genes contribute to fitness trade-offs between these environments (antagonistic pleiotropy) or whether they are conditionally neutral. Furthermore, knowledge of the genes contributing to adaptation in native populations provides insight into the genetic architecture of adaptation and whether adaptation is commonly a result of changes in a few genes of large effect (Orr 1998) or many genes of small effect (Fisher 1930). The current study identifies candidate flowering time genes such as *FLC* and *VIN3* that are strongly implicated in local adaptation in native populations of *Arabidopsis*. Identification of these genomic regions in conditions typical of the parental habitats, and the co-localization of the associated flowering time QTL with fitness QTL from the field is a significant step towards identifying the genetic basis of adaptation in this system.

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CHAPTER 2: LOCAL ADAPTATION AND FITNESS TRADE-OFFS IN THE CALIFORNIA ANNUAL, *LEPTOSIPHON PARVIFLORUS*

Introduction

Environmental heterogeneity often results in local adaptation (Hereford 2009) when populations evolve traits that confer greater survival and/or reproduction in their native habitat relative to foreign populations (Kawecki and Ebert 2004). When phenotypic optima vary among habitats, this process can lead to divergence (Walter et al. 2016; Hall and Willis 2006). The likelihood of such adaptive divergence depends on the strength of selection, level of gene flow, proximity of populations, and the existence of fitness trade-offs. Knowledge of how these factors affect population divergence is therefore important for understanding the origins of biodiversity.

As a homogenizing force, gene flow opposes divergence. Theory predicts that for divergence to occur under even moderate levels of gene flow, selection must be relatively strong in both environments (Endler 1973). This requires that adaptive traits have opposite fitness effects across environments, as might be expected if adaptation leads to trade-offs, when an adaptive response to one selective factor is linked to a detrimental response to another selective factor (Stearns 1989). Traits without costs in alternate habitats (i.e. conditionally neutral), will spread among interbreeding populations (Kawecki and Ebert 2004, Anderson et al. 2011) and therefore can only contribute to population differentiation in the absence of gene flow.

Despite the vital role trade-offs are expected to play in the initiation of divergence among interbreeding populations, empirical studies often lack evidence for them. Less than half of populations studied in reciprocal transplant experiments show evidence of costs associated with adaptation (Hereford 2009). Further, studies that examine the fitness effects of adaptive traits commonly find conditional neutrality, i.e. traits adaptive

in one environment are neutral in another (Anderson et al. 2011). Whether such empirical studies reflect a true lack of adaptive trade-offs or whether the detection of trade-offs is dependent on the spatial and/or temporal scale of study is still an open question. For example, trade-offs may be difficult to detect in short-term studies if selection changes across years (Siepielski et al. 2009). In *Boechera stricta*, the adaptive advantage of local phenotypes along an altitudinal gradient could only be detected by integrating fitness data across multiple years (Wadgymar et al. 2017). Similarly, among populations of *Arabidopsis thaliana*, the fitness disadvantages of southern populations in northern habitats depend on winter temperatures (Agren and Schemske 2012). Most reciprocal transplant studies are conducted in a single year (Gibson et al. 2016; Hereford et al. 2009), which may be insufficient to capture a population's long-term evolutionary advantage in its native habitat.

Evidence for divergence with gene flow has been found in a wide range of taxa that show elevated phenotypic differentiation relative to neutral genetic variation among populations (Cheng et al. 2012, Pespeni and Palumbi 2013, Linnen et al. 2009, Rosenblum 2006; Saint-Laurent et al. 2003; Schneider et al. 1999; Smith et al. 2001). However, the spatial scale of these studies range from ten to several thousand kilometers. With an environmental gradient that varies linearly across a large spatial scale, population differentiation is expected to occur under a wide range of conditions, simply because locally adapted phenotypes will be spatially segregated (Doebeli and Dieckmann 2003, Lenormand 2002). In contrast, phenotypic differentiation is less likely when steep environmental gradients lead to the close geographic proximity of different phenotypic optima (Doebeli and Dieckmann 2003). The minimum spatial scale over

which adaptive divergence can occur is therefore still an open question (Richardson et al. 2014).

Some of the best examples of local adaptation across small spatial scales include studies of plant populations adapted to different soil types. Soil characteristics such as nutrient content (Xu et al. 2014), salinity, substrate (Bennington et al. 2012; Ellis and Weis 2006), soil water content, the presence of heavy metals, or cation composition (Yost et al. 2012, Sambatti et al. 2007) often vary over short geographic distances (Brady et al. 2005) where gene flow across these soil gradients is likely. Differentiation among plant populations adapted to different edaphic environments is often attributed to strong selection in each habitat type, making them good systems for investigating the mechanisms that contribute to trade-offs.

Serpentine soil represents an extreme edaphic environment that is formed naturally from the weathering of ultramafic rocks (Kruckeberg 2002) and is characterized by low calcium to magnesium ratios, high levels of heavy metals, and low water holding capacity (Brady et al. 2005). Because these habitats are often colonized by unique plant species, serpentine soils in California contribute disproportionately to the floristic diversity of the region (Brady et al. 2005). High levels of endemism in serpentine-adapted species are believed to result from a trade-off between serpentine tolerance and competitive ability, which effectively limits serpentine-adapted species from colonizing more benign habitats (Anacker 2014, Brady et al. 2005).

Although a trade-off between stress tolerance and competitive ability is often proposed as a general mechanism maintaining plant species diversity and coexistence, (Grime 1974, 1977), the evidence for a competitive disadvantage associated with

serpentine adaptation is mixed (Anacker 2014, Burge et al. 2014). Classic greenhouse experiments using the serpentine endemic *Streptanthus* found that its growth was negatively impacted on benign greenhouse soil when competitors were present (Kruckeberg 1954). However, a greenhouse study on closely related species of *Cirsium* found no evidence for reduced competitive ability in the serpentine endemic *C. fontinale*, relative to its widespread, invasive congener, *C. vulgare* (Powell and Knight 2009). Similarly, ecotypes of *Achillea millefolium* living on adjacent serpentine and non-serpentine soils also had no difference in competitive ability in greenhouse studies that manipulated density (Higgins and Mack 1987). Some of these discrepancies may be a result of studying competitive interactions in the greenhouse, which can impact the structure and texture of serpentine soil relative to natural conditions (Wright et al. 2006, Moore and Elmendorf 2011). Further, precipitation patterns may mediate the effects of competitive interactions across edaphic environments (Fernandez-Going and Harrison 2013, Anacker and Harrison 2012). Studies that manipulate both competition and water availability in natural settings are needed to understand whether serpentine adaptation leads to trade-offs in competitive ability (Anacker 2014, Moore and Elmendorf 2011).

The current study investigates local adaptation at a small spatial scale and in the presence of gene flow, using a pair of adjacent populations of *Leptosiphon parviflorus*, an annual, self-incompatible wildflower native to CA. At Jasper Ridge Biological Preserve (JRBP) in San Mateo County CA (USA), populations of *L. parviflorus* grow on serpentine soil and sandstone soil in close proximity (<100 m.; figure 7). These soils differ in a number of characteristics including their levels of calcium and magnesium (figure 8). Interestingly, the population on serpentine at JRBP has exclusively pink

flowers, while the sandstone population has almost exclusively white flowers. Crossing studies reveal that this flower color polymorphism is controlled by a single locus.

Figure 7. Aerial view of the locations of the sandstone (left, white-flowered) and serpentine (right, pink-flowered) study populations at JRBP. Circles indicate the approximate boundaries of the populations.

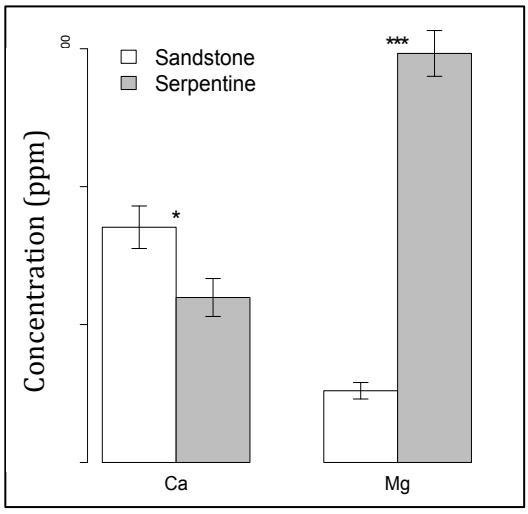

Figure 8. Concentration of Calcium and Magnesium measured on each soil type at JRBP.

Despite these differences in habitat type and flower color, there is evidence of gene flow among the study populations. Kay et al. (2011) used AFLP markers to determine that the average Fst between populations growing on the two different soils was 0.12, indicating that in most regions of the genome there is only moderate genetic differentiation between populations. The strong divergence in flower color among

populations over such a small spatial scale despite low genetic differentiation through most of the genome is consistent with the hypothesis that flower color plays a significant role in local adaptation to these habitats (Whitlock 2008).

Overall, this study 1) investigates whether local adaptation can occur at a small spatial scale and in the presence of gene flow; 2) estimates temporal variation in the magnitude of local adaptation, and 3) identifies the selective factors that contribute to adaptive trade-offs in these populations. To determine whether the populations are locally adapted to their respective soil types and whether trade-offs varied among years, four years of reciprocal transplant studies were performed in the native habitats of both populations. To determine whether variation in rainfall could mediate patterns of local adaptation, and if there was evidence of a trade-off between serpentine adaptation and competitive ability, the soil moisture and presence of competitors were manipulated on both soil types.

Methods

Local adaptation

Reciprocal transplant experiments were conducted at Jasper Ridge Biological Preserve (JRBP) in San Mateo county California during the spring growing seasons of 2012, 2013 2014, and 2015. Seeds from both populations were collected from >50 maternal plants grown in a common greenhouse environment. The maternal plants came from bulked collections of each population that had been maintained in the greenhouse through random intercrossing to minimize inbreeding. The same seed sources were used for all experiments described.

Seeds were germinated at the approximate time that seeds naturally germinate in the field (late December/early January). Seeds were surface sterilized and sown on petri plates containing a nutrient agarose medium. They were then placed in a cold (4°C) room at MSU in the dark for ten days, after which plates were put into an incubator set at 16 hour days, 22°C to encourage uniform germination and growth. After 7-10 days in the incubator cotelydons were visible on seedlings and plates were taken to JRBP.

Seedlings were first transplanted in randomized order into individual cells of plug trays (Hummert©, 12 x 24 cells) filled with serpentine or sandstone soil in a 4:1 mixture of field soil to perlite to allow soil aeration. This soil was collected haphazardly from disturbed gopher mounds in order to minimize disturbance to the grassland. An equal number of seedlings from each plate were transplanted into each soil type to minimize plate effects. Plate source was noted but later found to have no significant effect on fitness (data not shown). After transplanting, trays were placed in a sunny location outside the field station at JRBP and generously watered to allow seedlings to establish.

Seedlings were watered daily for one week and then seedling plugs were individually planted into field plots in the same randomized order, excluding seedlings that were weak or had died in the trays. Care was taken to minimize disturbance to the natural vegetation at each site to allow natural levels of competition. Transplanting was performed in the afternoon so that the seedlings were not exposed to a full day of sun immediately after transplant. To further minimize transplant shock, plots were covered with nylon tulle for 2 days after transplant and seedlings were watered for 1-2 weeks after transplant. This included a period of 7 days where watering frequency decreased before stopping completely. The length of watering depended on the temperature and rainfall in

that year (2013 and 2014 were years of extreme drought and high temperatures, and therefore plants were watered period longer in those years than in 2012 and 2015). Seedlings were censused ten days after transplant and any plants that died during this initial period were excluded from further analyses in order to remove any effects of transplant shock. Plants were censused every 2-3 days to record mortality and the day of first flowering. As plants began senescing, their lifetime production of flowers and fruits was recorded several times in case inflorescences were damaged in the interim. The largest recorded numbers of flowers and fruits were used for analyses. Fruits quickly dehisce and release seeds, but when possible, whole fruits were collected to count the number of seeds per fruit.

The performance of each parental population in each habitat was determined from several fitness components: survival to flowering, the number of fruits of survivors (hereafter fecundity) and total fitness (a composite measure of survival, number of flowers, and number of fruits). Analyses were conducted across years as well as within individual years to determine whether patterns of local adaptation varied.

Survival and fecundity were analyzed using generalized linear models (2012 and 2013) or generalized linear mixed effect models (2014 and 2015) all of which modeled survival and fecundity as binomial and Poisson distributed, respectively. Mixed effect models were used for 2014-15 because of the inclusion of a random effect of block nested within soil type, since replicate plots were planted in both soil types during those years. The four years of data were analyzed together using generalized linear mixed effect models that included a random effect of block (coded as one block for each soil type in 2012 and 2013). Least square means and standard errors were extracted from

these models to compare the performance of each population in each habitat across years. The day of transplant, field edge position, and tray edge position were determined to have no significant effects on fitness. Consequently, model results presented here do not include these factors.

Total fitness was analyzed using the statistical package ASTER (Shaw et al. 2008). ASTER models can incorporate multiple fitness components modeled with different distributions in a graph matrix framework where the value at each life stage predicts the next. Here, ASTER models included survival to flowering, number of flowers, and number of fruits, modeled as binomial, zero-truncated Poisson, and Poisson, respectively. All models tested the effects of population, soil and their interaction, on fitness.

Precipitation data were obtained from the JRBP weather station archive with records dating to 1983. In cases where rainfall data were not recorded, the data from the nearest weather station (Woodside Fire Station, Woodside, CA) were used. These data allowed a comparison of annual rainfall during the four years of study to that observed since 1983.

Watering treatments in the greenhouse

Greenhouse experiments were performed using soil collected from JRBP at both field locations as described above. Seeds were germinated using the same methods as described for the field experiment. After two weeks in an incubator, seedlings were transplanted to cone-tainers (Hummert©, RLC-4) filled with a mixture of sieved field soil (serpentine or sandstone) and perlite in a 4:1 ratio. To encourage establishment, seedlings

were top watered for 3 days after transplanting, after which plants were bottom watered only. Plants were initially grown in a haphazard arrangement for their first month of growth, after which they were randomly assigned to treatments. These treatments consisted of a full water treatment, where plants were continuously bottom watered throughout the duration of the experiment, or a drought treatment, where watering was ceased when the first plant began flowering, which occurred approximately one month after transplanting. This drought treatment was chosen to mimic the natural phenology in the field where plants begin flowering as the soil dries. Plants were censused every two days and the total number of flowers each plant produced was recorded at the end of the experiment. Because *L. parviflorus* is self-incompatible and relies on pollinators for fertilization, measuring fruit number is not relevant in greenhouse conditions. Instead, flower number, which is highly correlated with fruit number in the field (Pearson correlation coefficient=0.87, $p<0.0001$) was used as the measure of fitness.

In contrast to the field, plants in the greenhouse experiment had high survival (>85%). Therefore, the distribution of flower number was not zero inflated, allowing analyses to be conducted using the total number of flowers as the metric of fitness without incorporating a different distribution for survival as was employed with ASTER for the field experiment. The effects of source population, soil type, watering treatment, and their interactions on flower number were analyzed using generalized linear mixed effect models with a Poisson distribution. Tray was included as a random effect and treatments were replicated across trays but inclusion of tray nested within treatment and seed source (the time period seeds were harvested) did not contribute significantly to the

model fit and were not included. The least square means and pairwise significant differences were analyzed in a model that did not include non-significant terms.

A randomly chosen subset of plants from both greenhouse treatments were selected to estimate water use efficiency (WUE) using ^{13}C abundance . Samples were prepared by drying for 48 hours in a 60°C drying oven, then finely ground using a tissue homogenizer. Ground tissue was weighed and put into tin capsules before being sent to the UC Davis Stable Isotope Facility for testing. The same protocol was used on tissue from the 2014 field season. The resulting data were analyzed in a linear mixed effects model to determine the effects of soil type, population, and watering treatment on ^{13}C abundance. The model included a random effect of replicate nested within treatment nested within experiment (field vs. greenhouse).

Watering and weeding treatments in the field

An experiment evaluating the effects of soil moisture and competition was conducted in the field concurrently with the reciprocal transplant experiment in 2015. The fitness data reported above for this year come from the control (unmanipulated) plots. Each treatment was replicated in two locations on each soil type in order to minimize the influence of microhabitat variation on fitness measurements.

Seedlings were germinated and transplanted in the same manner as described for the reciprocal transplant experiment, except that field plots were randomly assigned to one of four treatments: control (unmanipulated), weeded, watered, and weeded + watered. A weeded + watered treatment was conducted to determine whether there was an interaction between the treatments. The plots assigned to the weeded or

weeded/watered treatment were prepared by removing all vegetation prior to seedling transplant and were maintained throughout the duration of the experiment. All seedlings were watered for three consecutive days after transplant, and then twice more over the next three days. Watering was performed by hand to minimize soil disturbance. At each watering, the soil surrounding the roots of each plant was saturated. Watering continued twice per week throughout the duration of the experiment for the plants assigned to the watering or weeding + watering. Survival to flowering and lifetime production of flowers and fruits was recorded for each individual.

The effect of population, soil type, watering treatment, weeding treatment and their interactions on survival, fecundity and total fitness were investigated using models that included a random effect of block nested within soil type. Survival and fecundity were analyzed using generalized linear mixed effect models following a binomial (survival) or Poisson (fecundity) distribution, while total fitness was analyzed using ASTER models that incorporated survival, total number of flowers, and total number of fruits. The significance of each model term was tested using a likelihood ratio test that compared the significance of models with and without individual terms. This process was iterative, such that insignificant terms were dropped from the full model and remaining interactions were then compared to the new model until only significant terms remained. The models that included only significant interaction terms were used to estimate the least square means (survival and fecundity) or total fitness (using ASTER's predict function). Tukey post-hoc tests for individual fitness components were used to test the significance of each pairwise interaction across treatments and soil types.

Results

Local adaptation

Across the four years of study, there was a significant population x soil type interaction for total fitness ($p<0.0001$) and evidence of local adaptation for each population on its native soil type (figure 9). However, the magnitude of local adaptation varied across years (figure 10). While the serpentine population consistently outperformed the sandstone population in its native habitat in all four years, the advantage of the sandstone population in its native habitat varied. In 2014, the sandstone population did not perform significantly better than the serpentine population in its native habitat and only had a small advantage in 2013.

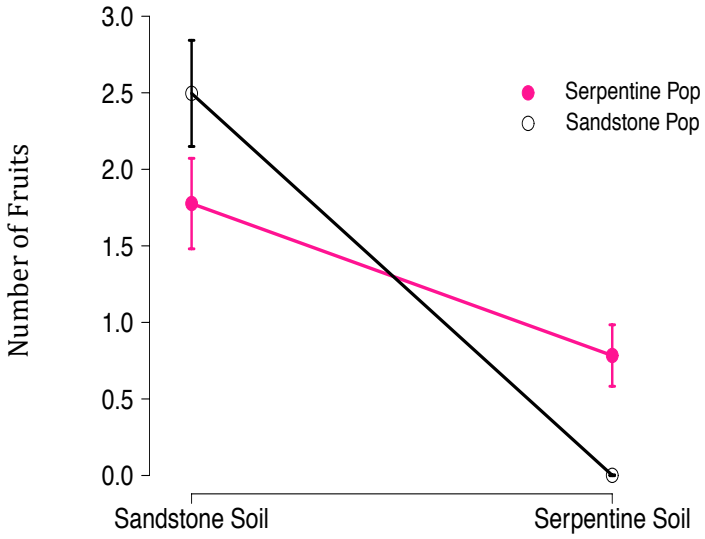

Figure 9. Fitness of the two parents in each soil type across the four years of study. Shown here are predicted results from ASTER (Shaw et al. 2008) models run separately for each soil type that incorporated survival, number of flowers, and number of fruits. Models included the fixed effects of year and spatial blocking factor.

The fitness components important for local adaptation differed among the soil types. On serpentine, the fitness advantage of the native population was due almost entirely to the differences in survival in this habitat (figure 10). Sandstone individuals

rarely survived to flower on serpentine. Across the four years of study, less than 1% of

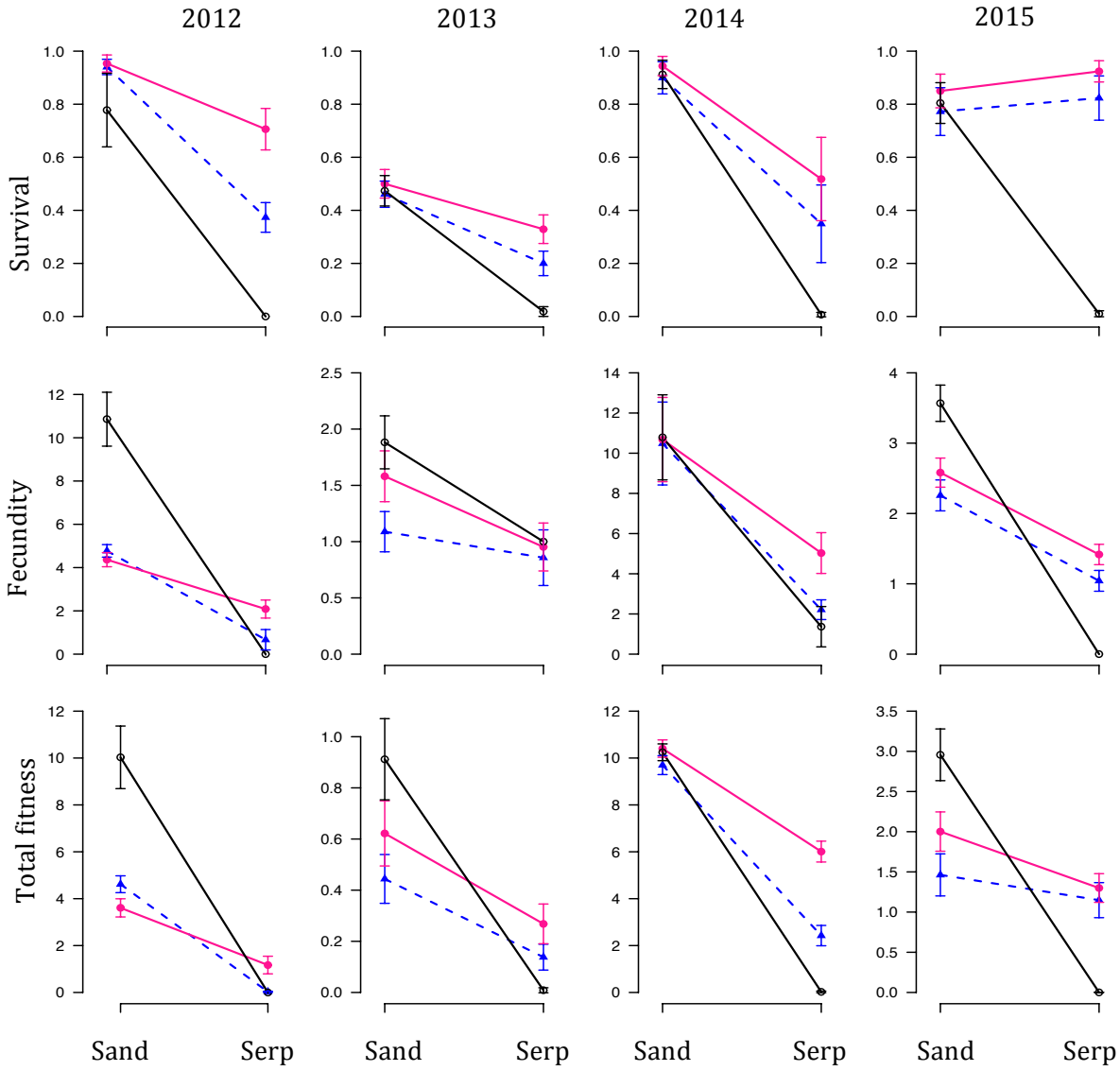

Figure 10. Survival, fecundity (number of fruits of survivors), and total fitness for each population in each soil type across the four years of study. Serpentine parents are indicated by the pink line (closed circles), sandstone parents are indicated by the black line (open circles), and F5 advanced-generation hybrids are indicated by the dotted blue line. Shown are the least square means +/- one standard error from generalized linear mixed models under a binomial distribution (survival) or poisson distribution (fecundity). Total fitness was estimated as the total number of lifetime fruits a plant produced using the predict function of the statistical program ASTER (Geyer 2015), in a model that incorporated survival, the number of flowers and the number of fruits each plant produced. Population x soil interactions were significant for survival in 2013 ($p<0.001$), 2014 ($p<0.0001$), 2015 ($p<0.0001$); fecundity in 2014 ($p<0.0001$); and for total fitness in all years ($p<0.0001$).

sandstone individuals survived to flower on serpentine, while the survival of the serpentine population on its native soil type ranged from 30-90%.

On sandstone soil, there were no significant differences in survival between the populations, and in most years, survival was relatively high, ranging from 70-90% in all years of study other than 2013, where it was approximately 50%. In contrast to the serpentine habitat, the advantage of the local population on sandstone was due to differences in fecundity (fruit production). Significant differences in fruit production between the populations on sandstone soil were observed in 2012 and 2015, while a marginally significant advantage was seen in 2013.

Table 2. Model results for survival, fecundity and total fitness for four years of reciprocal transplant experiments. F-values were obtained from generalized linear mixed effect models under a binomial distribution (survival) or Poisson distribution (fecundity).

Fixed Effect	F-value	
	Survival	**Fecundity**
Soil	10.5***	37.9***
Pop	15.2***	7.4*
Year	6.0*	47.7***
Soil x Pop	49.8***	2.5**
Soil x Year	4.3*	0.4
Pop x Year	1.2	16.1***
Soil x Pop x Year	1.5	0.4

***p<0.0001; **p<0.001; *p<0.05

Across all four years, a significant population x soil type interaction was observed for survival and fecundity (table 2). There was a significant interaction for soil x population x year for fecundity but not survival, reflecting the fact that the survival advantage of the serpentine population on its native soil was consistent, while the fecundity advantage of the sandstone population varied. The interaction between soil x population and the main effect of population had the greatest contribution to variation in

survival; reflecting the survival advantage of the serpentine population. The main effects of year and soil type had the greatest effect on variation in fecundity. Fecundity was significantly lower on serpentine than sandstone soil across the four years of study and varied considerably among years.

The fitness components of F5 advanced-generation hybrids were also analyzed relative to the parents (figure 10). Their survival on serpentine was intermediate between the parents, but their relative fecundity differed among years. Overall, the fecundity of F5s more closely resembled the serpentine population than the sandstone population, but was intermediate to the parents in three out of four years on serpentine and one out of four on sandstone.

The effects of water on local adaptation

Rainfall data collected from JRBP show that during the four years of field study, annual rainfall was well below average (figure 11). Because this subset of years may not reflect long-term conditions, manipulative field and greenhouse experiments were performed to determine how variation in precipitation could alter patterns of local adaptation.

Figure 12 demonstrates the effect of watering regime on patterns of local adaptation in the greenhouse. With constant water, the sandstone population exhibited a fitness advantage on its native soil type, and the serpentine population exhibited a non-significant advantage on its native soil type. However, in the dry treatment the serpentine population had higher fitness regardless of the soil type.

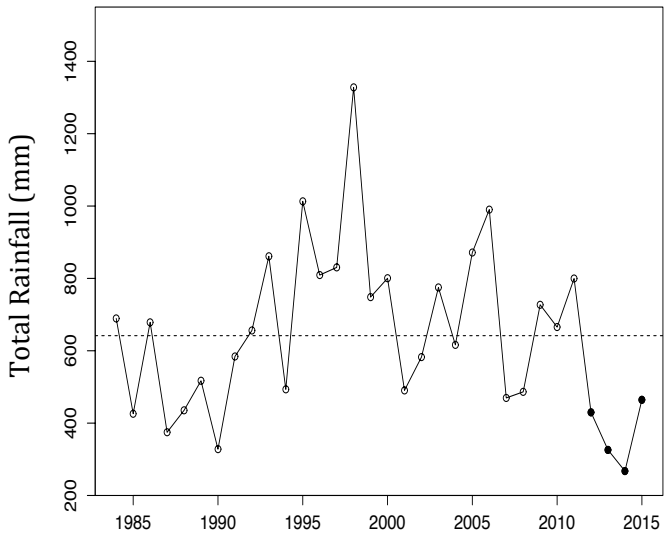

Figure 11. Total annual rainfall (mm.) recorded at JRBP for each year since 1984. The dotted line indicates the average annual rainfall. Solid black dots indicate the total rainfall in each of the four years of study performed.

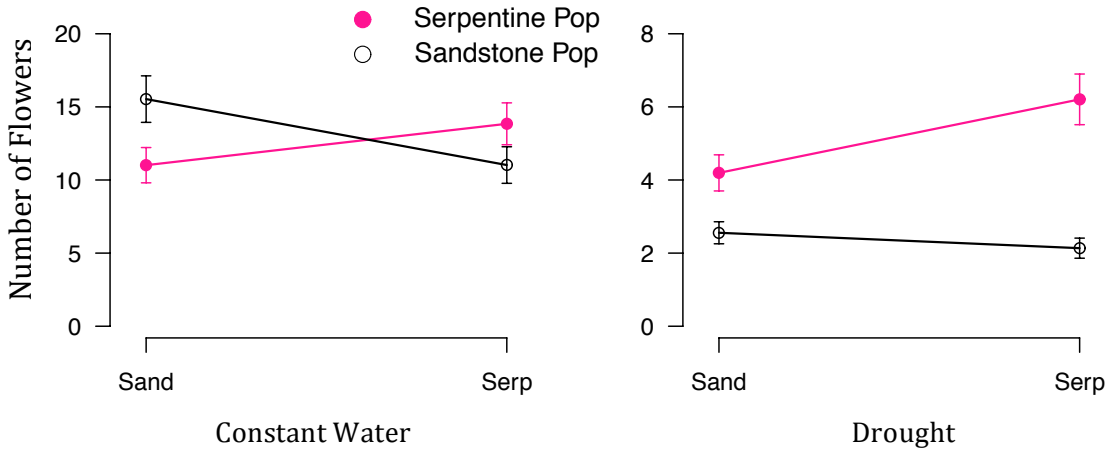

Figure 12. The least square means +/- one standard error for the total number of flowers produced by each population in each soil type under the two different watering regimes in the greenhouse. Results were obtained from generalized linear mixed models that did not include a non-significant 3-way interaction term among treatment x soil x population.

While survival in the greenhouse was higher than is typically observed in the field (>85%), most of the plants that died before flowering were from the sandstone population in the dry treatment, with a 57% survival on serpentine and 72% survival on sandstone. Table 3 shows the results of mixed effect models on this dataset

indicating that there are significant population x treatment, soil x treatment, and soil x population interactions for fitness. The large population x treatment interaction for fitness demonstrates that the fitness advantages of each population was impacted by watering regime, regardless of soil type.

Table 3. The effect of soil, population, and treatment on flower number in the greenhouse. Shown are F-values and their significance from a generalized linear mixed effect model using a Poisson distribution.

Fixed Effect	F value
Soil	0.01***
Pop	0.1***
Treatment	963.4***
Soil x Pop	13.4**
Soil x Treatment	15.8*
Pop x Treatment	107.5***
Soil x Pop x Treatment	0.8

***p<0.0001; **p<0.001; *p<0.05

The effects of watering and weeding on local adaptation in the field

Manipulative experiments were carried out in the field to further investigate the selective factors that could mediate local adaptation in these populations. Natural competitor removal and supplemental water were both found to significantly affect this pattern. Figure 13 shows the lifetime fitness of each population in each soil type across each treatment. Water addition had no effect on fitness differences among the populations on serpentine, but increased the advantage of the sandstone population on its native soil type. However, removing natural vegetation eliminated the advantage of the sandstone population on its native soil type, and the combination of watering and weeding gave the serpentine population a fitness advantage on sandstone. This is also evidenced by the significant population x soil x water x weed interaction for fitness (table 4).

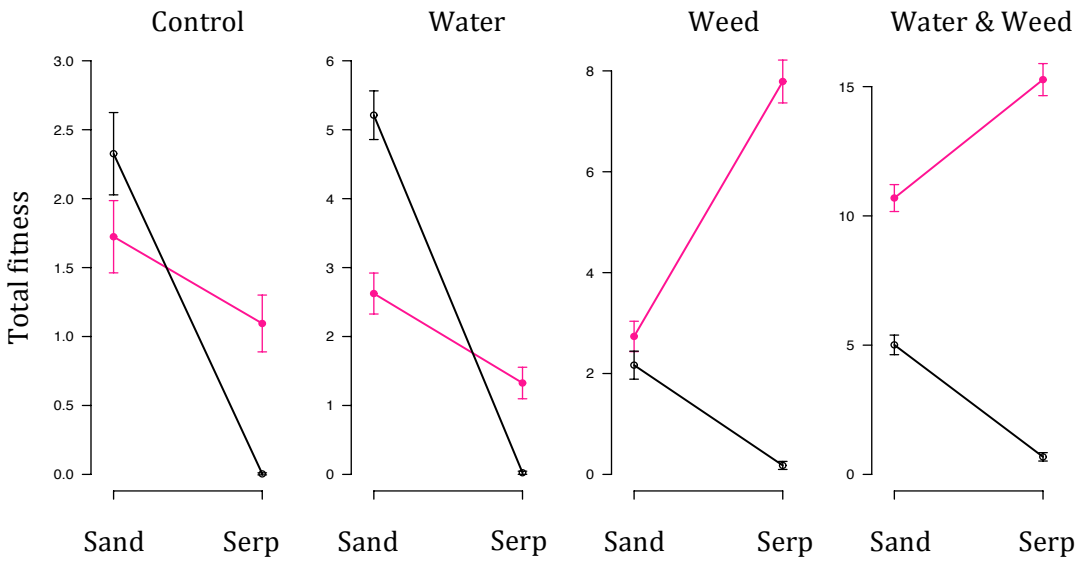

Figure 13. Lifetime fitness across treatments and soil types for each population, estimated from an ASTER model (Geyer 2015) that incorporated survival, number of flowers, and number of fruits.

Understanding the causes of these patterns requires an examination of the different fitness components. Figure 14 shows the survival and fecundity of each population in each soil type x treatment combination. Although absolute survival differs across treatments, the survival differences between populations remain largely unaffected by the treatments. In all cases, there is no significant difference in survival between the populations on sandstone, but a large survival advantage of the serpentine population on serpentine soil. Interestingly, vegetation removal affected plants differently in the different soil types. On sandstone soil, it had the effect of decreasing survival in both populations, while it increased the survival of the sandstone population on serpentine soil.

Although absolute survival was affected by the treatments, relative differences in survival among the populations within each soil type did not differ across treatments. However, the relative difference in fecundity between each population on sandstone did

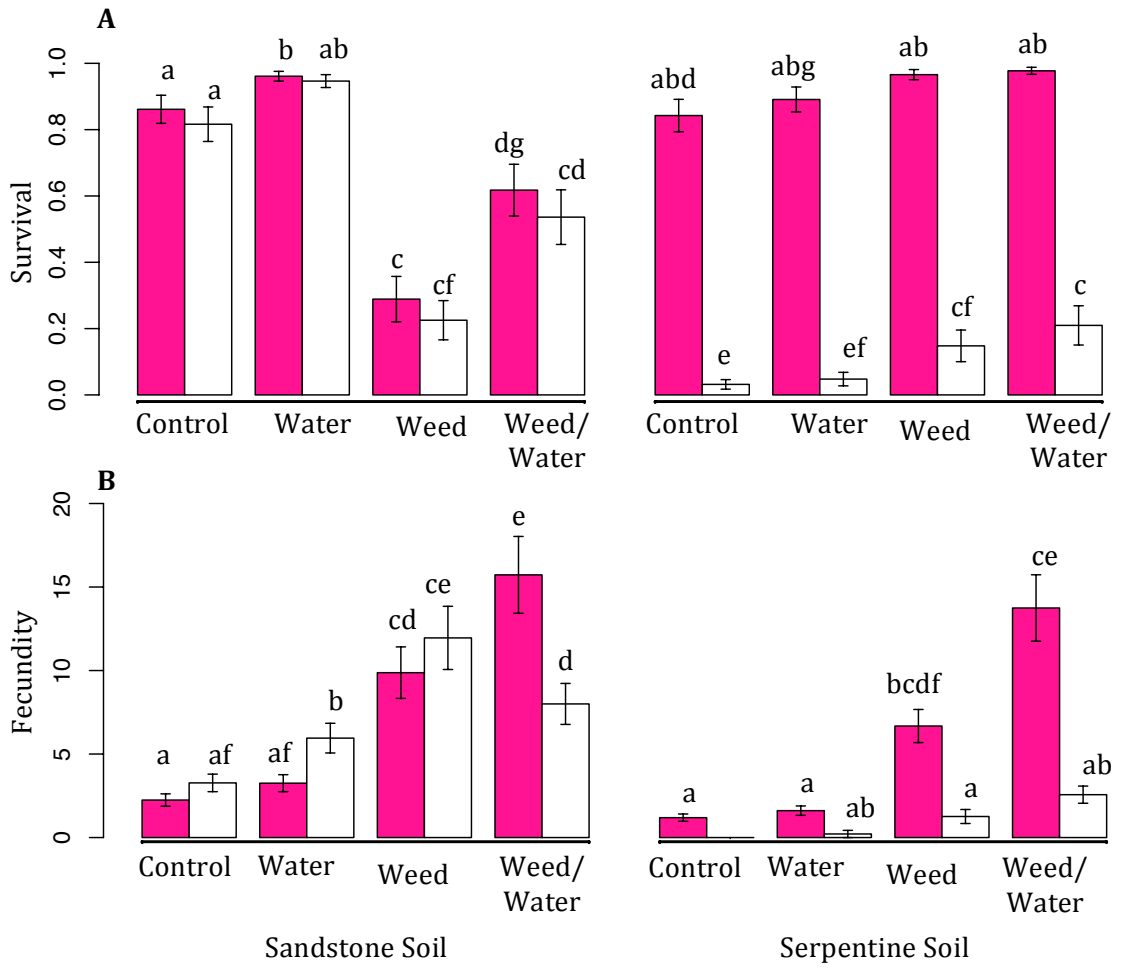

Figure 14. The least square means and standard errors for survival (A), and fecundity (B) for each population in each soil type and field treatment. Lower case letters indicate whether means are statistical different from one another based on pair-wise posthoc tests from models that include only significant interactions ($p<0.05$).

differ among the treatments. In the control treatment on sandstone, the difference in fecundity between the populations was only marginally significant ($p=0.085$ from Tukey pair-wise posthoc tests). However, supplemental water gave the sandstone population a greater advantage over the serpentine population on sandstone soil. In contrast, removing competitors eliminated the fitness advantages of the sandstone population in its native habitat. Further, the serpentine outperformed the sandstone population on sandstone soil when plots were both watered and weeded.

Table 4. The effects of population, soil type, field treatment, and their interactions on survival, fecundity and total fitness. F-values for survival and fecundity were obtained from generalized linear mixed models that included only significant factors. The significance of each factor on lifetime fitness was evaluated with ASTER models that tested the significance of interactions in nested ANOVA analyses. Also indicated is the deviance between full models and those without the factor indicated.

Fixed Effects	Survival	Fecundity	Total fitness
	F-value (significance)		Deviance (significance)
Pop	72.8***	92.2***	
Soil	0.8***	0.2***	3.18×10^{12}***
Water	6.4***	217.9***	
Weed	86.8***	1083.0***	
Pop x Soil	64.7***	154.0***	278.5***
Pop x Water		25.9**	17.0***
Pop x Weed			3.3 †
Soil x Water	2.8*		
Soil x Weed	98.2***	91.9**	123.7***
Water x Weed			
Pop x Soil x Water			
Pop x Soil x Weed			
Soil x Water x Weed		13.5***	
Pop x Soil x Water x Weed		71.9***	87.8***

***p<0.0001; **p<0.001; *p<0.05

Discussion

The current study demonstrates evidence for local adaptation to adjacent serpentine and sandstone soils in two populations of *L. parviflorus* that experience ongoing gene flow (figure 9). Across four years of study, each population performed best in its native soil type. On serpentine soil, selection against the foreign population was most significant for surival. Less than 1% of individuals from the sandstone population survived to flower. In contrast, differences in fitness among the parental populations on sandstone soil were due to differential fecundity. The sandstone population produced more fruits in three out of four years of study, and these fitness differences were more

variable across years (figure 10). This result demonstrates the importance of multi-year studies (Wadgymar et al. 2017, Agren and Schemske 2012), and the need to measure multiple fitness components (Wadgymar et al. 2017, Hereford 2009) to detect local adaptation

Although results demonstrate local adaptation among the populations, the fitness differences between the populations on sandstone are small relative to the differences on serpentine (figure 10). However, divergence with gene flow is predicted to occur only when selection is relatively strong in both environments (Endler 1973), and therefore fitness differences among the populations were expected to be of larger magnitude than was observed on sandstone. During the four years of study a severe drought occurred in this part of California and annual rainfall was lower than average in all years (figure 11). As a result of this unusually low rainfall, and because the advantage of the sandstone population in its native habitat was greatest in the two years with higher rainfall (2012 and 2015), experiments were established to determine whether greater soil moisture would increase the relative home-site advantage of the sandstone population. In a greenhouse experiment, the sandstone population had a fitness advantage on its native soil when plants were given constant water, but not in the dry treatment (figure 12). Manipulative experiments conducted in the field support this finding. A greater home-site fitness advantage was observed for the sandstone population when supplemental water was added (figure 13). Based on these results, it is predicted that the average fitness advantage of the sandstone population in its native habitat is greater than what was observed in the current study, and is likely related to the amount of rainfall that occurs in any given year. This result also indicates that continued drought conditions in California

as predicted by some climate models could have the effect of leading to the local extinction of this population.

A common feature of serpentine-adapted taxa is that they often perform as well or better on non-serpentine soils, leading to speculation that they are prevented from colonizing non-serpentine environments due to an external factor, such as interspecific competition (Anacker 2014). This pattern was also found in the current study where the serpentine population produced more fruits on sandstone soil then it did on serpentine soil (figure 9). To test whether competition was limiting the relative fitness of the serpentine population on sandstone soil, the populations were grown with and without competition. In support of this hypothesis, the removal of interspecific competitors allowed the serpentine population to perform as well as the sandstone population in sandstone soil (figure 13). Further, the synergistic effects of competitor removal and water addition unexpectedly led to a large fitness advantage of the serpentine population relative to the sandstone population on sandstone soil. It is possible that the early flowering phenotype of the serpentine population extended the length of the reproductive period without the usual associated costs related to insufficient water use or lack of competitive ability.

In summary, the current study demonstrates evidence for local adaptation to adjacent serpentine and sandstone soils among two populations of *L. parviflorus* that are in close geographical proximity and experiencing ongoing gene flow. While the population adapted to sandstone soil consistently dies before flowering on serpentine soil, it experiences greater fecundity on its home soil relative to the serpentine population. However, this fecundity advantage varies across years and is likely mediated by variation

in annual rainfall. In addition, this study lent support for the hypothesis that interspecific competition limits the ability of serpentine populations to colonize non-serpentine habitats. Future studies will investigate the role of flower color in contributing to local adaptation in this system.

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CHAPTER 3: THE CONTRIBUTION OF FLOWERING TIME TO ADAPTIVE DIVERGENCE

Introduction

Variation in life history strategies makes an important contribution to biological diversity and can be caused by fundamental trade-offs across different environments. In plants, flowering time is a critical stage in a plant's life history that directly affects reproductive output. A trade-off in the allocation of resources to vegetative growth versus reproduction may influence the optimal time to flower in a particular habitat (Weis et al. 2014; Aarssen 2008). This trade-off can result in differentiation in flowering time among populations (Hall and Willis 2006; Agren et al. 2016). Because differences in flowering time can also reduce gene flow between populations, the factors that affect variation in this trait may make an important contribution to adaptive divergence.

A variety of factors can impose selection on flowering time. Growing season length can be constrained by water availability and mediated by climatic variables such as the onset of drought (Hall and Willis 2006; Weis et al. 2014) or snowmelt (Inouye et al. 2003). In environments with short growing seasons, flowering early may ensure reproduction (Weis et al. 2014; Geber and Dawson 1990, Miller 1995). However, an early onset of flowering is sometimes associated with reduced vegetative biomass (Mitchell-Olds 1996, Fletcher et al. 2015, Houle 2002) and lower water use efficiency (McKay et al. 2003). Because of these growth-related costs, theory predicts that delayed flowering will be favored in environments with longer growing seasons, particularly when selection favors a greater investment in vegetative biomass before reproduction (Lindh et al. 2016, Cohen 1976, Ryti 1987) as might be expected in habitats with high levels of competition for light (Abrahamson and Gadgil 1973). In addition to the abiotic selective factors that impose limits on the length of the growing season, biotic factors

such as pollinator availability (Sandring and Agren 2009), herbivory (Ehrlen and Munzbergova 2009), seed predation (Valdes and Ehrlen 2017), or reproductive synchrony with potential mates (Dominguez and Dirzo 1995) can also impact the optimal time to flower in a particular habitat.

Because many factors can affect selection on flowering time, it is perhaps not surprising that heritable differences in this trait are commonly observed among populations occupying different habitats (Colautti and Barrett 2010; Weber and Schmid 1998, Agren and Schemske 2012) and are often implicated in divergent local adaptation (Hall and Willis 2006; Agren et al. 2016). Moreover, differences in flowering time are common reproductive isolating barriers among sister taxa (Lowry et al. 2008) and demonstrate the potential for reproductive isolation to arise as a byproduct of adaptive divergence (Bomblies 2010, Hendry et al. 2007).

However, differences in flowering time among populations can also be caused by phenotypic plasticity, as flowering time is often affected by factors that vary across habitats, such as seasonal environmental cues (Wilczek et al. 2009) or stress (Jordan et al. 2015). In contrast to divergent adaptation, environmentally mediated differences in flowering time can immediately lead to non-random mating across habitat types. Theory predicts that such habitat-induced flowering-time shifts can initiate divergence among populations, as long as they do not reduce fitness (Levin 2009, Gavrilets et al. 2007). It has been proposed that non-allopatric speciation in island palms occurred when substrate-induced flowering-time differences initiated non-random mating. Because this divergence in flowering time is believed to have also increased fitness in these habitats, subsequent

adaptive differentiation in flowering time may have further reduced gene flow (Savolainen et al. 2006).

Understanding the contribution of flowering time to reproductive isolation is relevant only in populations that might otherwise freely interbreed, such as those in close geographic proximity. Some of the best empirical examples of divergent adaptation in the presence of gene flow involve plant adaptation to different edaphic environments, for which divergence in flowering time is frequently observed (Ferris et al. 2017, Savolainen et al. 2006, Wright et al. 2006, Gailing et al. 2004, Rajakaruna & Whitton 2004, Gardner and Macnair 2000, Antonovics and Bradshaw 1970, Mcneilly and Antonovics 1968). These differences in flowering time could be a result of divergent selection (Peterson et al. 2013) and/or phenotypic plasticity (Jordan et al. 2015) and may have played a role in facilitating adaptive divergence, particularly if habitat-associated environmental factors caused flowering-time differences between populations that parallel the direction of divergent selection (Levin 2009).

The current study addresses the role of flowering time in adaptive divergence between populations of *Leptosiphon parviflorus* (Polemoniaceae), an annual, self-incompatible wildflower native to the western U.S. At Jasper Ridge Biological Preserve (JRBP) in San Mateo County, California, a pair of populations in close proximity (<100 m) inhabits different soil types, serpentine and sandstone. Serpentine soils are often used as model systems for the study of local adaptation over small spatial scales and are characterized by several stressors, including low calcium to magnesium ratios, low water holding capacities and high concentrations of heavy metals (Brady et al. 2005). In contrast, sandstone soils are relatively benign.

In *L. parviflorus*, plants from serpentine and sandstone populations can produce fully fertile hybrids when intercrossed (Dittmar, unpublished), and differentiation in AFLP markers (Fst= 0.12) indicates that a moderate amount of gene flow occurs among the populations (Kay et al. 2011). Nevertheless, the two populations are locally adapted to their native soil types (Dittmar, unpublished).

Differences in reproductive timing may have allowed adaptive divergence among these populations to occur in the face of gene flow. The peak flowering time of the serpentine population occurs 2-4 weeks earlier than the peak flowering of the sandstone population (Schmitt 1983 and pers. Obs) with only a moderate overlap in flowering duration (figure 21). The current study examines the degree to which differentiation in flowering time is genetically or environmentally mediated and whether plasticity in flowering time is caused by differences in soil moisture or soil type. In addition, the fitness effects of flowering time in each habitat were investigated over four years to determine whether divergent selection caused the genetic differences in flowering time among the populations. Integrating data across multiple years allows the cumulative effects of selection to be detected (e.g. Wadgymar et al. 2017). Selection was measured on a set of advanced-generation (F5) hybrids to expand the range of phenotypic variation in flowering time relative to either parental population. In addition, by recombining parental genotypes, the fitness effects of flowering time could be assessed independently of their genetic background (Lexer et al. 2003).

Methods

Field

Field studies were conducted at JRBP during the spring growing seasons in 2012, 2013, 2014, and 2015. Seeds from both populations were collected from >50 maternal plants grown in a common greenhouse environment. These maternal plants descended from >100 field-collected plants from each natural population which were maintained in the greenhouse across generations through random intercrossing to minimize inbreeding. The same seed source was used for all experiments described.

Seeds were sterilized at Michigan State University and sown in petri plates on agarose medium. The plates were immediately put into a dark room at 4°C for ten days, after which they were moved to an incubator set at 16 hour days, and 22°C to encourage uniform germination and growth. Seeds were germinated at the approximate time of year that they naturally germinate in their native habitat (late December/early January).

After 7-10 days in the incubator, when cotyledons were visible for all germinants, the plates were transported to JRBP. Seedlings were transplanted in randomized order into individual cells of plug trays (Hummert©, 12 x 24 cells) filled with field-collected serpentine or sandstone soil. A 4:1 mixture of field soil to perlite was used to improve soil aeration. An equal number of seedlings from each plate were transplanted into each soil type to minimize plate effects and subsequent analyses showed that there was no significant effect of plate on flowering time or fitness (data not shown).

Seedlings were watered daily for one week and then seedling plugs were individually planted into field plots in the same randomized order, excluding seedlings that were weak or had died in the trays. Care was taken to minimize disturbance to the

plots so that natural competitors could be maintained. The locations of field plots on each soil type varied slightly across years to minimize disturbance but were located within the boundaries of the natural populations.

To minimize transplant shock, plots were covered with nylon tulle for 2 days. Seedlings were watered for 1-2 weeks after transplanting, with the duration and volume based on the natural rainfall and temperatures in that year. Plants were censused every 2-3 days, and the flowering start date was recorded for each individual.

During the 2015 growing season, volumetric water content (VWC) was estimated using soil moisture sensors (Vernier© Model SMS-BTA) that measure soil capacitance. Sensors were calibrated to each field soil type to increase the accuracy of the relationship between soil capacitance and volumetric water content following the Vernier© two point calibration method. In brief, readings were taken on fully dry soil and after adding a known volume of distilled water to bring the volumetric water content to 45%. These readings were then used as calibration points to adjust the raw soil capacitance readings. A total of four sensors, each buried horizontally within the root depth of *L. parviflorus* (approximately 8 centimeters belowground) were deployed in pairs at two different locations on each soil type. Sensors were powered by portable solar power stations and continuously uploaded raw data through the field station's wireless mesh network at 5-minute intervals over the entire growing season (February – June). The means and standard errors of VWC on each soil type were calculated for each day based on the average of all observations across the four sensors for that day.

To test the fixed effects of soil type, source population, and year on the flowering start day, a linear mixed effects model (*lme*) was implemented in R (R core team, 2015)

using the *nlme* package (Pinheiro et al. 2017) that allowed for unequal residual variances among years. The flowering start day was analyzed relative to the first plant that flowered in that year. In two years (2014-2015), experiments were performed across multiple replicate plots in each soil type and therefore the model included a random effect of plot nested within year and soil type. A fixed effect of edge position (edge vs. non-edge) was also included. The high mortality of the sandstone population on serpentine soil (99%) precluded the analysis of a 3-way interaction between population x soil x year. The significance of each term was evaluated using maximum likelihood estimation that compared the Chi-square significance of models with and without individual terms. The model was also used to calculate least square means using the *lsmeans* package in R (Lenth 2016), for each study population in each soil type.

Greenhouse

Seeds were germinated using the same methods as described for the field experiment. After two weeks in an incubator, seedlings were transplanted to cone-tainers (Hummert©, RLC-4) filled with a mixture of sieved field soil (serpentine or sandstone) and perlite in a 4:1 ratio of field soil to perlite. To encourage establishment, seedlings were top watered for 3 days after transplanting, after which plants were bottom watered only. In each soil type, plants were randomly assigned to either a full water treatment, where they were continuously bottom watered throughout the duration of the experiment, or a drought treatment, where watering was ceased when the first plant began flowering, which occurred approximately one month after transplanting. This drought treatment was chosen to mimic the natural phenology in the field. Two flats of cone-tainers were used

for each treatment. Plants were censused every day and the first day each plant flowered was recorded.

The sources of variation in the day of first flower were investigated using generalized least squares (*gls*; Pinheiro et al. 2017) with REML estimation, allowing for unequal residual variances among treatments. Several random effects were evaluated for their significance to the overall model fit, such as the flat of each plant before being assigned to a treatment, and the flat nested within treatment, but likelihood ratio tests showed that adding these random effects made no significant improvement to the model, so they were not included. The means and standard errors of the day of first flower were determined for each source population in each treatment and soil type.

Selection on flowering time in the field

To expand phenotypic variation in flowering time, advanced-generation (F5) hybrids were created. Seeds were collected from 100 haphazardly chosen maternal plants in each population in the field. Crosses were performed between individuals from these populations and the resulting F1s were intercrossed. To minimize inbreeding, a bulk hybrid population was maintained through random intercrossing for four additional generations in a common greenhouse environment. At least 50 plants were used for each round of crossing where each plant was randomly crossed to another. The final F5 generation was used in all four years of field studies. These F5 hybrids were randomized and grown in the field on each soil type using the same methods described for the parental populations. The day each plant began flowering in addition to the number of flowers and fruits it produced were recorded. In 2012, the number of fruits and flowers

produced by F5s on serpentine soil were not obtained because of high herbivory and therefore selection analyses on this soil type do not include data from that year.

A significant interaction between the day of first flower x soil type was found in an ANCOVA that examined the fixed effects of soil type, year, and the day of first flower on relative fitness (table S1), and subsequent analyses investigated selection on flowering time for each soil type separately. To analyze selection on flowering time in the F5 hybrid population in each soil type, ANCOVAs were performed using linear mixed effects models (*lme*) in the nlme package in R (Pinheiro et al. 2017, R core team, 2015). The relationship between the day of first flower and relative fitness was investigated along with the fixed effects of year and edge position (edge vs. non-edge). Models included a random effect of block nested within year and allowed for unequal residual variances among years.

Relative fitness was calculated by dividing the number of fruits each plant produced by the average number of fruits produced in that soil type that year. The flowering day was calculated relative to the first plant that flowered that year and standardized to have a mean of zero and variance of one for each soil type in each year of study. The significance of adding a squared term for flowering day to the model was evaluated using likelihood ratio tests on models fit with maximum likelihood estimation. For each soil type, the shape of the fitness function was estimated by extracting coefficients from the mixed effect models as well as by fitting cubic splines with generalized additive models (*gam*) that used restricted maximum likelihood estimation to fit the smoothing parameters (mgcv package, Wood 2000). Mixed effect models were also used to analyze data from individual years.

Results

Sources of variation in flowering time

Results from four years of field studies demonstrate that flowering-time differences among the parental populations have both an environmental and genetic basis (table 5, figure 15). On sandstone soil, the day of first flower was significantly different among the populations ($p<0.0001$), with the serpentine population flowering an average of 15 days earlier than the sandstone population. In addition, the soil environment caused significant differences in flowering time in the serpentine population ($p<0.05$), with earlier flowering on serpentine soil occurring 9 days earlier than on sandstone soil. Because only two sandstone individuals survived to flower on serpentine soil, pairwise comparisons between the mean flowering times of each population on serpentine soil or between soil types for the sandstone population are not meaningful. Overall, the genetic and environmental effects on flowering time contributed to a 24-day difference in flowering onset between each population on its native soil type ($p=0.0001$).

Table 5. Effects of population, year, and soil type on the day of first flower in experimental populations across the four years of study.

Source of Variation	num df	F-value
Year	3	20.78***
Soil	1	58.98***
Pop	1	517.99***
Edge position	1	3.55[T]
Year x Soil	3	1.06**
Year x Pop	3	4.37**
Soil x Pop	1	0.62

[T]p<0.06; *P<0.05; **P<0.01: ***P<0.001

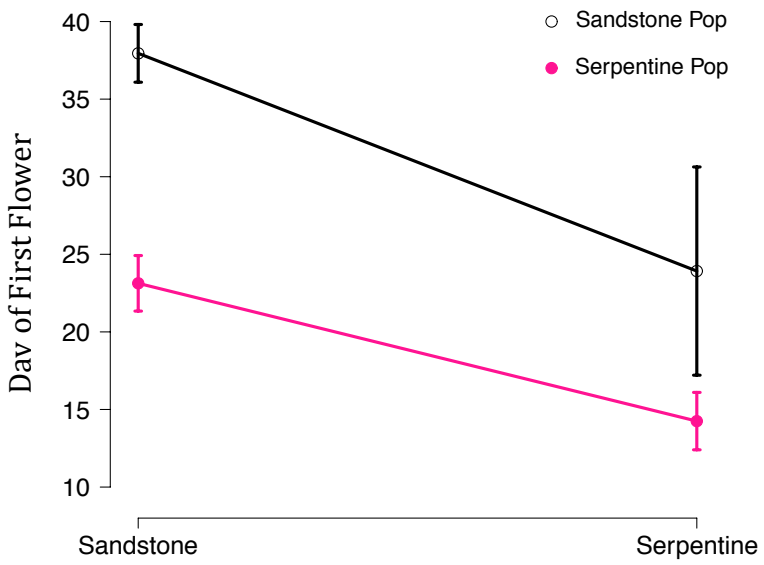

Figure 15. Flowering-time reaction norms of each population on each soil type. Closed pink circles represent the serpentine population and open black circles represent the sandstone population. Values shown are the least square means +/- one standard error across the four years of study using a linear mixed effects model. Pairwise post-hoc tests show that the flowering time differs significantly among the populations on sandstone soil ($p<0.0001$) and between the two soil types for the serpentine population ($p<0.05$). Pairwise comparisons with the sandstone population on serpentine soil are not significant because of low survival (N=2) of this population in this environment.

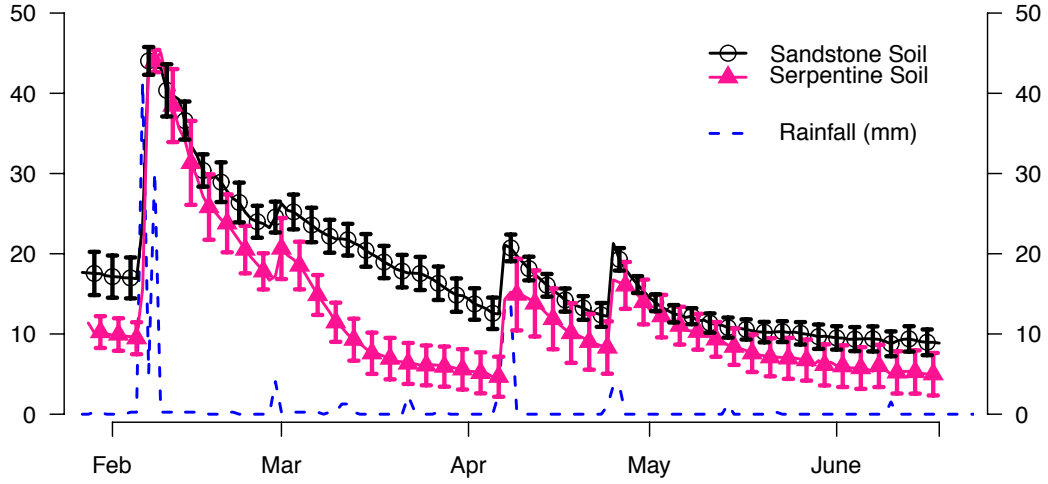

Figure 16. Volumetric water content (mean +/- one SE) on sandstone and serpentine soil across the growing season in 2015. Points shown are every third day. Rainfall data was obtained from JRBP weather station records

The change in soil volumetric water content (VWC) throughout the growing season differed among the habitats in 2015 (figure 16). Although soil moisture was similar across the two soil types during the period of highest rainfall in February (>40% VWC), the serpentine soil dried out faster than the sandstone soil and VWC decreased to below 10% by the beginning of April, a level not reached on sandstone soil until the very end of the growing season. Differences in VWC between the soil types were also observed during test deployments of the soil moisture sensors at the end of the 2014 growing season (figure 22).

Heritable differences in flowering time as well as phenotypic plasticity were also observed in both populations in greenhouse experiments (table 6, figure 17). However, in the greenhouse flowering time was affected by watering treatment rather than soil type, suggesting that the flowering-time differences observed in the field are caused by the different water holding capacities of the two soil types. There was also a significant treatment x population interaction for flowering time, with the sandstone population

Table 6. The effect of source population, treatment (constant water vs. drought), and soil type on the day of first flower in the greenhouse.

Source of Variation	num df	F-value
Soil	1	0.12
Pop	1	104.13***
Treatment	1	61.54***
Soil x Pop	1	0.56
Soil x Treatment	1	0.12
Pop x Treatment	1	12.34***
Soil x Pop x Treatment	1	0.20

*P<0.05; **P<0.01: ***P<0.001

exhibiting a greater degree of plasticity in flowering time between watering treatments. Relative to the fully watered treatments, the sandstone population flowered 6-7 days earlier in the drought treatment while the serpentine population flowered 3 days earlier.

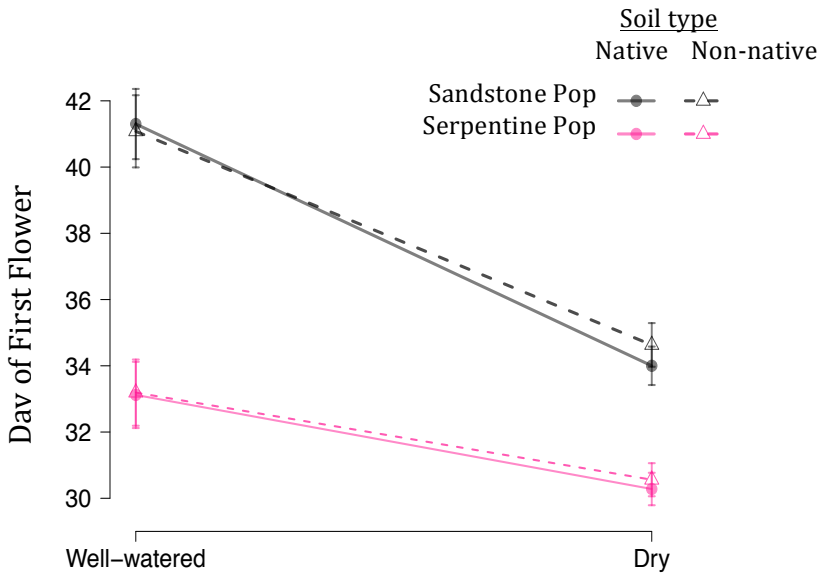

Figure 17. The mean (+/- one standard error) day of first flower for each population on each soil type measured in the greenhouse under well-watered (constant water) and dry (watering ceased when the first plant began flowering) conditions. Pink symbols represent the serpentine population and black symbols represent the sandstone population, while filled circles represent their native soil types and open triangles represent their non-native soil types. Flowering time differed among the populations in all treatment/soil type combinations ($p<0.0001$).

Selection on flowering time

The distribution in the day of first flower for the parental populations and advanced-generation hybrids (F5s) across the four years of study is presented in figure 18. On sandstone soil, the average day of first flower for F5s was intermediate to the parents. On serpentine soil, the high mortality of plants from the sandstone population prevented the comparison of its flowering-time distribution in this habitat. Results from an ANCOVA that examined the effects of flowering time (standardized) on relative

fitness among the F5s across the two soil types found a significant interaction between the day of first flower and soil type ($p<0.01$, table 8). Therefore, separate ANCOVAs were performed for each soil type to investigate the shape of the fitness function in each environment.

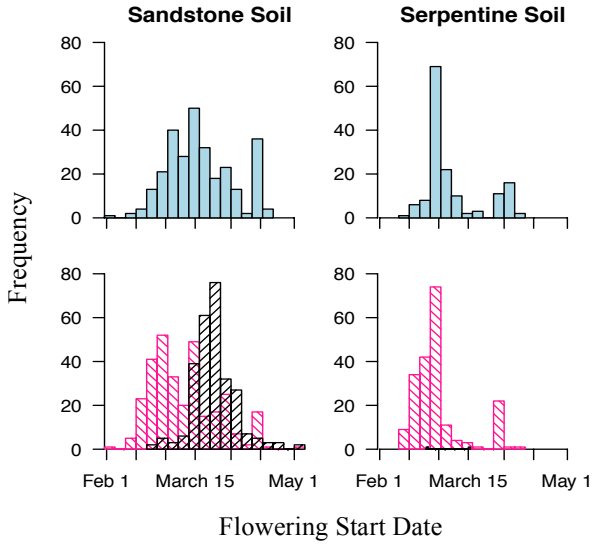

Figure 18. The distribution of the day of first flower among F5 advanced-generation hybrids on sandstone soil (top left) and serpentine soil (top right), shown in blue. The distribution in the parental populations is also shown on sandstone soil (bottom left) and serpentine soil (bottom right), with the serpentine population shown in pink, and the sandstone population shown in black. There was extremely low survival of the sandstone population on serpentine soil (N=2).

Table 7 lists all sources of variation analyzed and their significance with and without the inclusion of non-linear terms. The addition of a non-linear flowering time term and its interaction with year significantly improved the fit of the model on sandstone soil ($p=0.011$) and had a marginally significant effect on serpentine soil ($p=0.055$) based on a likelihood ratio test of the nested models. However, the main effect of the non-linear term for flowering time was only significant on sandstone soil (table 7).

Table 7. ANCOVA results that demonstrate the relationship between flowering time and relative fitness among advanced generation hybrids in the field in both soil types. Results from models including (A) only linear terms, or, (B) linear and quadratic terms are shown. Adding non-linear terms significantly improved the model fit for sandstone soil (p=0.011) and had a marginally significant effect on serpentine soil (p=0.055).

	Sandstone		Serpentine	
Source of Variation	**num df**	**F-value**	**num df**	**F-value**
(A). Linear Model				
Day of first flower	1	16.24***	1	11.10**
Year	3	0.73	2	4.42*
Edge Position	1	4.76*	1	6.10*
Day of first flower x Year	3	2.44	2	4.98**
(B). Non-linear model				
Day of first flower	1	16.43***	1	11.48***
Year	3	0.89$^{\mathrm{T}}$	2	4.67*
Day of first flower^2	1	8.75***	1	8.65
Edge Position	1	5.53*	1	4.24*
Day of first flower x Year	3	2.56	2	2.74*
Day of first flower^2 x Year	3	0.46	2	2.85*

$^{\mathrm{T}}$p<0.07; *p<0.05; **p<0.01; ***p<0.001

Visualizing the fitness surface using the regression coefficients from linear mixed effect models as well as by fitting a cubic spline to the data indicate that the optimal time to flower differed across habitats (figure 19). On serpentine soil, plants that flowered earlier than average had higher fitness than those that flowered later. In contrast, on sandstone soil fitness peaked among plants with intermediate flowering times, although the relationship between flowering time and fitness was asymmetrical around the mean such that fitness decreased more sharply among plants that flowered later compared to those that flowered earlier. Further, the estimated standardized selection differentials were higher on serpentine soil than on sandstone soil, indicating that selection on flowering time was much stronger in this habitat.

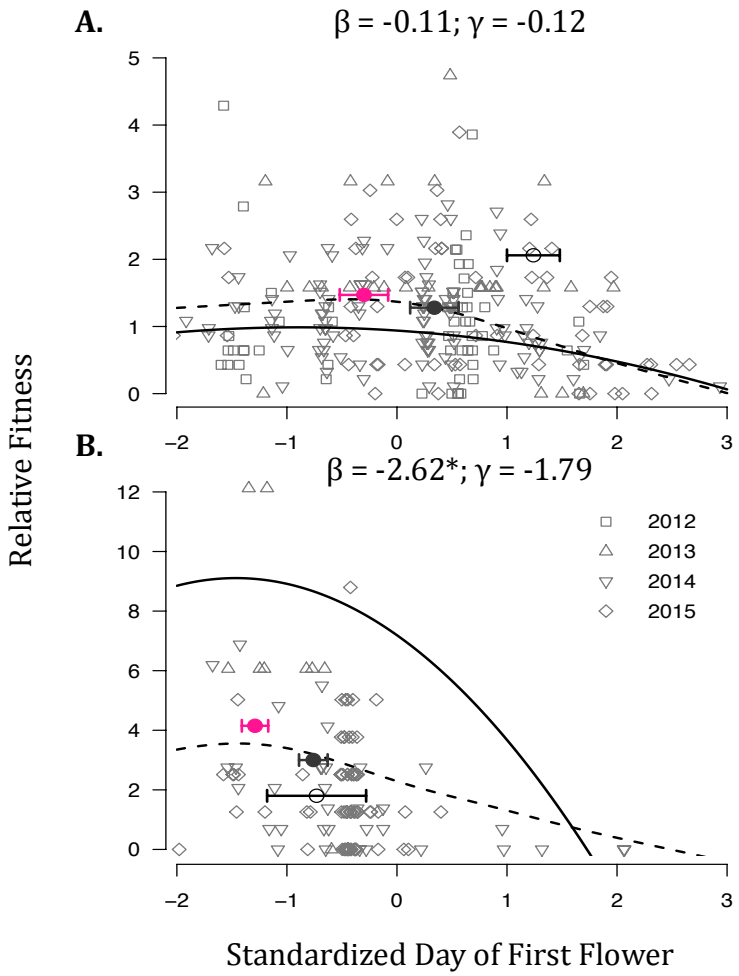

Figure 19. The relationship between the day of first flower and relative fitness for F5 hybrids on sandstone soil (A) and serpentine soil (B) across the four years of study. In order to compare the two habitats, the flowering time on the x-axis is standardized across soil types for a mean of 0 and variance of 1 within each year. Individual years are distinguished by different point shapes (see legend). The solid lines depict the relationship between standardized flowering time and relative fitness using linear mixed effect models for each soil type across all years. The regression coefficients from these models are shown (quadratic regression coefficients are not doubled). The dotted lines show the relationship between flowering time and relative fitness using generalized additive models. The mean flowering times +/- one standard error are shown for the serpentine population, F5 population, and sandstone population with pink closed circles, black closed circles, and black open circles, respectively. Y-coordinates of these points correspond to their mean relative fitness averaged across years using linear mixed effect models.

Years were also analyzed individually and the regression coefficients from these

models are presented in table 9. On serpentine soil, significant negative linear coefficients were detected in all years of study, indicating that early flowering is consistently associated with greater fitness in this habitat. A marginally significant ($p=0.08$) negative, non-linear selection coefficient was detected in one of the three years, suggesting some curvature in the relationship. On sandstone soil, regression coefficients were significant in only two of the four years of study, and a non-linear regression coefficient was significant in only one year. The statistical power to detect significant selection differentials was likely reduced relative to serpentine soil since the overall results indicate a weaker covariance between flowering time and fitness in this habitat. The sign of the linear and nonlinear regression coefficients on sandstone soil were consistently negative, suggesting that the weaker relationship between flowering time and fitness in this habitat was not caused by fluctuations in the direction of selection across years.

The mean flowering time of the serpentine parent in its native habitat occurred near the optimum flowering time as predicted by the models (figure 19). In contrast, the mean flowering time of the sandstone parent in its native habitat occurred later than was optimal during the four years of study. Despite this, the sandstone population had higher fitness than predicted by their sub-optimal flowering time (figure 19), suggesting that other traits may have been more important in contributing to their fitness advantage. The suboptimal flowering time of the sandstone population in its native habitat could have been caused by deviations in the selective factors operating on this trait. In particular, rainfall during the four years of study was consistently lower than average (figure 20). While total precipitation from November 1 – May 31 was on average 602 mm (standard

error = 42 mm.), between 2012-2015 it was just 404 mm., 313 mm., 240 mm., and 463 mm, respectively.

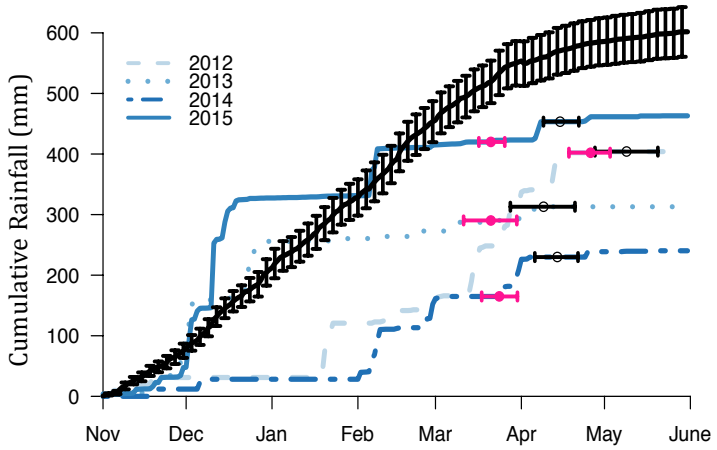

Figure 20. The cumulative rainfall since November 1 across the four years of study (in blue) compared to the average cumulative rainfall (+/- one standard error) from the past 31 years, obtained from JRBP weather station records. The day of first flower for each population on its native soil type in experimental plots is also shown for each year, with serpentine represented by pink, closed circles and sandstone by black, open circles.

Discussion

Flowering time is a trait of particular interest to evolutionary biologists because of its potential to contribute to reproductive isolation among populations or species that otherwise might interbreed (Lowry et al. 2008). The current study demonstrates that divergence in flowering time can occur across strikingly short geographic distances (<100 m.), and illustrates the potential for habitat differences to directly contribute to adaptive differentiation among populations. In the current study, flowering-time differences among populations were shown to be a result of both phenotypic plasticity and heritable genetic differentiation (figure 15). Plasticity in flowering time was affected

by soil moisture (figure 17) and is likely influenced by the different water holding capacities of the soil types (figure 16). In addition, differences in selection on flowering time among the habitats (figure 19) indicate that the heritable genetic differentiation among the populations in flowering time may be caused by local adaptation.

Sources of variation in flowering time

On average, the day of first flower in the two populations of *L. parviflorus* in their native habitats differed by about 24 days (figure 15). These differences were driven by both habitat-mediated environmental factors as well as genotypic differences among the populations. Results from the current study suggest that 62% of this difference in flowering time has a genetic basis, while 38% of this difference is caused by environmental factors.

In the greenhouse, flowering time was affected by watering regime rather than soil type for both populations (figure 17). This indicates that the different water holding capacities of the two soils (figure 16) may be causing the observed habitat-associated flowering-time differences. However, the magnitude of the effect of watering treatment on flowering time in the greenhouse was smaller than observed between soil types in the field. Greenhouse conditions were more benign (in the dry treatment, 56% of sandstone individuals survived to flower on serpentine soil compared to less than 1% in the field), indicating that the degree of stress present in the field was not fully captured in the greenhouse. This difference could have been caused by the finer and more homogenous physical texture of the sieved field soil used in the greenhouse, which may have increased its water holding capacity. However, soil moisture was not measured in the greenhouse

so it is not possible to determine whether it dried at a different rate than the field. Wright et al. (2006) found similar results in *Collinsia sparsiflora*, where non-serpentine ecotypes performed better on serpentine soil in the greenhouse than in the field. It seems likely that the degree of stress imposed by serpentine soil under natural conditions is difficult to replicate in the greenhouse. Nevertheless, it is clear that soil moisture plays a role in mediating flowering-time differences among these populations.

Plasticity in life history traits as a response to water stress is common to taxa that occupy arid environments (Heschel et al. 2017, Jordan et al. 2015, Des Marais et al 2012, Franks 2011, Aronson et al 1992). Although genetic variation for flowering-time plasticity in response to water deficits is common, it is not often clear to what extent this plasticity is adaptive (Franks 2011, Des Marais et al. 2012, Aronson et al. 1992). A plastic response to water deficits may be adaptive in environments where water availability varies (Heschel et al. 2017), but could also simply be a physiological consequence of stress (Des Marais et al. 2012).

The non-serpentine population of *L. parviflorus* at JRBP exhibited a greater degree of flowering-time plasticity in response to watering regime than the serpentine population (figure 17, table 6). In contrast, a study conducted on serpentine and non-serpentine populations of *Clarkia gracilis* found that the serpentine populations exhibited greater plasticity in their acceleration of flowering time in response to drought (Heschel et al. 2017). Additionally, a study on the fitness consequences of plasticity in seedling emergence timing and leaf turnover in *Erodium cicutarium* found that plasticity was favored on serpentine soil and disfavored on nonserpentine soil, presumably because of the finer-grained scale of environmental heterogeneity on serpentine (Baythavong 2011).

In the current system, plasticity could be favored on sandstone soil if the amount of winter rain has a greater effect on the length of the growing season than on serpentine soil, which may dry up more predictably. Understanding whether variation in annual rainfall affects the soil types differently deserves further consideration. Unfortunately, precipitation in all four years of this study was consistently lower than average, which limits the ability to investigate the effects of rainfall variation on flowering-time plasticity in each habitat. However, based on the minor differences in rainfall across the four years, there is no evidence that the sandstone population has a greater degree of flowering-time plasticity across years (Figure 20).

Regardless of the adaptive value of phenotypic plasticity, theoretical models demonstrate that a direct effect of the environment on flowering time can contribute to non-allopatric divergence when it is associated with a new ecological niche (Gavrilets and Vose 2007). In this case, a flowering-time shift associated with the colonization of serpentine soil in *L. parviflorus* may have facilitated adaptive divergence by reducing the amount of gene flow among the populations. Differentiation in flowering time is commonly associated with adaptive divergence among serpentine and non-serpentine ecotypes (Brady et al. 2005), although the degree to which these differences are environmentally or genetically controlled varies among taxa. In populations of *Collinsia sparsiflora*, both serpentine and non-serpentine ecotypes flower about two weeks earlier when grown on serpentine soil (Wright et al. 2006), but do not exhibit heritable genetic differences in flowering time, nor does there appear to be divergent selection acting on this trait (Wright and Stanton 2007). In contrast, serpentine-adapted populations of *Helianthus exilis* flower earlier than non-serpentine populations regardless of soil type,

indicating that this difference is mostly under genetic control (Sambatti and Rice 2007, Sambatti and Rice 2006). Similarly, among adjacent and overlapping species of *Lasthenia* that grow on different parts of a serpentine ridge, the 7-10 day difference in their flowering time appears to be entirely genetically based (Rajakaruna and Bohm 1999). Only environmentally mediated flowering-time differences will be involved in the initiation of divergence among populations. However, because flowering time can sometimes evolve rapidly (Franks 2011), adaptive differentiation in this trait may still play an important role in the maintenance of divergence among populations adapted to different edaphic environments.

Selection on flowering time

While habitat-associated flowering-time shifts can have immediate effects on the extent of gene flow between habitats, differences in selection on flowering time may further promote divergence among populations, particularly when selection occurs in the same direction as the plastic response (Levin 2009). To determine whether divergent selection was operating on flowering time in these populations, a set of F5 advanced generation hybrids were grown in both habitats across four years. While early-flowering plants had higher fitness than late-flowering plants in both habitats, the strength of the association between flowering time and fitness was much stronger on serpentine soil (figure 19). Additionally, the optimal flowering time on serpentine soil was earlier than on sandstone soil, indicating that parental differences in flowering time may be a result of local adaptation.

On serpentine soil, strong directional selection for early flowering was observed. Early flowering may be favored in this habitat because of its faster rate of moisture loss (figure 16) and thus shorter growing season. Models of optimal flowering time predict that early flowering will be favored in environments with short growing seasons (Weis et al. 2014). Many studies of edaphic adaptation in plants find that ecotypes adapted to harsher, drier soils flower earlier than neighboring congeners (Ferris et al. 2017, Gailing et al. 2004, Rajakaruna and Whitton 2004, Gardner and Macnair 2000), indicating that this is a common strategy for coping with stressful edaphic habitats.

In contrast, a combination of weak directional selection for early flowering and non-linear selection was found on sandstone soil. This resulted in an asymmetric fitness function, with a large fitness cost to late flowering and little to no cost to early flowering (Figure 19). Because the effect of flowering too late is expected to have more severe consequences for fitness than flowering too early, these results are consistent with theory predicting that stabilizing selection on flowering time will often be asymmetrical (Austen et al. 2017, Weis et al. 2014). Such asymmetry also means that selection against early flowering is more difficult to detect because of weaker selection (Austen et al. 2017). Therefore, it's possible that fitness costs to early flowering on sandstone soil are underestimated in the current study.

Regardless, the results indicate a lack of major fitness costs associated with early flowering on sandstone soil. This result is puzzling, since heritable genetic differences in flowering time among these populations have been maintained in the face of gene flow (Kay et al. 2011), indicating the presence of strong divergent selection on this trait (Endler 1973). Directional selection favoring early flowering is similarly common across

many plant taxa (Munguia-Rosas 2011), despite observations that populations often harbor considerable heritable variation for flowering time (Austen et al. 2017). Rather than the presence of ubiquitous directional selection for early flowering, Austen et al. (2017) suggests that experimental limitations such as not accounting for multiple fitness components or multiple years could limit the detection of stabilizing selection. Underscoring the need to account for variation in climatic conditions, a study by Wadgymar et al. (2017) demonstrated that stabilizing selection on flowering time in *Boechera stricta* was only detected when data were integrated across multiple years.

Although the current study integrated four years of data, unusually low levels of rainfall occurred in all years, and therefore the four years of study were not a representative subsample of the variation in annual rainfall for this climate (Figure 20). This may have had a significant effect on selection in these habitats, and a recent meta-analysis found strong associations between patterns of precipitation and variation in selection across terrestrial plant and animal systems (Siepielski et al. 2017). Because drought can impose selection for early flowering by decreasing the length of the growing season (Franks 2011), the advantages to early flowering may be greater during dry years. The average flowering time of the native sandstone population was later than the optimal flowering time in this habitat during the four years of study (Figure 19), which may be closer to the optimal time to flower over the long-term.

The non-linear selection on flowering time observed on sandstone soil (Figure 19), does suggest that there may be some advantages to intermediate flowering time in this habitat, such as a greater requirement for investing in vegetative growth before reproduction. Because sandstone soils are more benign than serpentine, they often

support a greater density of inter-specific competitors (Harrison 1997). Increased investment in vegetative biomass is often associated with greater competitive ability (Miller 1995, Llancourt and Tielborger 2009), and can be negatively correlated with flowering time (Bolmgren and Cowan 2008). Individuals from the sandstone population are taller and have more vegetative biomass than the serpentine population (pers. obs), consistent with the hypothesis that there is stronger selection for investment in growth in this habitat. In a study of plant responses to abiotic stress, Stanton et al. (2004) found that increased competition decreased the strength of selection for early flowering. In a QTL study between a serpentine adapted species and its congener, Gailing et al. (2004) found a direct trade-off between early flowering and leaf production. Serpentine adaptation is widely believed to come at a cost of competitive ability, thus limiting the ability of serpentine adapted species to colonize more benign habitats (Anacker 2014). The degree to which flowering time contributes to this trade-off is worthy of further exploration.

In addition, some evidence suggests that early flowering is negatively correlated with water use efficiency (WUE) (Kenney et al. 2014; McKay et al. 2003). Because early flowering is a common drought avoidance strategy (Heschel and Riginos 2005; Stanton et al. 2000), drought can sometimes lead to selection for early flowering at the expense of WUE (Franks et al. 2011, Donovan et al 2009). However, dry habitats may also select for high WUE (Heschel et al. 2002). Whether drought avoidance or high WUE are favored likely depends on the length of the growing season, level of competition, and whether drought conditions occur early or late in the season (Geber and Dawson 1990, Heschel and Riginos 2005). Early flowering on serpentine soil is likely to be a drought avoidance strategy related to the high rate of moisture loss in this habitat. On sandstone soil

however, a growth strategy that leads to greater WUE but delays flowering may be more selectively advantageous in some years.

Pollinator availability can also contribute to selection on flowering time (Sandring and Agren 2009). The peak flowering time of the sandstone population at JRBP was found to have more overlap with pollinator abundance than the serpentine population, leading to greater rates of visitation (Schmitt 1983). However, because this species can also reproduce through wind pollination (Goodwillie 1999), reproduction is not necessarily limited by pollinator availability. Further, a similar pattern of selection in both habitats was found when using flower number as a fitness metric instead of fruit number, implying that pollinator limitation is not the underlying cause of selection on flowering time in these populations.

Future studies will investigate additional traits that are likely to play a role in local adaptation of these populations. Despite the suboptimal flowering time of the sandstone population in its native habitat across the four years of study, its fitness remained high relative to the serpentine population (figure 19), indicating the involvement of other traits in local adaptation to this habitat. Flower color is a trait of particular interest for future investigation as it is highly differentiated among the populations, although uncorrelated with flowering time in the F5 population (Dittmar, unpublished). It is also possible that unmeasured traits that are correlated with flowering time are driving the patterns of selection observed in the current study (Lande and Arnold 1983).

Overall, the results indicate that selection on flowering time differs among the habitats. On serpentine soil, there was strong selection for early flowering, while on sandstone soil a combination of weak directional selection and non-linear selection was

detected. These differences in selection may have caused the heritable differences in flowering time between the populations and thus contributed to a reduction in gene flow among them. Future avenues of research will investigate whether variation in annual rainfall mediates selection on flowering time and the mechanisms that cause non-linear selection on flowering time on sandstone soil. In addition, the role of other traits important for local adaptation in these populations, such as flower color, will be studied.

Conclusions

The current study found that flowering-time differences among closely adjacent, locally adapted populations of *L. parviflorus* are a result of both genetic and environmental factors. Because these populations are self-incompatible, share pollinators, and are in close geographic proximity, a difference in flowering time may have been instrumental in facilitating adaptive divergence among them. Identifying the factors affecting flowering time in this system thus provides the first step towards investigating the process of adaptive divergence among these populations, and contributes to an understanding of the mechanisms involved in the initiation and maintenance of adaptive differentiation generally.

APPENDIX

APPENDIX

Table 8. ANCOVA results that demonstrate the relationship between flowering time and relative fitness among advanced generation hybrids in the field across both soil types.
Results are shown from models that include (A) only linear terms, or (B) linear and quadratic terms. Adding non-linear terms significantly improved the model fit ($p<0.0001$).

Source of Variation	numDF	F-value
(A.) Linear Model		
Day of first flower	1	24.68***
Year	3	2.86
Soil	1	1.27
Edge Position	1	8.07**
Year x Soil	3	2.53
Day of first flower x year	3	2.27$^{\mathrm{T}}$
Day of first flower x soil	1	9.15**
Day of first flower x year x soil	3	2.43$^{\mathrm{T}}$
(B.) Non-linear Model		
Day of first flower	1	24.85***
Year	3	2.74
Soil	1	1.24
Edge Position	1	8.24**
Day of first flower^2	1	10.60**
Year x Soil	3	2.61
Day of first flower x year	3	1.15
Day of first flower x soil	1	11.41***
Day of first flower^2 x year	3	0.18
Day of first flower^2 x soil	1	0.12
Day of first flower^2 x Year x Soil	3	3.47662*

$^{\mathrm{T}}p<0.1$; *$p<0.05$; **$p<0.01$; ***$p<0.001$

Table 9. Regression coefficients from linear models (2012-13) and linear mixed effect models (2014-2015) that analyzed the relationship between flowering time and relative fitness within years and soil types. Nonlinear terms improved the model fit on sandstone in 2014 and 2015 and had a marginal effect on the model fit on serpentine in 2015. Quadratic regression coefficients are not doubled.

		Linear	Nonlinear	
	N	**β**	**β**	**γ**
Sandstone				
2012	56	-0.05744	-0.095	-0.1466
2013	28	-0.4551	-0.1029	-0.5509
2014	115	-0.19322**	-0.21906**	-0.17443**
2015	57	-0.5883**	-0.6608**	-0.1007*
Serpentine				
2012	2	*N/A*	*N/A*	*N/A*
2013	10	-3.848*	-1.0466*	-2.9156
2014	34	-1.0476***	-1.501***	0.1736
2015	74	-0.2873	-0.4409 $^{\mp}$	-0.4163 $^{\mp}$

$^{\mp}$p<0.1; *p<0.05; **p<0.01; ***p<0.001

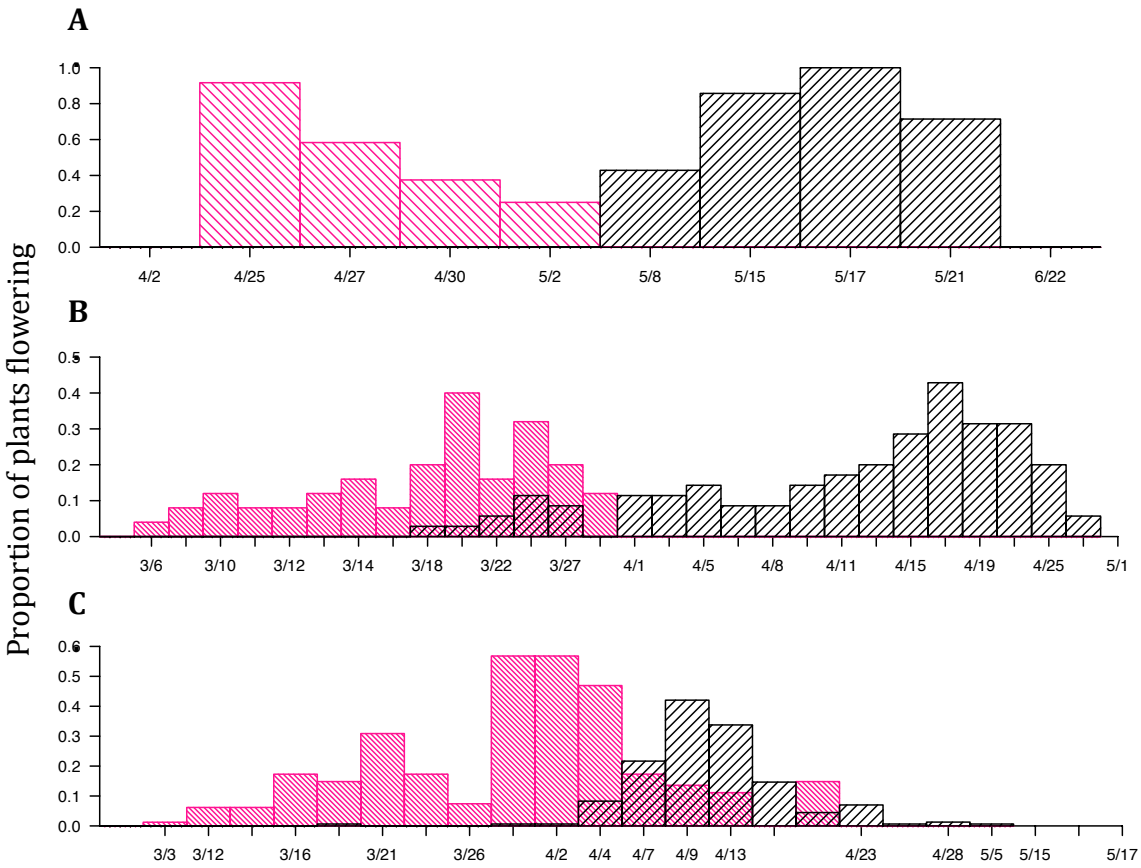

Figure 21. The proportion of individuals flowering (out of the total number of individuals that flowered) in experimental plots for each population on its native soil type across the growing season. Shown are data from 2012 (A), 2013 (B), and 2014 (C) (this data was not collected in 2015). Plants from the serpentine population on serpentine soil are shown in pink and plants from the sandstone population on sandstone soil are shown in black.

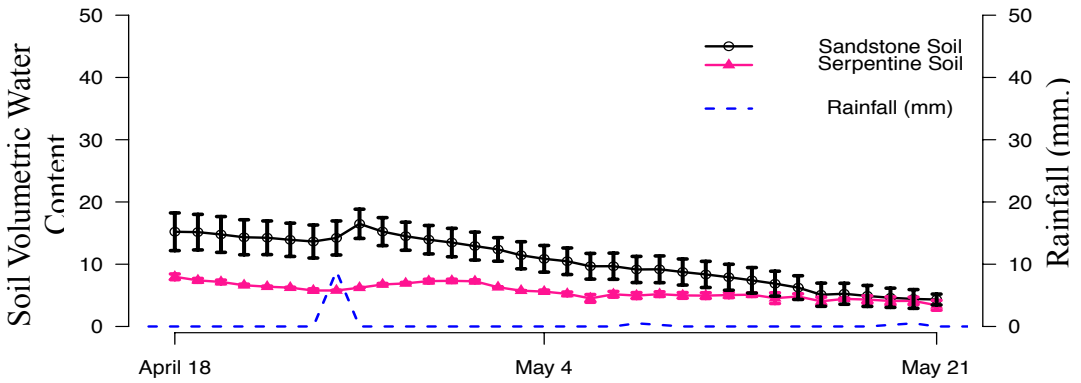

Figure 22. Volumetric water content (mean +/- one SE) on sandstone and serpentine soil from test deployments of the soil moisture sensors at the end of the 2014 growing. Points shown are every third day. Rainfall data recorded directly from JRBP.

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CHAPTER 4: THE ADAPTIVE SIGNIFICANCE OF FLOWER COLOR

Introduction

The dazzling array of floral diversity among angiosperms is often attributed to pollinator-mediated selection (e.g. Bradshaw and Schemske 2003; reviewed in Fenster et al. 2004). However, mounting evidence suggests that flower color may sometimes be associated with stress tolerance in some plant systems (reviewed in Strauss and Whittall 2006). Studies of the biochemistry of floral pigment synthesis have shown that the anthocyanin biochemical pathway shares genes that are involved in other, seemingly unrelated physiological processes (Koes et al. 2005), demonstrating the potential for pleiotropic effects of floral color changes on other traits (Rausher 2008). Recent studies have found a relationship between flower color and herbivore damage (McCall et al. 2013), mating system (Fehr and Rausher 2004), and survival (Coberly and Rausher 2008), for example. However, no study has yet conclusively demonstrated the abiotic selective agents operating on flower color (Rausher 2008) and understanding the potential for flower color to be under selection by abiotic factors would yield insight into evolutionary constraints to floral evolution (Streisfeld and Rausher 2010).

At Jasper Ridge Biological Preserve (JRBP) in San Mateo County, California, populations of *Leptosiphon parviflorus* grow in close proximity on two very different soil types, serpentine and sandstone soils. The serpentine population has exclusively pink flowers, while the sandstone population has almost exclusively white flowers and crossing studies reveal that this flower color polymorphism is controlled by a single locus, with pink dominant to white. Four years of comprehensive field and greenhouse studies demonstrate that the populations of *L. parviflorus* are locally adapted to their native soil types despite their close proximity (Dittmar, chapter 2). Previous research

using genetic markers showed low genetic differentiation among these populations (Kay et al. 2011), indicating frequent gene flow between populations. Despite this, flower color is the most differentiated of all traits examined in these populations (Kay et al. 2011). This strong divergence in flower color among populations over such a small spatial scale despite low genetic differentiation through most of the genome is consistent with the hypothesis that flower color plays a significant role in local adaptation to these habitats.

The current study thus aims to understand the adaptive significance of flower color in these populations. A set of advanced-generation F5 hybrids were grown in the field to determine whether the local flower color morph was associated with increased survival or fecundity in its native habitat. In addition, the flower color visitation patterns of pollinators were assessed through observational studies conducted on the experimental plots to determine whether there was evidence for selection on flower color by pollinators.

However, several lines of evidence suggest that it is unlikely that pollinators are playing a role in selection on flower color in these populations of *L. parviflorus*. First, both populations are pollinated primarily by beeflies (Dittmar pers. obs.). Further, if there was pollinator preference for flower color, we would expect a greater degree of genetic differentiation throughout the genome, and the relatively low Fst values observed indicate that this is not the case. Finally, a closely related self-fertilizing species, *Leptosiphon bicolor*, also exhibits a pink and white flower color polymorphism, with pink morphs on serpentine soil and white morphs on neighboring sandy soils, despite not requiring pollinators for reproduction (Goodwillie 2000). The striking parallelism in flower color differentiation between this species and *L. parviflorus*, despite their differences in the

importance of pollinators, suggests that edaphic factors, not pollinators, are responsible for the observed spatial pattern of the color morphs.

Therefore, instead of being driven by pollinator-mediated selection, flower color variation in this system may instead be related to some aspect of the abiotic environment associated with serpentine soil. While no study has yet demonstrated a connection between floral pigmentation and serpentine adaptation, flower color polymorphisms are associated with serpentine habitats in other systems. A serpentine population of *Collinsia sparsiflora* was found to have consistently darker petals than a nearby non-serpentine adapted population (Wright and Stanton 2007). Further, flavonoid profiles (pigments that cause yellow coloration) and differential tolerance to excess metal ions were correlated among parapatric races of *Lasthenia californica* growing on serpentine soil, (Rajakaruna et al. 2003).

To determine whether flower color affects survival and/or reproduction on either soil type in the absence of pollinators, a growth chamber study was performed on soil collected from the field using a set of Near Isogenic Lines (NILs). These NILs were created by introgressing the pink flower color phenotype into the sandstone genetic background, thus allowing for the study of flower color in isolation. NILs are beneficial for exploring adaptive variation for several reasons. First, one can measure selection on a trait as if it had been an early step during an adaptive walk. Second, fitness trade-offs as a result of this trait can be directly compared between environments, instead of indirectly inferred from the direction of selection in each environment.

Although multiple stressors are associated with serpentine soils including low water holding capacity, high concentrations of heavy metals, and low levels of nutrients, the low concentrations of calcium and high concentrations of magnesium are arguably one of the most stressful characteristics of these habitats (Kazakou et al. 2008, Brady et al. 2005, Walker et al. 1955). While low levels of calcium, an essential nutrient, and toxicity from high magnesium are both inherently stressful to plants, there is evidence that it is the ratio of these cations that is more important to plant performance than absolute concentrations (reviewed in Brady et al. 2005), and calcium has been shown to ameliorate the toxic effects of magnesium (Johnston and Proctor 1981, Walker et al. 1955). Understanding the physiology underlying serpentine tolerance may provide an explanation for the apparent trade-off between serpentine adaptation and competitive ability in non-serpentine environments (Anacker 2014).

As a first step towards investigating the abiotic selective factors operating on flower color, the current study investigated whether flower color variation was associated with magnesium tolerance. As is typical, the serpentine soil at JRBP has much higher magnesium and lower calcium than the neighboring sandstone soil (Dittmar, chapter 2). Selection on serpentine soil is so strong that the sandstone population consistently dies before flowering on serpentine soil (a survival rate of less than 1%). To determine whether the flower color phenotype has pleiotropic effects on magnesium tolerance, NILs were grown with parental populations in hydroponic assays that experimentally manipulated the concentration of magnesium in nutrient solutions. In addition, to determine whether calcium and magnesium had synergistic effects or additive effects on

plant performance, the parental populations were grown in solutions that varied both the absolute concentrations and the ratios of these cations.

Methods

Flower color and pollinator preferences

Advanced-generation (F5) hybrids were grown in the field with both parental populations. The creation of this hybrid population is described in chapter 2. Flower color and fitness were recorded for all F5 plants that survived to flower. To determine whether flower color had any effect on performance in either environment, two approaches were taken. First, the ratio of pink-flowered F5s observed in each soil type across the four years of study was compared to the expected ratio of 75% (due to its dominant mendelian inheritance). Any significant deviation from this expected proportion indicates that mortality occurred non-randomly with respect to flower color. Second, the number of fruits produced by pink and white-flowered morphs on each soil type was compared to determine whether there was any relationship between flower color and fecundity.

Pollinator observations were performed at experimental plots across multiple years of study. Most observations were performed on sandstone soil due to the more equal proportions of pink and white flowered plants on this habitat. Plots were composed of both parental populations and the F5 hybrid population. Observations were conducted at 30-minute intervals during which the order and number of visits to pink and white-flowered plants was recorded for each pollinator. Because preference and constancy could be skewed by a small number of flowers visited, metrics were assessed on pollinator visitation bouts to seven flowers or more (N=55 unique bouts). The numbers of

pink and white flowers visited by each pollinator were compared to the number of pink and white flowered plants 'available' to determine whether there was evidence of a preference for either flower color morph. Constancy was assessed by comparing the number of transitions between pink and white flowers relative to what would be expected under random foraging. Preference and constancy metrics followed Jones (1997).

Growth chamber field soil study

The effects of flower color on field soil were also explored using Near Isogenic Lines (hereafter NILs). Through several rounds of crossing, NILs were created to introgress the pink flower color phenotype into the sandstone genetic background. Because *L. parviflorus* is self-incompatible, NILs had to be created by crossing from different maternal families. First, maternal lines from each population were intercrossed to make a set of F1s. Each F1 was maintained as a separate lineage and were back-crossed to multiple sandstone (white-flowered) individuals. The resulting F2s were a mixture of approximately 50:50 pink:white-flowered plants. Pink-flowered F2s were then backcrossed again to a new group of sandstone individuals and this process was repeated until lines had been backcrossed for seven generations. Seventh-generation backcross lines from different lineages were then crossed to each other and unique individuals from different sets of crosses were intercrossed. These plants were also crossed to white flowered plants, since the offspring of these crosses would allow determination of the pink-flowered genotype. If all offspring were pink, the parent was homozygous at this locus, while if some offspring were white, the parent was heterozygous. Offspring from homozygous parents were then grown and intercrossed between two homozygous lines.

In addition, offspring from heterozygous crosses were grown so that white-flowered offspring could be intercrossed to provide a control for the pink-flowered NIL genotype. The resulting pink-flowered and white-flowered NILs are expected to have mostly the sandstone genetic background and differ only at the pink flower color locus, allowing an examination of the effects of this locus on performance.

Field soil assays were performed with the serpentine and sandstone parental populations as well as pink-flowered NILs and white-flowered NILs that had identical genotypes except for the flower color locus. Seedlings were first sown on petri plates using filter paper saturated with water that had been mixed and then decanted from both field soil types. The plates were put into a 4°C dark room for 10 days, and then moved to an incubator (22°C with 12 hour days) for 1 week before transplanting. Seedlings were then transplanted to 2,000 μL pipette tips filled with a 1:1 mixture of sieved field soil and perlite to allow soil aeration and randomized with respect to soil type and population across fourteen 4 x 6 tip boxes. Tip boxes were put into a growth chamber (22°C with 12 hour days) and randomized throughout the duration of the experiment. Plants were censused for survival to flowering, the day of first flower, and number of lifetime flowers produced. The effect of genetic background, flower color, and soil type on survival was determined using linear mixed effect models (*lmer*) that incorporated a random effect of tip box and cross type (parent versus NIL).

Hydroponic Assays

Hydroponic assays were performed using Rockwool sheets of 1” x 1” cubes (©Grodan). Sheets were soaked in deionized water until fully saturated, and then seeds

from both parental populations and the two NIL flower color genotypes were sowed directly into each cube in a randomized design. These trays were placed in the dark at 4°C for ten days, after which they were transferred to a growth chamber set at 22°C, 12 hour days. During this period, the rockwool was kept moist using ½ strength Hoagland's solution and supplemental spraying of deionized water. After approximately five days in the growth chamber, trays were censused to record the positions of seeds that had failed to germinate. Seedlings were allowed to grow in half-strength Hoagland's solution for an additional five days before being randomly assigned to treatments. Randomization occurred at the level of the ½ rock wool sheet, with multiple replicate blocks for each treatment.

Table 10. Concentrations (mM) of Magnesium sulfate and calcium nitrate used for the hydroponic treatments

	$MgSO_4$ (mM)	$Ca(NO_3)_2$ (mM)	Ca:Mg
Control	1	3.6	3.6
High Mg	100	3.6	0.036
Low Ca	1	0.036	0.036
Low Ca + Low Mg	0.01	0.036	3.6

Both parental populations were grown in all four treatments, while the NILs were grown only in the control and high magnesium treatment (table 10). The control treatment was watered with half-strength Hoagland's solution and the other treatments were identical to the control except for differences in $MgSO_4$ and/or $Ca(NO_3)_2$ (table 10). Flats were kept well-watered and solutions were replaced two times per week. Plants were censused twice a week for flowering and survival and at the end of the experiment the total lifetime production of flowers was counted for each individual. The effects of genetic background, flower color, and treatment on survival and the total number of

flowers produced was determined using linear mixed effect models with a random effect of treatment replicate and cross type (parent versus NIL). ANOVA results came from models fitted with restricted maximum likelihood (REML) and the significance of fixed effects was evaluated using log likelihood tests on models with maximum likelihood. To investigate the effects of varying both calcium and magnesium and potential interactions with source population on performance, a linear mixed effects model was performed on the total number of flowers produced by the parents across all four treatments with the random effect of treatment replicate.

Results

Field

The fitness and flower color of F5 advanced generation hybrids were analyzed to determine whether flower color had an effect of survival or fecundity in either habitat. While the proportion of pink-flowered F5 hybrids was higher than expected on serpentine and lower than expected on sandstone, neither of these differences were statistically significant (figure 23). The fecundity of pink and white flowered morphs relative to the parental populations are shown in figure 24. The only significant differences in fecundity were observed on sandstone soil in 2015, where the white-flowered F5s had greater fecundity than pink-flowered F5s, and on serpentine soil in 2014, where pink-flowered plants outperformed white-flowered plants on serpentine soil.

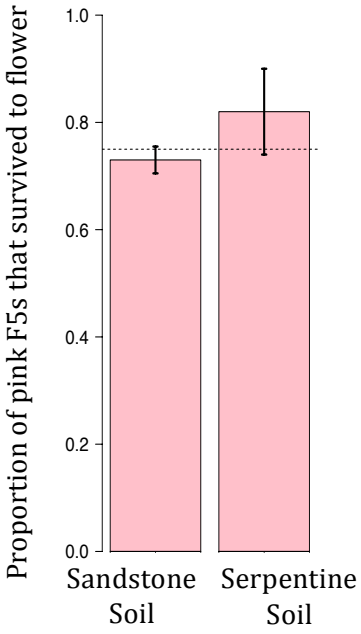


Figure 23. The proportion of pink-flowered F5 advanced-generation hybrids that survived to flower on each soil type across the four years of study. The expected proportion is 0.75, indicated by the dotted line.

Beeflies were the predominant visitors to *L. parviflorus*. Because they made contact with the stigma during visits and large numbers of pollen grains were observed on their legs and proboscis (pers. obs), they are believed to be effective pollinators for this species. The mean flower color preference did not differ significantly from zero (t=-1.0, p=0.3) indicating that there were no preferences towards either pink or white flowers. The constancy of pollinators in visiting pink or white-flowered plants was also evaluated. The mean constancy (likelihood of visiting two plants with the same flower color in succession) was not significantly different from zero for pink-flowered plants (mean= -0.033, p=0.22), but was significantly different for white-flowered plants (mean = -0.17, p=0.002). Although visitation to pink flowers was not significantly different from what would be expected under random foraging, the null hypothesis of random foraging was rejected for white-flowered plants. The negative mean value for constancy indicates that

pollinators were more likely to visit a pink-flowered plant after visiting a white-flowered plant (they had less constancy than expected by chance).

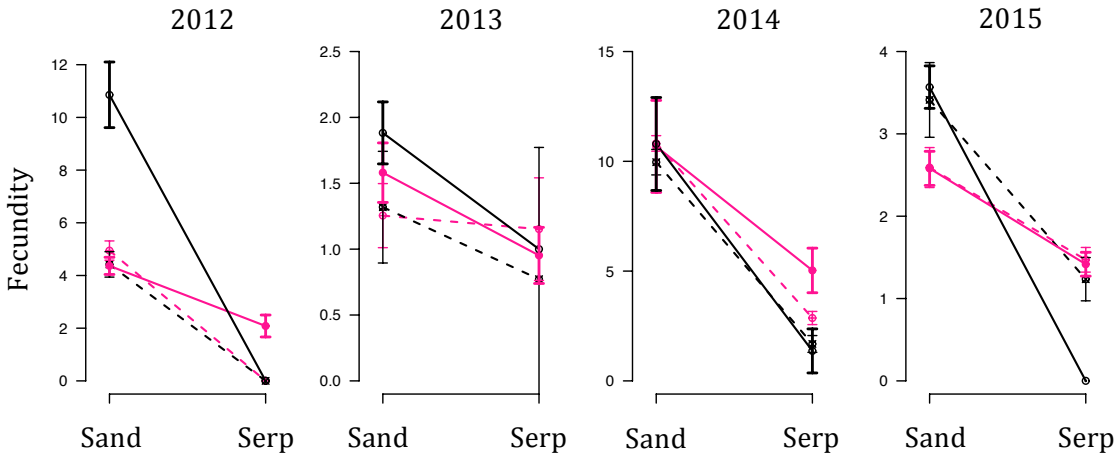


Figure 24. The fecundity (total number of flowers produced by survivors) of serpentine parents (solid pink line), sandstone parents (solid black line) and pink-flowered (dotted pink line) and white-flowered (dotted black line) F5s. Shown here are least square means from generalized linear mixed effect models.

NIL studies

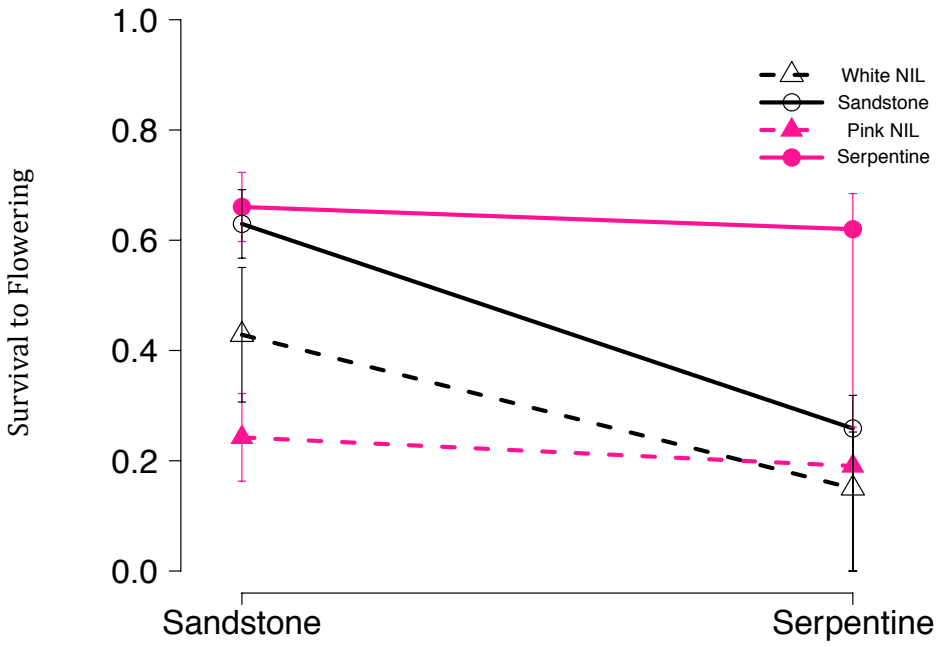
A significant interaction for soil x flower color on survival was found in field soil assays (table 11). The white-flowered NIL exhibited a survival advantage on sandstone soil relative to the pink-flowered NIL (figure 25). While the pink-flowered NIL had no survival advantage on serpentine soil, the slope of its reaction norm across the two soil types was similar to the serpentine parent (close to zero). In contrast, both the white-flowered NIL and the sandstone parent experienced a reduction in survival on serpentine soil relative to sandstone soil.

Table 11. Sources of variation and their significance in the field soil and hydroponic assays for survival and total number of flowers

Sources of Variation	F-values	
	Survival	Number of Flowers
Field Soil Assay		
Soil	13.86**	*NA*
Background	12.91**	*NA*
Flower Color	1.42$^{\mp}$	*NA*
Soil x Background	3.74	*NA*
Soil x Flower Color	5.32*	*NA*
Hydroponic Assay		
Treatment	10.82**	20.12**
Background	18.07***	7.2
Flower Color	5.56*	5.02*
Treatment x Background	5.27	6.26
Treatment x Flower Color	0.76	13.70***

$^{\mp}$ p<0.08; *p<0.05; **p<0.01; ***p<0.001

Figure 25. The reaction norms for survival across both soil types for both parental populations and pink and white-flowered NILs



In the hydroponic assays, there was a significant interaction between flower color and treatment on total flower number (table 11). The sandstone population exhibited a fitness advantage over the serpentine population in the control treatment (half-strength Hoagland's) for both trials. Although the serpentine population outperformed in the high magnesium treatment, this difference was only significant in one of the two trials (figure 26). In addition, the white-flowered NIL has greater fitness than the pink-flowered NIL in the control treatment (figure 26), but there was no significant difference between the flower color morphs in the high magnesium treatment. Although the pink flower color morph survived for a longer period of time in the high magnesium solution its final fitness did not differ from the white-flowered control genotype.

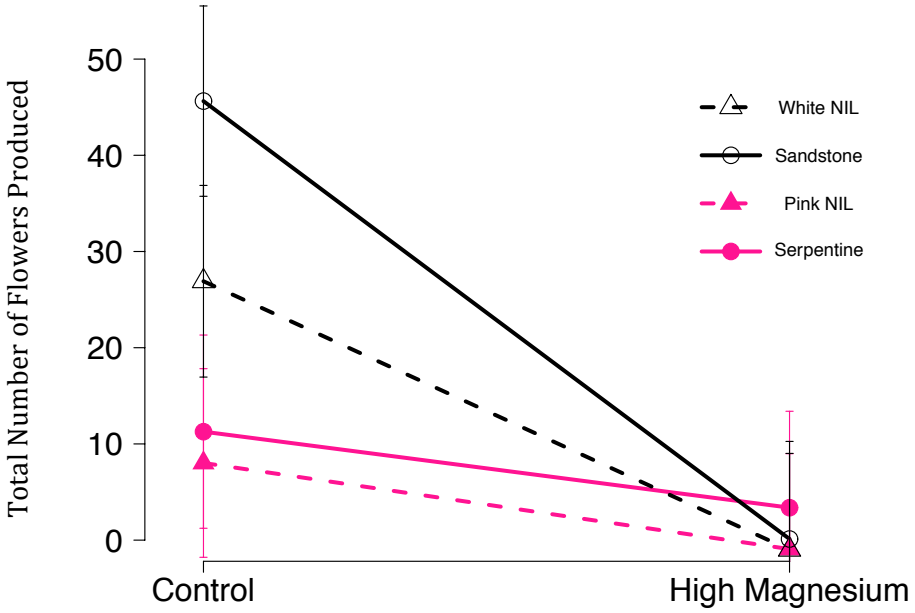


Figure 26. The reaction norms for the total number of flowers produced in both hydroponic experimental treatments for both parental populations and pink and white-flowered NILs

When the concentrations of calcium and magnesium were varied simultaneously for the parental populations, the serpentine population had greater survival in all but the

control treatment (figure 27). However, the performance advantage of the serpentine population based on total flower number was not significant in the high magnesium and low calcium treatments, and only marginally significant in the low magnesium + low calcium treatment (p=0.07, figure 27). There was no significant interaction between magnesium x calcium on plant performance (table 12). Instead, it appears the effects of these two cations are additive.

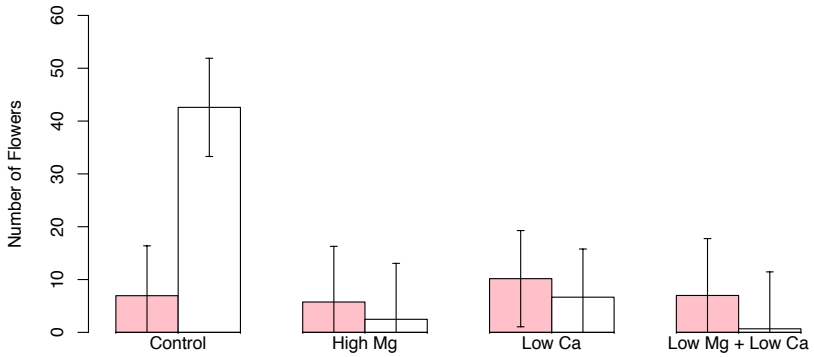


Figure 27. The total number of flowers produced by the serpentine (pink) and sandstone (white) populations across solutions with different concentrations of calcium and magnesium (see table 10). Shown here are least square means +/- 1 standard error from linear mixed effect models fit with REML estimation.

Table 12. Effects of magnesium concentration, calcium concentration, and their interactions with population (serpentine versus sandstone) on total flower number in hydroponic assays

Sources of Variation	F value
Mg	1.20*
Ca	4.20*
Population	0.71
Mg x Ca	0.09
Mg x Population	1.56***
Ca x Population	61.38***
Mg x Ca x Population	0.52

*p<0.05; **p<0.01; ***p<0.001

Discussion

Overall, the results find moderate support for a relationship between flower color and stress tolerance in the populations of *L. parviflorus* at JRBP. Pollinator observations found no pollinator preference for either flower color. Further, constancy metrics indicate that pollinators exhibited random foraging with respect to flower color, and a small bias that made them significantly more likely to visit two different flower colors in succession. These results indicate that flower color differentiation among these populations is not due to pollinator-mediated selection. More pink-flowered individuals survived to flower than the expected proportion of 75%, but across the four years of study, this difference was not statistically significant. (figure 23). No difference was observed on sandstone soil, although this was expected due to the high survival common to both parental populations in this habitat. In 2014, the fecundity of pink-flowered plants was greater than white-flowered plants on serpentine soil and in 2015, fecundity was greater for white-flowered plants on sandstone soil (figure 24). However, across most years and soil types, the differences in fecundity were not significantly different among the flower color morphs. These results are difficult to interpret in an adaptive context because significant differences in the fecundity of survivors of the parental populations were also not observed in all years and soil types. Several aspects of using this F5 population to study the fitness effects of flower color present difficulties. First, individuals represent a mixture of both parental genomes, potentially confounding the results if other genomic regions are important for survival and reproduction in either habitat. Second, the phenotypes of individuals that die before flowering are unknown. Finally, homozygote and heterozygote pink-flowered individuals cannot be distinguished

phenotypically, and it's unknown whether the genotype influences fitness in either habitat. Studies that use NILs that have the pink flower color phenotype introgressed into the sandstone genetic background and are homozygous for the pink flower color locus overcome these limitations and increase the statistical power to detect the fitness effects of this trait by incorporating data from individuals that die before flowering.

Using this approach, the current study found that the white-flowered NIL had a significant survival advantage on sandstone soil relative to the pink-flowered NIL (figure 25). While the mean survival of the pink-flowered NIL was higher on serpentine soil, this difference was not statistically significant. However, the reaction norm of this genotype resembled the serpentine population, in that survival did not decrease on serpentine soil relative to sandstone soil. Counting the number of flowers to measure fecundity on both soil types is currently in progress. In addition, the white-flowered NIL had a significant fitness advantage in control solutions relative to the pink-flowered NIL (figure 26). While the pink-flowered NIL did not have a fitness advantage in the high magnesium treatment, it lived longer in this treatment than the white-flowered NIL (data not shown). More experiments are needed to determine the mechanism underlying these patterns.

To our knowledge, this is the first study that has demonstrated an association between flower color and magnesium tolerance. Future studies will be aimed at understanding whether this association is caused by pleiotropy or linkage. The genes that regulate anthocyanins in floral tissue are also involved in other stress tolerance pathways (Koes et al. 2005) and therefore allelic variation in a gene that controls expression of this entire pathway could have pleiotropic effects on magnesium tolerance. Other studied cases of flower color variation in natural populations have repeatedly found the same transcription

factor, the R2R3-MYB, to underlie putatively adaptive flower color variation (Wu et al. 2013). However, there has been no demonstrated relationship between variation at the R2R3-MYB and tolerance to edaphic stressors. Furthermore, the finding that allelic variation in this gene repeatedly affects flower color in natural populations has led some to believe that it has fewer pleiotropic effects relative to other genes in the anthocyanin biochemical pathway (Streisfeld and Rausher 2011).

Another possibility is that the gene affecting flower color variation is physically linked to one affecting magnesium tolerance, particularly if both genes are in an area of suppressed recombination such as a chromosomal inversion or centromere. A growing body of evidence suggests that chromosomal inversions may play an important role in local adaptation (Lowry and Willis 2010) and the linkage of adaptive alleles is expected to be favored when gene flow among differentially adapted populations is ongoing (Yeaman and Whitlock 2011). Future studies will investigate the genetic basis of this flower color variation and the mechanisms that lead to its association with magnesium tolerance. This will provide the first demonstration of causal abiotic selective forces operating on flower color and will yield information as to how pleiotropy can contribute to fitness trade-offs generally.

Finally, the current study provides support for the common finding that low Ca:Mg ratios are a selective factor operating on serpentine soil (reviewed in Brady et al. 2005). In hydroponic treatments that varied the concentrations of calcium and magnesium, the sandstone population had higher fitness in benign nutrient solutions while the serpentine population exhibited a fitness advantage (although not statistically significant) in treatments with high magnesium, low calcium, and low levels of both

cations. Although differences were not significant in the assay shown, similar experiments have found a significant survival advantage of the serpentine population in high magnesium treatments (not shown). These results indicate that the serpentine population has adapted to the stressful concentrations of these cations in its native soil.

The ability of plants to survive in soils with low calcium to magnesium ratios may be caused by a greater tolerance to high levels of foliar magnesium, or an increased ability to discriminate between magnesium and calcium cations and/or prevent their transport into leaf tissue. Some studies have shown that serpentine-adapted taxa and their non-serpentine congeners contain similar amounts of calcium and magnesium in their foliar tissue across a range of Ca:Mg ratios (Pakdaman et al. 2013, Palm et al. 2012), indicating that these species are able to tolerate high levels of foliar magnesium. Some taxa even exhibit a greater requirement for magnesium, and their performance is positively associated with magnesium concentrations (Dehart et al. 2014, Pakdaman et al. 2013, Palm et al. 2012, Asemaneh et al. 2007, Marrs and Proctor 1976). A physiological basis for this adaptive strategy has been suggested by Bradshaw (2005) who found that a loss of function mutation in the *CAX1* gene allowed *Arabidopsis* mutants to survive on solutions with low calcium to magnesium ratios that were inhospitable for the wild type. Because *CAX1* functions to maintain cell calcium homeostasis, a loss of function mutation may prevent nonselective cation channels from opening in response to low levels of calcium, thereby preventing the magnesium poisoning that would occur when concentrations of magnesium are high. However, these nonfunctional cation channels would also lead to a higher magnesium requirement and make plants more vulnerable to

calcium toxicity, which explains the increased performance of some serpentine-adapted plants in environments with increased magnesium.

Alternatively, instead of a physiological tolerance or greater requirement for foliar magnesium, some plants may instead be better able to discriminate between calcium and magnesium cations, and thus take up less magnesium relative to their non-serpentine congeners at the root level or selectively transport less magnesium from their roots to shoots (Arnold et al. 2016, Asemaneh et al 2007, O'Dell et al 2006). Support for this mechanism come from studies that find a higher ratio of Ca:Mg in vegetative tissue among serpentine plants even in the presence of low Ca:Mg environments (Arnold et al. 2016, Sambatti and Rice 2007, O'Dell et al. 2006, Rajakaruna et al. 2002, Walker et al. 1955). The ability to discriminately transport magnesium and calcium cations explains how some plants maintain consistent performance across a range of Ca:Mg ratios (O'Dell et al. 2006, Rajakaruna et al. 2002, Walker et al. 1955). Future studies will be aimed at understanding the physiological mechanisms underlying serpentine tolerance in this system.

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