Spring 5-2017

Divergent allocation strategies between annuals and perennials in the field

Genevieve Pilch
Syracuse University

Abstract

Within a species, geographic populations often diverge as they adapt to their local environments. Such divergence can be an early step in speciation, especially when it results in reproductive isolation and a reduction of gene flow. In the wildflower *Mimulus guttatus*, local adaptation is observed with variation in life history strategies. Annual plants flower early and invest very little in vegetative growth, whereas perennials spend more time growing vegetatively before reproducing. My study investigates the genetic basis of these alternative strategies in the field using an F4 cross between parent populations representative of the two ecotypes. I grew 24 replicates of each inbred parent and 537 F4 offspring. Phenotypic observations taken in the field assessed allocation to vegetative versus reproductive growth, as well as overall fitness. DNA collected in the field, extracted in the lab, and sequenced at the University of Rochester will be combined with phenotypic data for Quantitative Trait Loci (QTL) mapping. This will identify regions of the genome that explain phenotypic variation, and comparisons of the QTL for multiple traits will indicate the role of linkage or pleiotropy in the evolution of *M. guttatus*. Current results come from correlations in the phenotypic data. They show that while some traits that were correlated in parent populations are broken up by the rounds of recombination in the F4 population (e.g. stolon number and flower number); other traits remain linked (e.g. flowering date and senescence). These results suggest that evolution may act on combinations of traits as populations become adapted to their environments.

Executive Summary

Biodiversity is generated and maintained through evolutionary processes like selection and adaptation. A better understanding of these mechanisms gives insight into how current species came about and can also be used to evaluate future evolutionary responses. *Mimulus guttatus*, a common wildflower, provides a great opportunity for such studies. Local adaptations are observed in this species through variation in life cycle and resource allocation, with plants falling into one of two main categories. Annual plants complete their entire life cycle in one year, then die. They tend to flower early and invest very little in vegetative growth. Perennials can survive over multiple years and generally spend more time growing vegetatively before reproducing. Annual and perennial populations of *M. guttatus,* coexist throughout its range. By looking at genetic differences between representative populations, we can examine the influence of genetics on the traits that have diverged during their evolution.

The genetic architecture of traits can affect their evolution. For example, genes that are located closer together on a chromosome are seldom broken up by recombination and therefore their evolutionary fate may be linked. Another example is when a single gene controls multiple traits, so that the fate of those traits cannot be independent. These types of genetic interactions make it impossible for selection to act on a single trait without affecting other traits, which can accelerate or constrain evolution. By crossing plants from divergent populations for multiple generations, we can expect to shuffle the genes for many of the traits found in the parent plants. Therefore, traits that remain correlated in the offspring of this cross indicate either that the genes are physically close together, or that a single gene controls multiple traits. Analysis of the combined genetic and phenotypic data from this cross can map traits onto specific regions of the

genome and determine the role of gene interactions play in evolution. I recorded phenotypic data while my plants were growing in the field, and DNA was collected in the field, extracted in the lab, and sequenced at the University of Rochester. Analysis of this genetic data is ongoing, so my current results are based solely on the phenotypes observed.

These results show evidence of linkage between some traits. For example, first flowering date and senescence remained correlated in the mapping population, with early-flowering plants dying sooner and late-flowering plants living longer. This difference is not large enough to prevent mating between the two groups and produce reproductive isolation on its own, but it may contribute to divergence. Other trait combinations usually correlated in the parent populations, like allocation to vegetative versus reproductive growth, appear to have broken from original patterns and therefore do not show evidence of linkage. These findings will help to focus research on interesting trait combinations once the genetic data has been analyzed and combined with phenotypic measurements, which increases our understanding of how selection acts on combinations of traits and populations become adapted to their environments.

Table of Contents

List of Figures

Acknowledgements

I'd first like to thank the Biology Department for funding my research over two summers and the Renée Crown Honors Program for providing the funds for genetic sequencing through one of their Crown Awards.

I also could not have completed my capstone without the other members of the lab I work in. Dr. Jannice Friedman accepted me into her lab during my sophomore year, worked with me to develop my project, and always supported and guided my research. Matt Rubin taught me many of the methods necessary to carry out this research and answered all of my questions along the way. And Kelly Schmid spent a summer with me in the field, helping with measurements and tissue collections. I'd also like to thank all of the other members of the Friedman lab for making research a very positive part of my undergraduate experience.

Introduction

How and when to reproduce are some of the most basic questions faced by all life forms. An organism's fitness is determined by its ability to pass its genes on to the next generation. Mating and reproduction can also be incredibly costly; for plants, such costs include the energetic investment in structures such as flowers and fruits (Snow and Whigham, 1989). Due to limited resources, investment in vegetative and reproductive growth potentially trade off with one another (Watson, 1984). If they are not allocated appropriately, the organism may run out of resources prior to reaching its reproductive phase and die without passing on its genes. Or if the shift to reproductive growth is timed incorrectly, reproduction may be unsuccessful due to an absence of mates, pollinators, or poor weather, resulting in a fitness of zero. Numerous factors are involved in this successful timing of reproduction, including pollination and fertilization as well as the development, dispersion, and germination of seeds at appropriate times (Rathcke and Lacey, 1985).

The conditions of a plant's environment influence the optimal allocation strategy, which can result in local adaptation and, over time, population divergence (van Kleunen, 2007). Understanding the process of local adaptation remains one of the central questions in evolutionary biology (Hoban et al., 2016). Focusing on the earliest stages of divergence creates a foundation for the origins of biodiversity. How does selection produce divergent reproductive strategies, and what are the traits involved? A robust answer to this question requires an understanding of both phenotypic and genotypic divergence. In particular, we need to understand how many genetic changes underlie adaptive changes and how such underlying genetic

architecture can accelerate or constrain evolution (Orr, 2005; Ågren et al., 2013). Can these factors lead to general evolutionary patterns across species?

One widespread evolutionary transition in plants is that between annual and perennial life history strategies (Stebbins, 1974). Annuals are plants that complete their entire life cycle in one year, generally flower quickly, and have small flowers, whereas perennials continue growing over many years and exhibit traits such as increased vegetative growth, delayed flowering, and large flowers (Friedman and Rubin, 2015). It is thought that annual populations evolved from perennials as an adaptive response when spreading into harsher climates (Stearns, 1976). Annuals are generally found in drier or more frequently disturbed areas where they have a limited amount of time to spread their seeds before death, whereas perennials tend to be found in less stressful environments (Barbier et al., 1991; Evans et al., 2005; Cruz-Mazo et al., 2009). These differences in environment result in different selective pressures that may lead to local adaptation and population divergence.

Mimulus guttatus (common monkey flower) is a model system for ecological and evolutionary genetics (Clausen et al., 1940; Vickery 1952; Clausen and Hiesey, 1958; Twyford et al., 2015). Found naturally on the west coast of North America, it is a highly variable species containing both annual and perennial populations (Pennell, 1947; Lowry et al., 2008). An illustration of this divergence can be seen in the plants in Figure 1, grown in a common field environment. The annual life history strategy likely evolved as *M. guttatus* spread into Mediterranean climates that experience intense summer drought, and rapid flowering provided a drought-avoidance mechanism (Stearns, 1976; Hall and Willis, 2006). Perennials are generally found near permanent bodies of water (Lowry et al., 2008).

This system provides an excellent opportunity to incorporate evolutionary genetics into the study of reproduction, elucidating how genetic variation leads to phenotypic differences and ultimately how selection causes evolutionary transitions between alternative reproductive strategies. In particular, my research uses quantitative genetics, exploring the genetic architecture of complex traits and their interactions over the entire genome as opposed to simply identifying one or two genes or interest (Hill, 2010). This is a more inclusive approach that simultaneously assesses an individual's fitness and explores its genetic architecture (Stinchcombe and Heokstra, 2008). The combination of genetic and phenotypic data is necessary for understanding evolutionary trajectories as both can act as constraints on selection (Lande, 1982). An understanding of these processes can be further applied to predict the ability of specific species to adapt to changing environments or selective pressures in the future. In my research, I will address the following questions: How did natural selection drive differences between annuals and perennials? Did suites of traits evolve together, in a correlated way? And do just a few genomic regions underlie the variation, or are many smaller-effect genetic loci involved?

Methods

Study system

Mimulus guttatus exhibits extensive variability in growth and reproduction throughout its natural range, including both annual and perennial populations (Pennell, 1947). These ecotypes are interfertile, allowing us to create recombinant mapping populations to investigate the genetic architecture underlying phenotypic traits of interest (Vickery, 1978). In this study, I use a mapping population created from the parental populations, SWB and LMC. SWB is a coastal perennial population found in Irish Beach, Mendocino Co., CA (coordinates: 39º 02' 09" N, 123º 41' 2" W). LMC is an annual population from Yorkville, Mendocino Co., CA (coordinates: 38º 51' 50" N, 123º 05' 02 W) (Lowry et al., 2008). These sites are about 50 km apart, with a similar overall environment, but varied growing seasons due to a difference in water availability (Figure 2).

Generation of parental and F4 mapping populations

Previously, seeds were collected from the LMC and SWB parental populations in the field and self-pollinated for several generations through single seed descent (LMC-G8, SWB-G7) to remove heterozygosity (Friedman and Willis, 2013). Inbred SWB and LMC were then crossed to create a single F1 heterozygote, which was self-pollinated to generate 1200 F2 individuals. F2s were randomly paired and cross-pollinated to create 600 F3 plants. Two seeds from each F3 were used to produce plants that were then paired to create 600 F4s. This crossing design generated a mapping population referred to as Recombinant Inbred Advanced Intercross Lines, RIAIL (Rockman and Kruglyak, 2008). The benefit of this design is that individuals are outbred and have multiple recombination breakpoints (Figure 3), which facilitates genomic

mapping of traits of interest. Here I use 275 of the seed families with 1-2 replicates from each F3 cross for a total of 537 F4 plants.

Plant care

In addition to the 537 F4 offspring, I grew 24 replicates of each inbred parental line. I planted approximately 4 seeds for each individual in a 96 well plug tray filled with Fafard 4P growing mix. I cold treated the seeds at 4°C in the dark for 10 days, then moved them to a walk-in growth chamber set to a 16-hour photoperiod with 21/18°C day/night temperature cycles and 50% humidity. I misted the plants two times daily and bottom watered daily for 60 minutes. Following germination, I bottom watered plants daily for 60 minutes. Three days after peak germination, I thinned to one seedling per pot. Five days later, I began to gradually acclimate the plants to sunlight in preparation for field transplantation by exposing them to 1hr of outdoor conditions on day one, then 4, 8, and 16hr for three consecutive days, respectively. By the fifth day, the plants remained outside full time.

Field experiment

Once acclimated, I transplanted all 585 plants into a field site at Syracuse University, (Syracuse, NY, USA; 43° 0' 48" N, 76° 7' 8" W; Figure 4). I laid a ground cloth to limit weed growth, and cut 10 cm square holes every 30 cm, assigned plants to random locations, and transplanted them into the grid. The field site was fenced to prevent damage from large herbivores. I watered the plants with overhead sprinklers for fifteen minutes twice daily at dawn and dusk. I removed weeds from experimental blocks once a week to reduce competition and encroachment by other plants.

Phenotypic trait measurement

Over the duration of the field experiment, I measured a suite of morphological, phenological, floral, and fitness phenotypes. Ten days after transplant, I recorded longest leaf length as an initial measure of vegetative growth. At the same time, I recorded each plant's overall anthocyanin content on a scale from 1 (green) to 3 (red) as an indicator of stress from transplantation. On the day a plant first flowered, I measured another suite of traits: the date of first flower, traits assessing vegetative growth, including longest leaf length (cm) and stolon number, plus floral traits including flower corolla width (cm) and anther-stigma separation (cm) as measures of allocation to reproduction and fitness. Anther-stigma separation was measured as the distance between the upper whorl of anthers and the height of the stigma lobe. Three weeks after flowering, I recorded stolon number and height again as measures of vegetative growth and overall size, and total flower number as a measure of reproductive fitness. For the last ten weeks of data collection, I measured senescence with a rating of 1(flowering) to 3 (dead) each week. During the field season, many plants were damaged by weather conditions or small animals, so in addition, I recorded level of damage for each plant.

One-to-seven days after first flowering, I collected all 4 anthers from an unopened flower on each plant for pollen quantification; following the protocol I developed (Appendix 1). Unopened flowers were selected to ensure that no pollen had been dehisced. I dried the samples for 24 hours at room temperature and stored them in 1mL of 70% ethanol in microcentrifuge tubes. To prepare the samples for pollen counts, I submerged the tubes in an ultrasonic bath for two minutes and mixed them on a vortex for ten seconds. Immediately after vortexing, I drew a 10uL representative sample from the middle of the tube and placed it onto a microscope slide. I added a drop of basic fushcin to stain pollen grains (Beattie, 1972). I allowed the pollen

subsample to dry for one minute on the slide, then photographed the slide using a Leica S8 APO microscope at 1.25X magnification (Figure 5). Using Photoshop, I increased clarity and hand drew grid lines, then counted pollen samples using a handheld counter. I multiplied the number of pollen grains in the subsample by the total volume in the microcentrifuge tube to estimate the total number of pollen grains for a single flower.

DNA extraction, quantification, and sequencing

During the field season, I collected tissue from each plant in the form of 3-4 floral buds or young leaves and froze them in dry ice in the field. I stored samples in a -80°C freezer. Later, I used a modified CTAB method to extract genomic DNA (Friedman and Willis, 2013). I determined DNA concentrations with the Quanti-iT^{TM} PicoGreen dsDNA Assay Kit and a BioTek fluorescent plate reader. The output from this analysis is relative fluorescence values (Table 1). Samples with known concentrations of DNA were used to create a standard curve, which was then used to calculate the concentrations for the rest of the samples from their fluorescence readings (Figure 6). I then diluted each sample to have the same DNA concentration.

Next, I digested the DNA with the ApeKI enzyme and tagged each sample with a unique barcode (Elshire et al., 2011). The 288 uniquely tagged individuals were pooled into a single sample and indexed with Illumina barcodes. This library was sent to the University of Rochester Genomics Research Center, where a quality assurance step was performed to confirm that fragment lengths fit our target values. BioAnalyser data showed significant amounts of fragments outside of our target range (Figure7). PippinPrep (Sage) was used to remove fragments less than 150 base pairs long so none of our reads were wasted. These small fragments would either be missing a barcode, or have little DNA to sequence after the barcode. The

remaining DNA was sequenced on an Illumina Hi-Seq 2500. Over 230 million reads were recorded, and about 97% pass quality control, which provides sufficient material to perform genetic analyses.

Data analysis

I used Microsoft Excel 2007 to perform my analyses of phenotypic data and generate figures. To visualize the distribution of trait values in the F4 mapping population, I generated histograms for each trait. I also overlaid parent means and standard deviations to show the relationship between the parent lines and the F4s. I used correlation analyses to test for relationships between pairs of traits and ANOVA and t-tests to evaluate differences across categorical variables and determine the significance of results.

JMP Pro was used to calculate residuals (JMP®, Version *13*. SAS Institute Inc., Cary, NC, 1989-2007). Because this experiment was carried out in the field, variation between microsites could alter phenotype expression. To account for this effect, I separated individuals into 16 blocks based on their positions in the field. Means for each trait were then calculated within blocks and compared to overall means. These values were used to adjust individuals within each block and remove the effect of location. I then used the transformed data to create matrices of Pearson correlation coefficients, again in JMP Pro.

Castle-Wright estimator of gene number is a statistic used to approximate the number of loci controlling a trait when only phenotypic data is available. It is calculated using the following equation (Lynch and Walsh, 1998):

$$\#\text{ of loci} = \frac{(\text{high parent mean} - \text{low parent mean})^2}{8 \times [\text{variance of F4s} - (\text{variance of high parent} + \text{variance of low parent})/2]}$$

Results

Phenotypic data distributions

Distribution curves for 8 traits of interest in Figure 8 shows that parental phenotypes (green and orange lines) always fall within the range of F4 variation (blue curves). This is expected because the F4s contain various combinations of parental alleles and should cover the full range of both parents. However, contrary to expectations, the parents did not always fall on either side of the distribution and in some cases the F4 phenotypes covered a much wider range than that seen in the parent plants.

An example of the type of distribution I expected is shown for stolon number (Figure 8F). Stolons are mainly a feature of perennial populations and are a form of clonal reproduction. These stems spread out from the main plant, root, and establish themselves as new plants genetically identical to the parent. Annuals rarely produce stolons since they exist in harsher environments where clonal offspring may not be able to survive for very long (Dole, 1992; Lowry et al., 2008). In these conditions it is more beneficial to produce seeds which will grow quickly following rainfall than to produce clones with a low probability of survival. The parents in my experiment fit these expectations, with LMC averaging 1.25 stolons per plant and SWB 5.46 (Table 2). The F4 distribution covers this entire range, and peaks between the two parent means, as predicted.

A trait that does not exhibit this pattern is total flower number (Figure 8G). Both parent means fall well below the majority of the F4 offspring. Annuals, since they depend more on seeds than stolons, are expected to produce more flowers than perennials (Lowry et al., 2008; van Kleunen, 2007), but in my experiment the opposite was true. I expected the peak of F4s to be

intermediate, but it falls beyond both parent ranges. This latter observation may be due to gene interactions from recombination, but the former is concerning and could be an artifact of my experimental design. These possibilities are addressed in the discussion.

Another conclusion can be made based on leaf lengths 1 and 2, showing a change in vegetative growth over time (Figures 8C and 8D). Relative to the F4 curve, the LMC mean stays in roughly the same position from one graph to the other. But SWB's value moves much farther to the right, indicating a change in allocation to vegetative growth. Because perennials generally experience moister climates where they are less stressed, their growth tends to be slower, but more substantial than annuals (Lowry et al., 2008). Since leaf length was first measured ten days after transplantation to the field, at this time annuals were nearing their reproductive stage and most of their investment in vegetative growth had already finished while perennials were just beginning to grow. By the time plants were flowering, perennials had been growing steadily for weeks or months, pulling ahead of the annuals. This trend can be observed in the F4 population as well. The average leaf length stays about the same between the two measurements, but the right tail of the graph extends a full centimeter farther, suggesting that many plants followed the annual strategy favoring reproductive growth while some more perennial-like individuals focused more on vegetation.

The various distribution widths also provide information about my data. Some traits like height (Figure 8B) show very steep, narrow curves while others like pollen (Figure 8H) are much wider and more gradual. This variation could be due either to differences in genetic architecture or environmental effects influencing the traits, or a combination of the two. Evidence of environmental influence can be seen by observing the parental phenotypes. The LMC and SWB individuals grown in the field came from the same plant used in the F4 cross and underwent the

same 6-7 generations of inbreeding. Because of this, the replicate plants should have nearly identical genomes. If there was no environmental influence, their phenotypes would also be identical, but the standard deviation bars show that this was not the case. It appears as though the narrowly distributed traits with smaller standard deviations, like height, are influenced less by environmental factors than traits like pollen, with large standard deviations.

Quantitative genetic architecture of traits

The genetic sequencing data is still being analyzed and is not ready to be combined with the phenotypic measurements yet. These are the data that will be used to estimate the number of loci controlling traits of interest, and comparing the genetic architecture of different traits will offer insight into the observed phenotypic correlations. However the results and conclusions I can present in this paper will have to be based on the phenotypic observations made during the field experiment.

One way to estimate the number of loci underlying a trait using only phenotypic data is with the Castle-Wright estimator. When I substitute my values into this equation, the results were not what I expected. Except for total flowers, all of my numbers were negative, and total flowers was less than one (Table 2). This statistic generally falls in the single digits, and getting a negative result does not make sense as a trait cannot be controlled by a negative number of loci. Most of the Castle-Wright estimators are negative because of high variance, which is just the nature of the data. This statistic was not expected to give us highly accurate results, researchers are skeptical about the usefulness of Castle-Wright estimators, especially considering the wide variety of techniques now available that provide detailed genetic data (Zeng et al., 1990). I had hoped that it would give an estimate of the number of loci, but unfortunately that was not possible with the data.

Genetic correlations

Correlation coefficients were calculated within F4, LMC, and SWB populations, for all traits of interest (Table 3). One trend seen in these tables is that measurements like leaf length, branch number, and height are positively correlated (blue shading). This provides evidence for the genetic linkage of various vegetative growth traits, indicating that a genetic correlation influences the phenotypes.

Other traits that stand out are days to flower and senescence, which are both negatively correlated with nearly every other trait (red shading). Therefore, if a plant is shorter and has smaller leaves, it takes a longer time to reach its first flowering date. Additionally, plants with fewer flowers and less pollen tend to die earlier.

Trade-offs among traits

Some correlations between traits that correspond to trade-offs between annual and perennial allocation strategies do not fit with predictions. I expected traits representative of vegetative and reproductive allocation to show a negative correlation, indicating a trade-off. Because resources are limited and selective pressures vary over space, different strategies are optimal for different ecotypes. Perennial-like plants would invest more in vegetative growth and make fewer flowers, and vice versa for the annual-like ones (van Kleunen, 2007). To test this, I examined the correlation between leaf length and flower number (Figure 9A). Contrary to my expectations, these traits showed a positive relationship.

Similarly, stolon number and flower number showed a positive correlation (Figure 9B). Here, stolon number is a measure of clonal reproduction while flower number represents sexual reproduction. Because of resource limitation, I expected plants to invest preferentially in one or the other, annual-like plants making more flowers, and perennial-like individuals more stolons

(Watson, 1984). This would result in a negative correlation between the traits, illustrating a trade-off, but again we found a positive correlation instead.

Reproductive isolation

Reproductive isolation is a key component of evolution. It prevents gene flow between populations or species, mediated through pre and/or post zygotic barriers. This process can promote the divergence of neutral genes over time and accelerate speciation (Widmer et al., 2009; Coyne and Orr, 1998). In *Mimulus*, several traits have been shown to facilitate reproductive isolation. Divergent flower colors attract different pollinators and flowering time determines available mates (Bradshaw et al., 1998; Hall and Willis, 2006). By comparing days to flower and senescence, I was able to look for signs of reproductive isolation between annual-like and perennial-like plants in the F4 population. If these traits remained linked in F4 offspring, the expected pattern would be early-flowering plants dying earlier (like annuals) and later-flowering plants living longer (like perennials).

Results from ANOVA showed a significant effect of senescence on flowering time (F=14.01, P<0.0001), with plants that flowered later also senescing later (Figure10). The senescence measurement used in these analyses grouped plants based on whether they were still flowering; not flowering, but still green; or dead at the end of the field season. Pairwise comparisons showed that plants that were already dead had flowered significantly earlier than the other two groups (Flowering vs. Green: t=0.86, P=0.19; Green vs. Dead: t=4.74, P<0.0001; Flowering vs. Dead: t=3.85, P<0.0001).

Physical damage

The main advantage of a field experiment is that it gives a closer representation of the results that would be seen in nature. However, I was also concerned about possible drawbacks of

this method. Over the course of the field season, many plants were damaged, most of the time on their apical meristem, which is the top of the central stem where growth is focused. This led to concerns that randomly sustained damage could alter total flower number, interfering with the main fitness measurement. If it had only been a few plants I would have removed them from the analyses, but by the end of the experiment nearly 200 plants had been damaged in this way. Removing all of these individuals would have greatly reduced the sample size and the significance of the results. To examine the effects of damage in the field, I looked at the distributions of flower number in damaged versus undamaged plants (Figure 11). Damaged plants actually made a few more flowers than undamaged ones on average, but the amounts were not significantly different (P=0.32). So, damage does not affect this fitness measure. I observed that, although damaged plants made fewer flowers on their apical meristems, they made up the difference with more stolon flowers. Therefore, damage resulted in a change in the location, but not total number, of flowers. These results are consistent with a release from apical dominance.

Discussion

With this research, I have identified several trait interactions warranting further investigation. These include trait correlations in the F4 population consistent with parental phenotypes (suggesting linkage) as well as unexpected F4 trait distributions that indicate some type of gene interaction. In addition, the results highlight the benefits and drawbacks of a field study's experimental design and how it compares to other techniques.

Phenotypic data distributions

The expectation for parent means of traits was that they would fall on either side of the F4 curve, but for many traits that was not the case (Figure 8). One possible explanation for the observed pattern is transgressive segregation, a natural phenomenon that produces extreme phenotypes as a result of genetic interactions in the mating of distinct parental populations (Rieseberg et al., 1999). This is a common occurrence, and is seen more frequently in plants, inbred populations, and intraspecific crosses, all of which describe my experiment (Rieseberg et al., 2003). Looking at Figure 8A, it is possible that gene interactions such as epistasis or complementary alleles are altered when segregating parents are crossed, resulting in unexpected F4 phenotypes.

An alternative explanation for parents not falling at either end of the F4 distribution is that the parental phenotypes observed in the field are not accurate representations of those populations. As described previously, the parental plants were self-pollinated for 6-7 generations before being used to create the cross, then these highly inbred parent plants were grown in the field experiment. With each generation heterozygosity is removed, fixing more of the genome, which is the goal for the parents of a cross. Nevertheless, there are negative side effects as well.

Inbreeding depression is the reduction of fitness that results from mating with closely related individuals (Charlesworth and Charlesworth, 1987; Charlesworth and Willis, 2009). This is also due to the loss of heterozygosity, because as more of a genome is fixed, more of the deleterious alleles that were present in the original plant will become homozygous (Charlesworth and Willis, 2009). *Mimulus guttatus* is known to suffer from inbreeding depression for traits including germination success, aboveground biomass, and total flower number (Dudash et al., 1997). This is observed in Figure 8G where both inbred parents produced fewer flowers than their F4 offspring. The effects of inbreeding depression tend to become more severe with harsher conditions as well, so my plants grown in the field suffered greater fitness costs than we would have seen in a greenhouse study (Armbruster and Reed, 2005). To avoid this issue, I could have planted field seed collected from the parent populations in California instead of the inbred seed.

In the summer following the main experiment, I grew some of these non-inbred parents to assess fitness traits, but these plants cannot be compared directly with my F4 populations since they were grown in different years and experienced different environmental conditions.

Trade-offs among traits

Positive correlations between traits where we would expect to find evidence of trade-offs indicate either that plants are not receiving the same amount of resources or that these resources are being allocated to tissues that we are not measuring (Figure 9). Our expectations of trade-offs assume that all plants have equal access to resources, then "decide" where to allocate them, but that is unlikely to be the case in a field experiment (Watson, 1984). A positive correlation between vegetative and reproductive growth, or between clonal and sexual reproduction, might indicate an overall vigor response (Van Noordwijk and de Jong, 1986; Caruso et al., 2012). This means that certain plants are simply better at acquiring resources than others and they end up

with more resources to invest in both options. When considering the relationship between leaf length and flower number, this explanation makes sense. If a plant invests in larger leaves, it will be able to fix more carbon through photosynthesis and generate more resources for itself.

While a vigor response may explain the results found in this experiment, it raises some larger questions as well. If there are plants that invest more in vegetative growth, clonal reproduction, and sexual reproduction, they are clearly much more fit than other plants. So why do the individuals at the other end of the spectrum, with lower vegetative, clonal, and sexual growth, even exist? Shouldn't plants with superior resource acquisition outcompete those which invest less in every aspect of fitness? These questions are especially pressing given that annual and perennial ecotypes generally correspond to different sides of the trade-offs. Perennials invest more in vegetative and clonal growth, whereas annuals opt for faster flowering with less of a focus on growth.

The explanation for these patterns may be attributable to the field conditions. Although the experiment was carried out in the field, the conditions were not 100% natural. Plants faced little competition due to the ground cloth and regular weeding, and daily watering prevented any drought stress. In my experiment, an individual that used up lots of resources, spent time growing vegetatively, and made both stolons and flowers, would do very well. But in a completely natural environment, competition or drought could alter this result. Plants that depend on a large quantity of resources might never acquire enough to reach their reproductive phase, giving them a fitness of zero. In this situation, the smaller plants which invest less in vegetative and reproductive growth might not make many stolons or flowers, but they would still be able to make a few before they died, giving them the advantage.

Reproductive isolation

A significant difference between flowering times observed for plants with different life spans does not necessarily indicate reproductive isolation. The difference between average flowering times for these groups was only about three days. After a plant has flowered, it will usually continue for at least a week, sometimes several, so an average distance of three days doesn't mean that the earlier-flowering, short-lived (annual) plants never had an opportunity to mate with the later-flowering, long-lived (perennial) ones. Therefore, gene flow could continue between these populations, working against divergence. But much of the time reproductive isolation arises from several contributing factors, not just one (Bradshaw et al., 1998). So, the slight linkage between flowering time and senescence may still play a role in divergence, even if it is not solely responsible for separating ecotypes.

Physical damage

My observations of increased stolon growth as a result of apical meristem damage are consistent with previous reports. When present, apical meristems focus a plant's growth upward, helping it compete for light, pollination, and seed dispersal (Aarssen, 1995). The mechanism for this apical dominance is the production of hormones in the apical meristem that suppress other buds (Cline, 1991). But when this meristem is removed, plants exhibit a change in architecture, switching from vertical to lateral growth (Aarssen, 1995; Huhta et al., 2000). Since the main function of apical dominance is to help a plant compete with its neighbors, individuals in highly competitive environments are more likely to experience fitness costs whereas those in less competitive environments face reduced costs, or even benefits from the release of apical dominance (Aarssen and Irwin, 1991). If a plant has no need to grow vertically to ensure access to light and pollinators, growing from all meristems at once has the potential to produce more

flowers and fruits. From the fitness components measured in my experiment, there was no detectable cost from apical meristem damage. Since my field experiment removed most competition, these results are consistent with the expectations based on past studies.

Further Research

The combination of the phenotypic data presented here with the genetic data that is currently being processed facilitate exploration of genetic interactions in greater detail. A QTL analysis will reveal which regions of the genome explain the variation in traits of interest. I expect traits with correlated phenotypes to show overlapping QTL, confirming that they are controlled either by the same gene (pleiotropy) or genes close to each other on the genome (linkage). The trait combinations identified here will serve as a foundation for these future efforts.

Tables

Table 1. Raw output from a BioTek fluorescent plate reader. Relative fluorescence values are shown for one plate of samples, with standards in columns 1 and 12. Darker blues indicate higher DNA concentrations.

	1	2	3	4	5	6	7	8	9	10	11	12
A	44	2547	4113	239	4375	3744	5467	5359	9012	5740	4779	41
B	1260	5650	4560	4075	4850	2872	3948	8425	6089	5228	4732	1194
C	2412	4696	3571	5269	40	1398	5915	5896	4440	7778	4372	2488
D	4749	5479	3105	6493	2302	3723	8479	2544	8455	6447	6814	4743
E	9887	5827	2107	5022	31	8705	29	2043	8320	7079	9642	9174
F	19828	7921	7931	9103	4379	10817	13654	12576	10380	7995	34	18307
G	25196	4749	6795	13467	5093	7656	40	10512	5053	10220	11021	23661
H	46232	7038	7667	34	6459	8479	11176	5600	3812	8083	1829	48616

Table 2. Means and standard deviations for the traits of interest in LMC, SWB, and F4 populations. The final row shows values for the Castle-Wright estimator of gene number.

		Days to Flower	Leaf Length 1	Leaf Length 2	Height	Branches	Stolons	Total Flowers	Pollen per Flower
LMC	**μ**	49.94	1.62	1.64	14.19	4	1.25	27.5	21393
	σ	4.18	0.48	0.68	5.63	1.07	1.61	14.96	12031
SWB	**μ**	64.62	1.79	2.85	21.32	4.45	5.35	39.45	41930
	σ	11.93	0.49	0.4	5.46	1.96	1.63	27.80	12585
F4	**μ**	48.82	2.4	2.65	24.69	4.41	3.58	76.96	36113
	σ	6.27	0.33	0.59	5.89	1.87	1.42	40.31	11721
CW Estimator		-0.447	-0.050	-1.381	-0.473	-0.029	-1.300	0.057	-0.636

Table 3. A matrix of Pearson correlation coefficients shows relationships between traits of interest for the F4 population, LMC parents, and SWB parents. Darker blue and red highlight more positive and negative values, respectively.

F4	Leaf Length 1	Node	Leaf Length 2	Corolla Width	Anther/ Stigma	Stolons	Branches	Pollen	Days to Flw	Total Flw	Height	Senescence
Leaf Length 1	1	0.16	0.79	0.28	0.05	0.13	0.28	0.19	-0.41	0.46	0.40	-0.07
Node	0.16	1	0.30	0.39	-0.01	0.39	0.48	0.21	0.46	0.39	0.27	-0.29
Leaf Length 2	0.79	0.30	1	0.39	0.11	0.25	0.33	0.24	-0.28	0.52	0.49	-0.08
Corolla Width	0.28	0.39	0.39	1	0.13	0.23	0.32	0.28	-0.04	0.22	0.40	-0.10
Anther/Stigma	0.05	-0.01	0.11	0.13	1	-0.04	0.03	-0.11	0.00	-0.02	0.07	-0.01
Stolons	0.13	0.39	0.25	0.23	-0.04	1	0.10	0.09	0.21	0.33	0.19	-0.31
Branches	0.28	0.48	0.33	0.32	0.03	0.10	1	0.19	-0.15	0.42	0.48	-0.08
Pollen	0.19	0.21	0.24	0.28	-0.11	0.09	0.19	1	-0.17	0.18	0.27	-0.06
Days to Flw	-0.41	0.46	-0.28	-0.04	0.00	0.21	-0.15	-0.17	1	-0.01	-0.29	-0.22
Total Flw	0.46	0.39	0.52	0.22	-0.02	0.33	0.42	0.18	-0.01	1	0.48	-0.17
Height	0.40	0.27	0.49	0.40	0.07	0.19	0.48	0.27	-0.29	0.48	1	-0.10
Senescence	-0.07	-0.29	-0.08	-0.10	-0.01	-0.31	-0.08	-0.06	-0.22	-0.17	-0.10	1

LMC	Leaf Length 1	Node	Leaf Length 2	Corolla Width	Anther/ Stigma	Stolons	Branches	Pollen	Days to Flw	Total Flw	Height	Senescence
Leaf Length 1	1	0.51	0.67	0.21	0.33	0.65	0.42	0.38	-0.40	0.49	0.61	-0.44
Node	0.51	1	0.35	0.25	-0.08	0.22	0.43	0.00	0.02	0.24	0.06	-0.34
Leaf Length 2	0.67	0.35	1	0.55	0.47	0.46	0.32	0.38	-0.35	0.60	0.77	-0.37
Corolla Width	0.21	0.25	0.55	1	0.19	0.08	0.20	0.19	-0.31	0.28	0.26	-0.02
Anther/Stigma	0.33	-0.08	0.47	0.19	1	0.56	-0.33	0.63	-0.07	-0.05	0.64	-0.17
Stolons	0.65	0.22	0.46	0.08	0.56	1	-0.01	0.77	0.01	0.30	0.68	-0.47
Branches	0.42	0.43	0.32	0.20	-0.33	-0.01	1	0.01	-0.30	0.56	0.20	-0.50
Pollen	0.38	0.00	0.38	0.19	0.63	0.77	0.01	1	-0.31	0.35	0.80	-0.42
Days to Flw	-0.40	0.02	-0.35	-0.31	-0.07	0.01	-0.30	-0.31	1	-0.23	-0.34	0.08
Total Flw	0.49	0.24	0.60	0.28	-0.05	0.30	0.56	0.35	-0.23	1	0.64	-0.60
Height	0.61	0.06	0.77	0.26	0.64	0.68	0.20	0.80	-0.34	0.64	1	-0.55
Senescence	-0.44	-0.34	-0.37	-0.02	-0.17	-0.47	-0.50	-0.42	0.08	-0.60	-0.55	1

SWB	Leaf Length 1	Node	Leaf Length 2	Corolla Width	Anther/ Stigma	Stolons	Branches	Pollen	Days to Flw	Total Flw	Height	Senescence
Leaf Length 1	1	-0.09	0.43	0.25	0.19	0.23	-0.10	0.23	-0.67	0.25	0.18	0.01
Node	-0.09	1	0.49	0.51	-0.15	0.36	0.28	0.24	0.82	0.24	-0.09	-0.54
Leaf Length 2	0.43	0.49	1	0.59	-0.01	0.62	-0.08	0.35	-0.13	0.69	0.35	-0.14
Corolla Width	0.25	0.51	0.59	1	0.43	0.48	0.06	0.35	0.20	0.24	0.34	-0.52
Anther/Stigma	0.19	-0.15	-0.01	0.43	1	-0.09	-0.23	-0.09	-0.15	0.00	0.26	-0.25
Stolons	0.23	0.36	0.62	0.48	-0.09	1	-0.42	-0.05	0.22	0.31	0.16	-0.40
Branches	-0.10	0.28	-0.08	0.06	-0.23	-0.42	1	0.52	0.10	-0.10	0.05	0.09
Pollen	0.23	0.24	0.35	0.35	-0.09	-0.05	0.52	1	-0.34	0.46	0.49	0.27
Days to Flw	-0.67	0.82	-0.13	0.20	-0.15	0.22	0.10	-0.34	1	-0.09	-0.24	-0.38
Total Flw	0.25	0.24	0.69	0.24	0.00	0.31	-0.10	0.46	-0.09	1	0.50	0.12
Height	0.18	-0.09	0.35	0.34	0.26	0.16	0.05	0.49	-0.24	0.50	1	0.11
Senescence	0.01	-0.54	-0.14	-0.52	-0.25	-0.40	0.09	0.27	-0.38	0.12	0.11	1

Figures

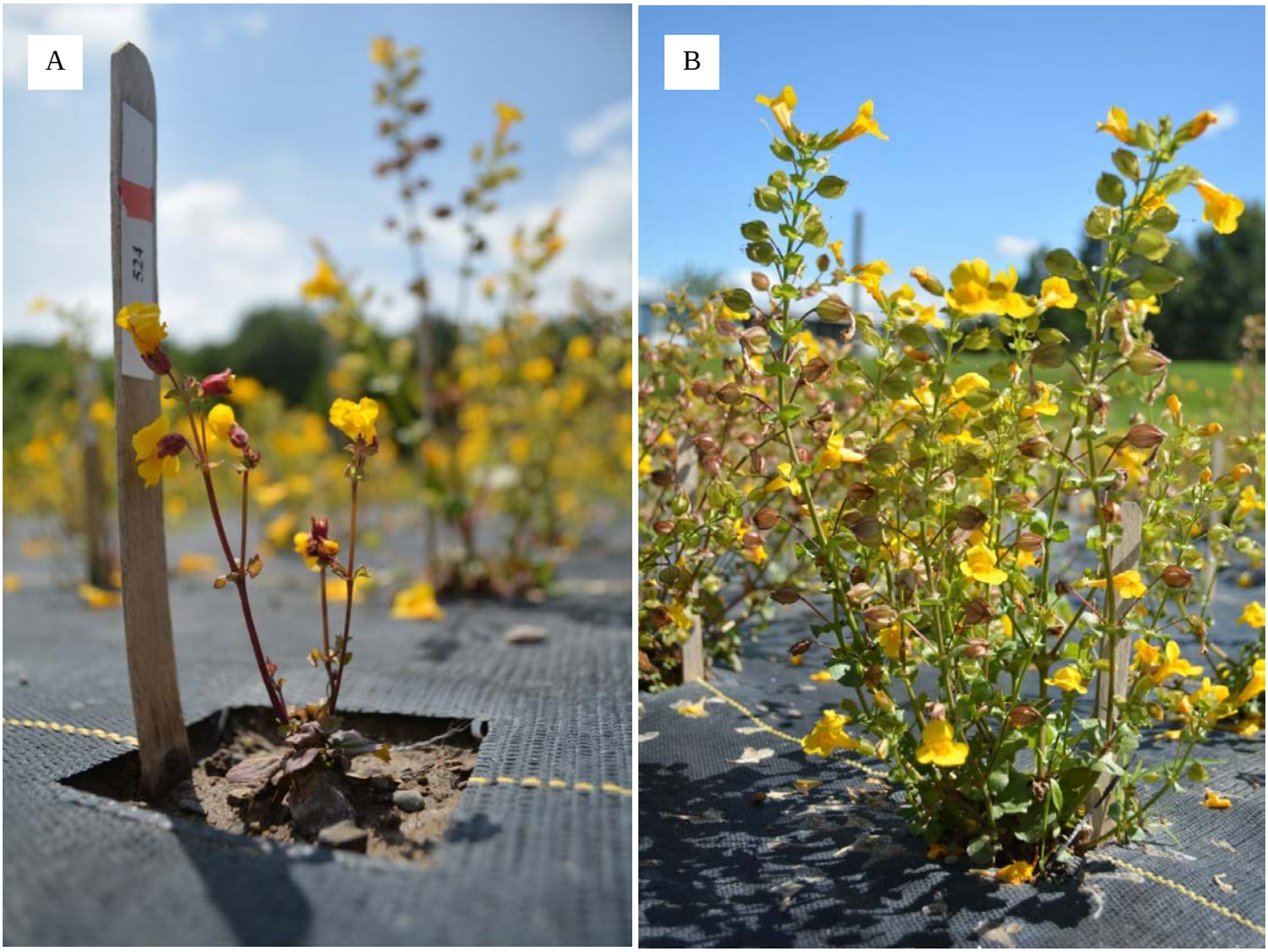


Figure1. Annual (A) and perennial (B) individuals of *Mimulus guttatus* grown in a common field experiment at Syracuse University, summer 2015.

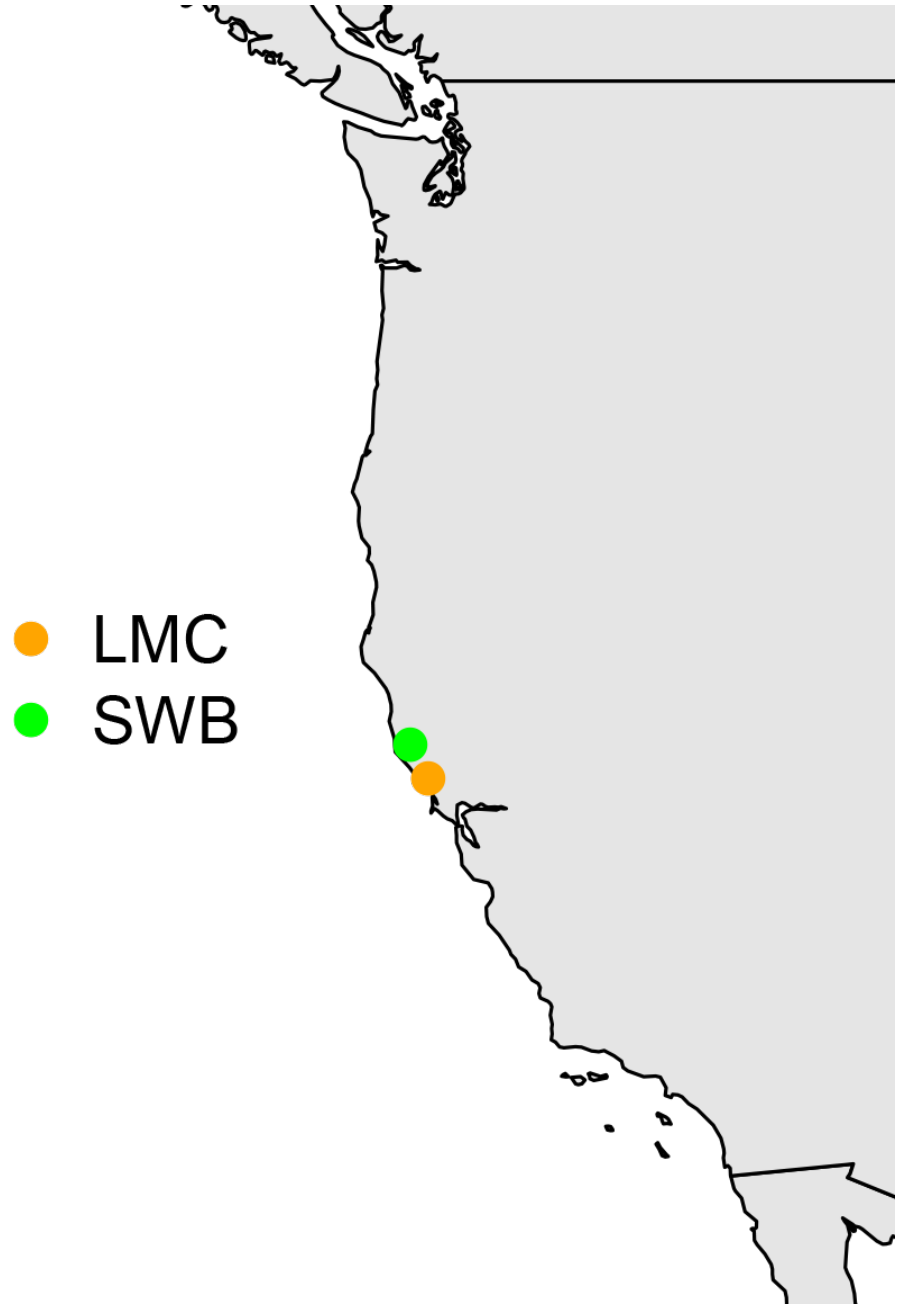
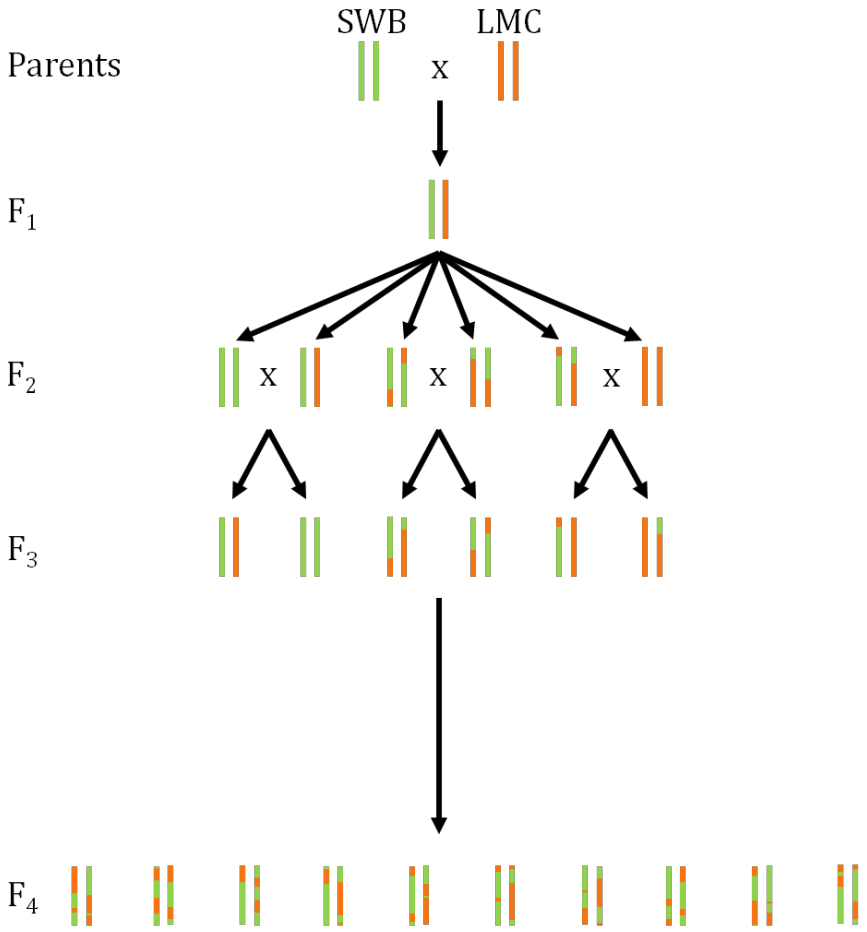


Figure 2. The locations of parental populations (LMC and SWB) on the coast of California, USA. The two populations are about 50 kilometers apart.

Figure 3. A diagram of genetic recombination in an F4 cross between inbred parents LMC and



SWB. Colored bars show representative chromosomes of each individual's genome, with colors indicating which parent each genomic region comes from. Black arrows represent seeds from a cross grown for the next generation. X's fall between mating pairs. Each round of crossing results in more recombination, which can be seen in the increased number of breakpoints in later generations.

Figure 4. Syracuse University field site, August 2015, showing the experimental setup.

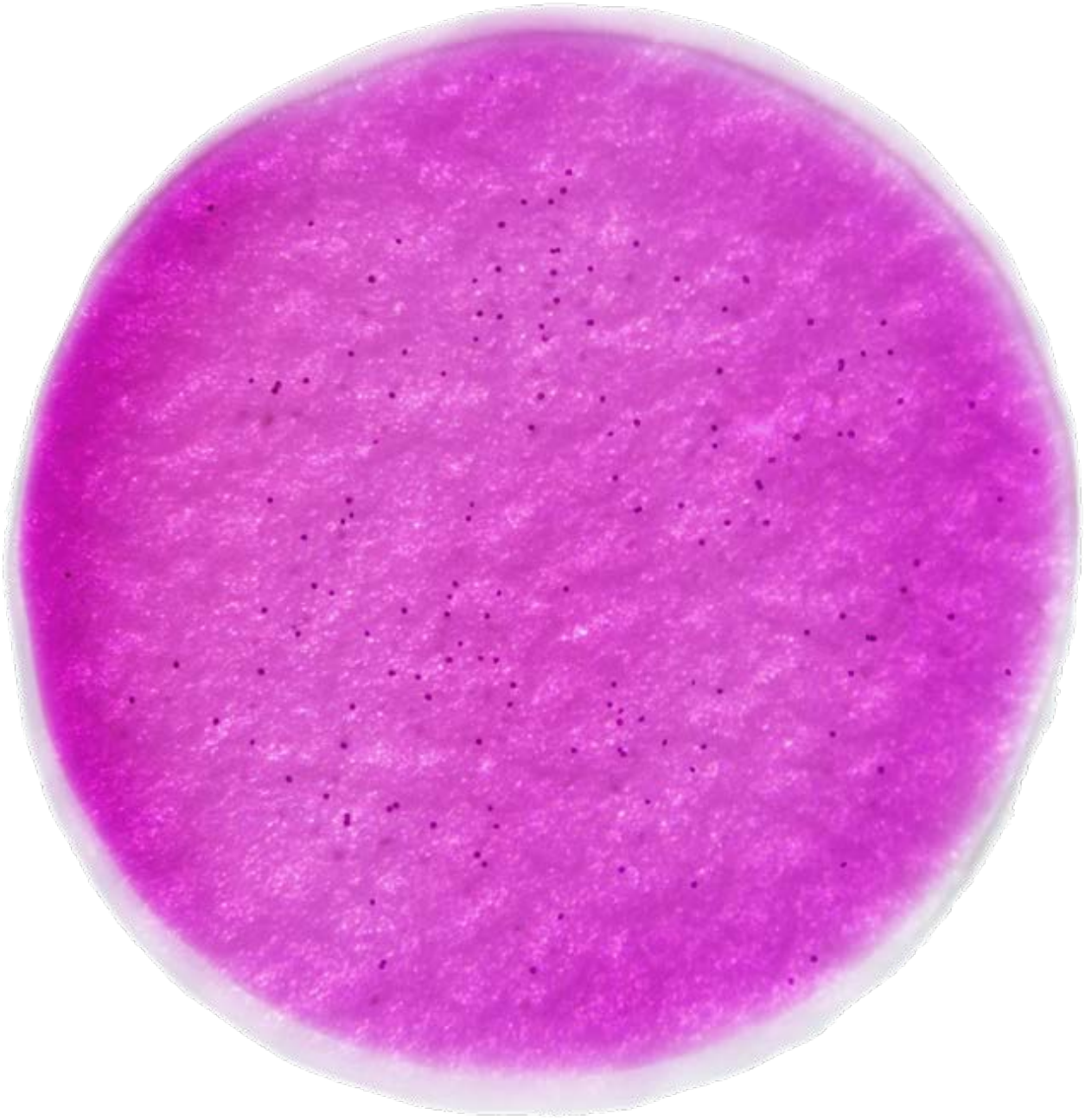


Figure 5. A photomicrograph at 1.25X magnification of pollen grains (small dark purple dots) immersed in ethanol with a basic fuschin stain.

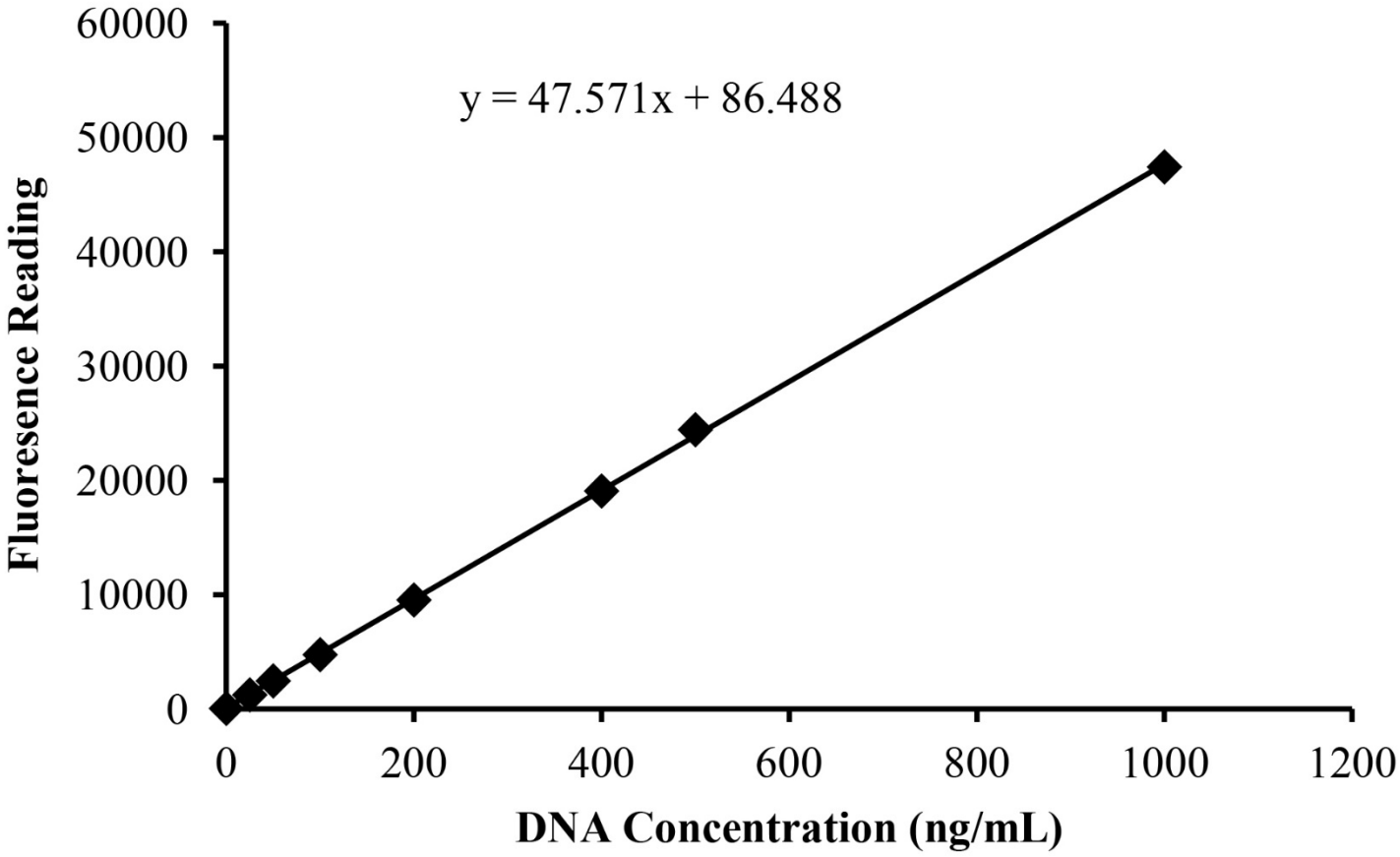


Figure 6. A standard curve for PicoGreen DNA quantification. The equation of the line is used to calculate concentrations of sample DNA from their fluorescence readings.

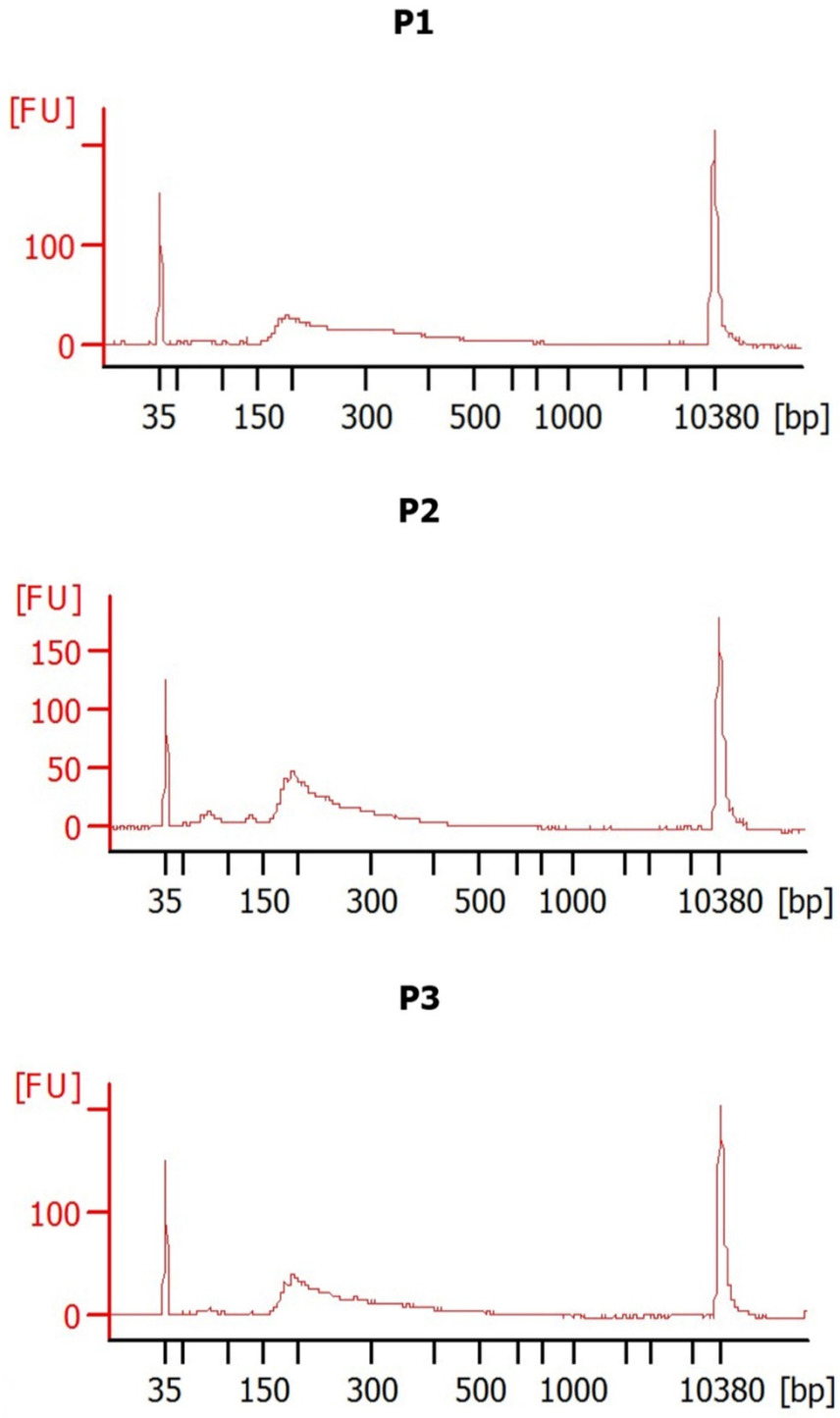
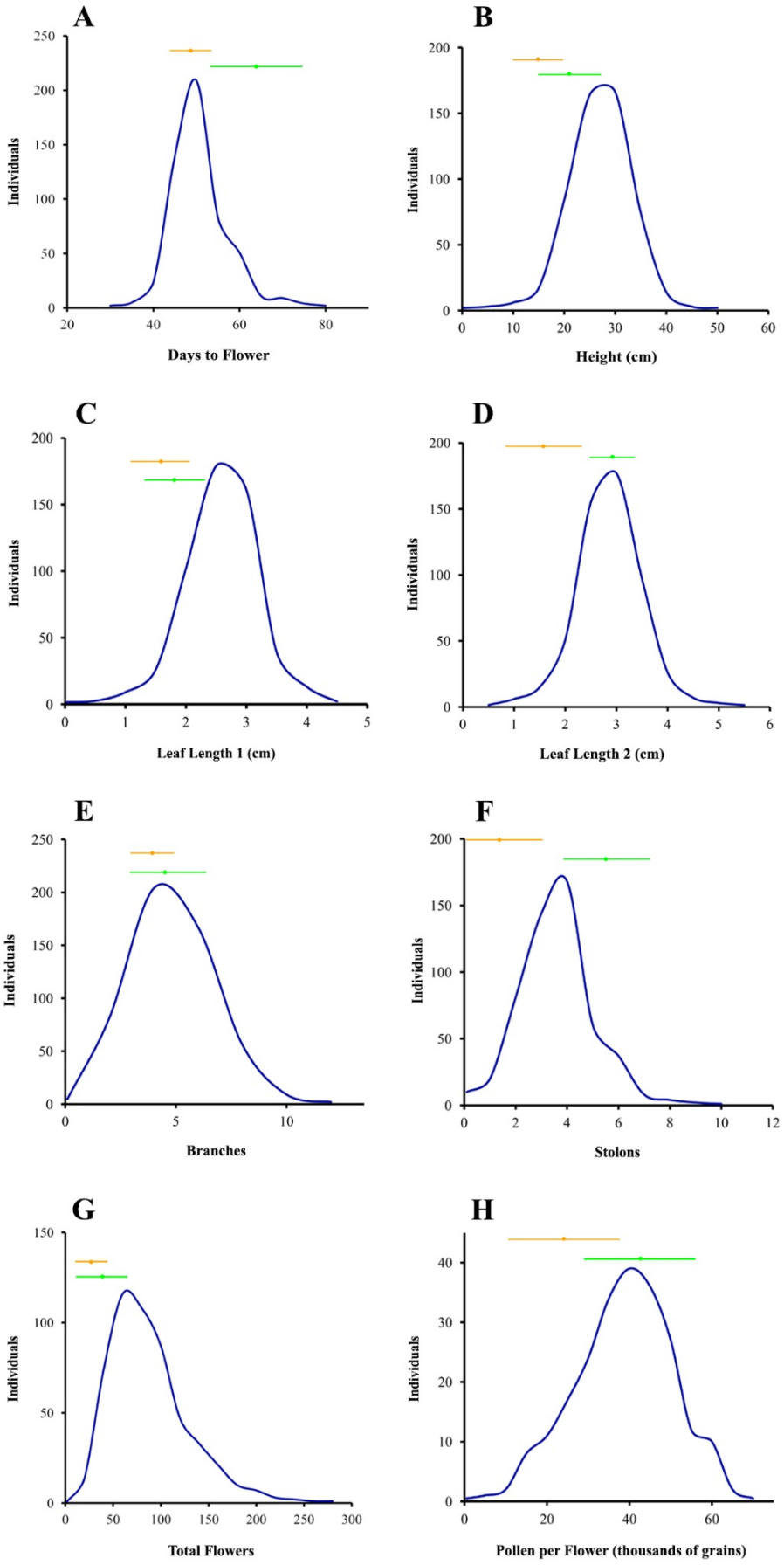


Figure 7. BioAnalyser data from the University of Rochester Genomics Research Center's quality control step for the three plates we sent to be sequenced. The number of fragments (measured in fluorescence units) present for a range of base pair lengths are shown.

aits. Blue curves represent F4



distributions, while orange (LMC) and green (SWB) lines represent parent means with standard deviation. Leaf length 1 was taken a week after transplantation and leaf length 2 on the day of first flowering.

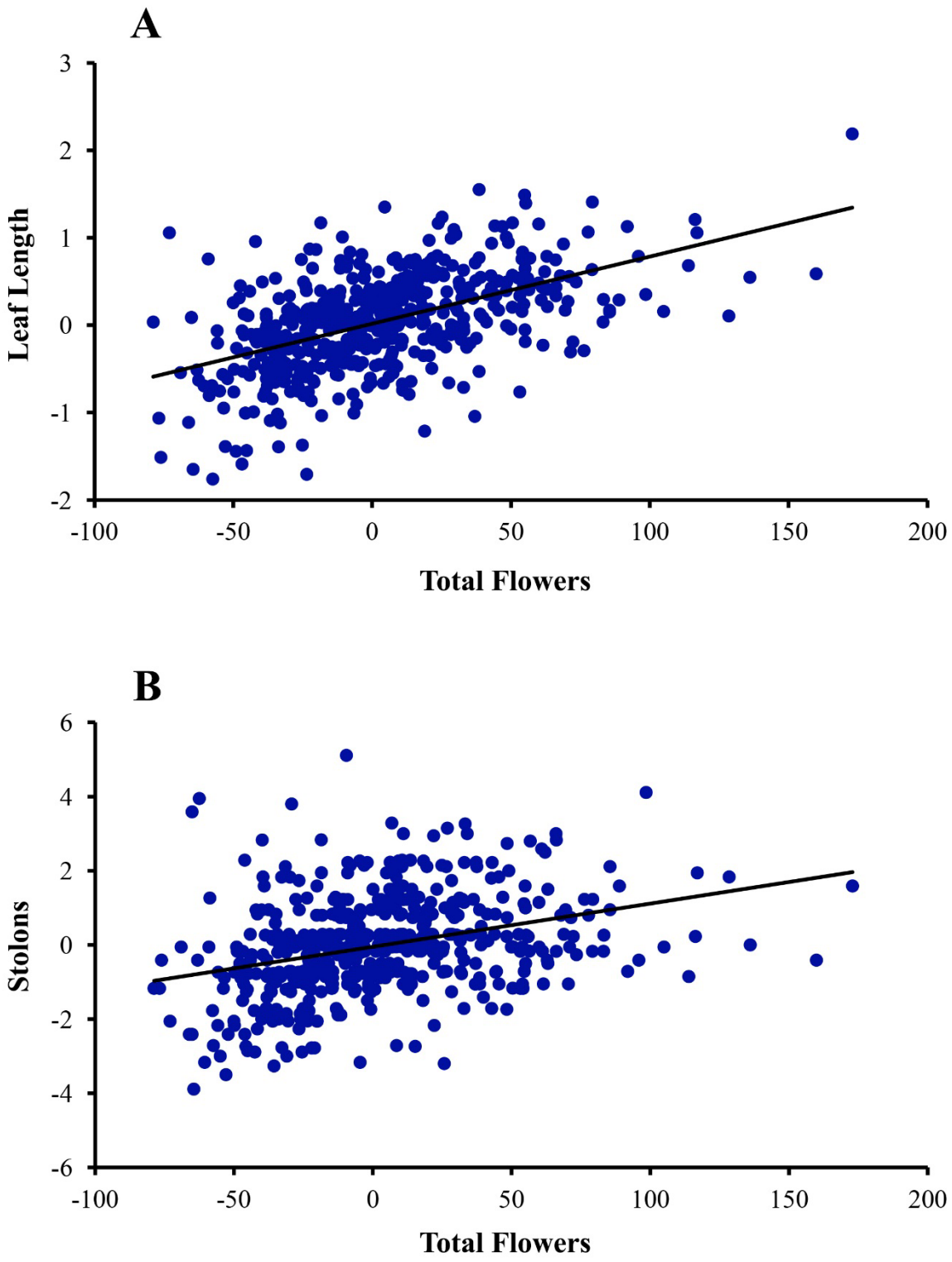


Figure 9. Correlations investigating trade-offs in the field are shown. In A, leaf length 2 (vegetative growth) is compared to total flower number (reproductive growth), ($r^2 = 0.26$, $p <$

0.0001). In B, stolon number (clonal reproduction) is compared to total flower number (sexual reproduction), ($r^2 = 0.11$, $p < 0.0001$).

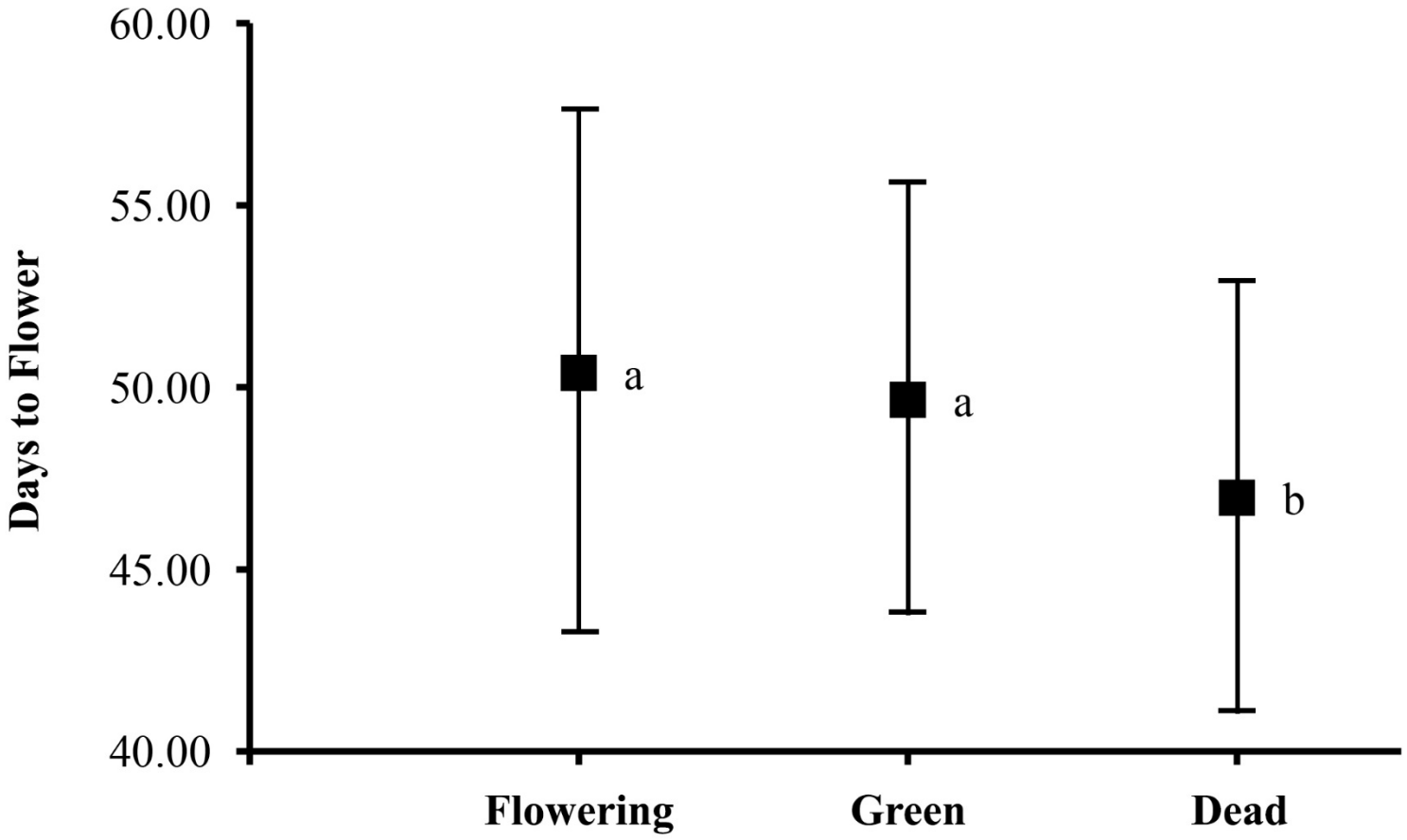


Figure 10. Average days to flower (with standard error) is shown for plants that were still flowering; done flowering, but still green; and dead, on the last day of data collection. Lowercase letters indicate significant differences between groups.

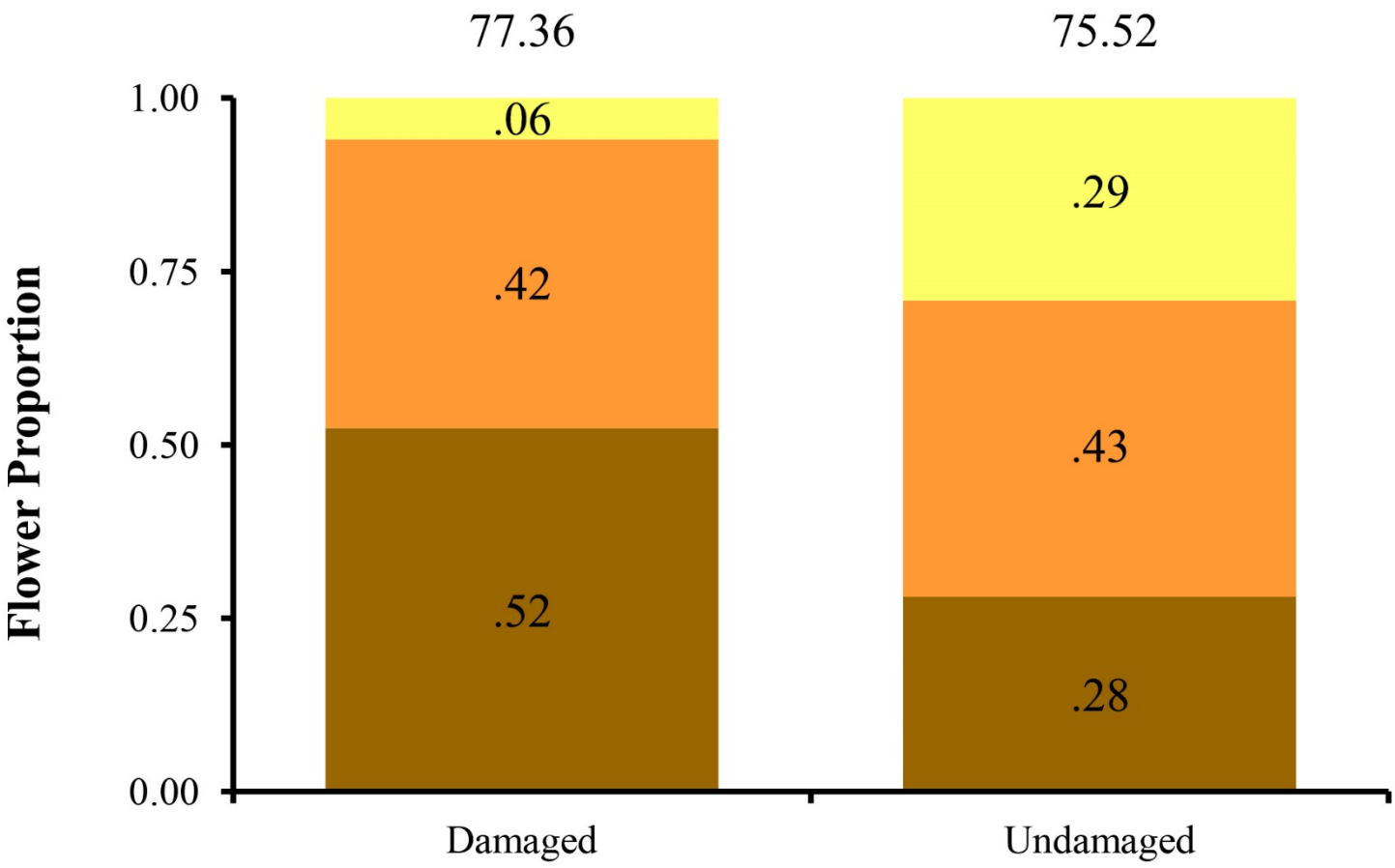


Figure 11. Proportion of total flowers on stolons (brown), branches (orange), and apical meristems (yellow) are shown for damaged and undamaged plants. Mean total flower number for each group is included above.

Appendices

Appendix 1

Pollen Quantification Protocol

Collection

1. Collect all 4 anthers from unopened flowers (close to flowering; collect one day before flower would open) in the field in microcentrifuge tubes
2. Leave samples to dry for 24 hours at room temperature (keep lids closed)
3. Add 1mL of 70% ethanol and store at room temperature

Quantification

1. Ensure that samples are still in exactly 1mL of ethanol (if not refill to 1mL)
2. Put samples in a microcentrifuge tube holder (white with legs) and sonicate for about 2 minutes, water level should exceed ethanol level in tubes
3. Vortex individual samples for 10 seconds
4. Use a pipette to draw 10uL from the center of the tube immediately after vortexing, before pollen has a chance to settle
5. Place onto a microscope slide and add one drop of basic fushcin stain
6. Allow one minute for the pollen grains to stop moving the stain to work
7. Photograph the entire drop through the microscope's photo tube
8. With a computer draw in grid lines and count the grains in each sample
9. Multiply count by 100 for total pollen grains made by one flower

References

Aarssen, L. W.
(1995). Hypotheses for the evolution of apical dominance in plants: implications for the interpretation of overcompensation. *Oikos*, 149-156.

Aarssen, L. W., & Irwin, D. L.
(1991). What selection: herbivory or competition?. *Oikos*, *60*(2), 261-262.

Ågren, J., Oakley, C. G., McKay, J. K., Lovell, J. T., & Schemske, D. W.
(2013). Genetic mapping of adaptation reveals fitness tradeoffs in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences*, *110*(52), 21077-21082.

Armbruster, P., & Reed, D. H.
(2005). Inbreeding depression in benign and stressful environments. *Heredity*, *95*(3), 235-242.

Barbier, P., Morishima, H., & Ishihama, A.
(1991). Phylogenetic relationships of annual and perennial wild rice: probing by direct DNA sequencing. *Theoretical and Applied Genetics*, *81*(5), 693-702.

Beattie, A. J.
(1972). A technique for the study of insect-borne pollen. *The Pan-Pacific Entomologist*, *47*(1).

Bradshaw, H. D., Otto, K. G., Frewen, B. E., McKay, J. K., & Schemske, D. W.
(1998). Quantitative trait loci affecting differences in floral morphology between two species of monkeyflower (*Mimulus*). *Genetics*, *149*(1), 367-382.

Caruso, C. M., Maherali, H., & Benscoter, A. M.
(2012). Why are trade-offs between flower size and number infrequently detected? A test of three hypotheses. *International Journal of Plant Sciences*, *173*(1), 26-35.

Charlesworth, D., & Charlesworth, B.
(1987). Inbreeding depression and its evolutionary consequences. *Annual review of ecology and systematics*, *18*(1), 237-268.

Charlesworth, D., & Willis, J. H.
(2009). The genetics of inbreeding depression. *Nature Reviews Genetics*, *10*(11), 783-796.

Clausen, J., Keck, D. D., & Hiesey, W. M.
(1940). Experimental studies on the nature of species. I. Effect of varied environments on western North American plants. (520).

Clausen, J., & Hiesey, W. M.
(1958). Phenotypic expression of genotypes in contrasting environments. *Phenotypic expression of genotypes in contrasting environments.*

Cline, M. G.
(1991). Apical dominance. *The Botanical Review*, *57*(4), 318-358.

Coyne, J. A., & Orr, H. A.
(1998). The evolutionary genetics of speciation. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, *353*(1366), 287-305.

Cruz-Mazo, G., Buide, M. L., Samuel, R., & Narbona, E.
(2009). Molecular phylogeny of *Scorzoneroides* (Asteraceae): Evolution of heterocarpy and annual habit in unpredictable environments. *Molecular phylogenetics and evolution*, *53*(3), 835-847.

Dole, J. A.
(1992). Reproductive assurance mechanisms in three taxa of the Mimulus guttatus complex (Scrophulariaceae). *American Journal of Botany*, 650-659.

Dudash, M. R., Carr, D. E., & Fenster, C. B.
(1997). Five generations of enforced selfing and outcrossing in Mimulus guttatus: inbreeding depression variation at the population and family level. *Evolution*, 54-65.

Elshire, R. J., Glaubitz, J. C., Sun, Q., Poland, J. A., Kawamoto, K., Buckler, E. S., & Mitchell, S. E.
(2011). A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PloS ONE*, *6*(5), e19379.

Evans, M. E., Hearn, D. J., Hahn, W. J., Spangle, J. M., & Venable, D. L.
(2005). Climate and life-history evolution in evening primroses (Oenothera, Onagraceae): a phylogenetic comparative analysis. *Evolution*, *59*(9), 1914-1927.

Friedman, J., & Willis, J. H.
(2013). Major QTLs for critical photoperiod and vernalization underlie extensive variation in flowering in the *Mimulus guttatus* species complex. *New Phytologist*, *199*(2), 571-583.

Friedman, J., & Rubin, M. J.
(2015). All in good time: understanding annual and perennial strategies in plants. *American journal of botany*, *102*(4), 497-499.

Hall, M. C., & Willis, J. H.
(2006). Divergent selection on flowering time contributes to local adaptation in *Mimulus guttatus* populations. *Evolution*, *60*(12), 2466-2477.

Hall, M. C., Basten, C. J., & Willis, J. H.
(2006). Pleiotropic quantitative trait loci contribute to population divergence in traits associated with life-history variation in *Mimulus guttatus*. *Genetics*, *172*(3), 1829-1844.

Hill, W. G.
(2010). Understanding and using quantitative genetic variation. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, *365*(1537), 73-85.

Hoban, S., Kelley, J. L., Lotterhos, K. E., Antolin, M. F., Bradburd, G., Lowry, D. B., Poss, M. L., Reed, L. K., Storfer, A., & Whitlock, M. C.
(2016). Finding the genomic basis of local adaptation: pitfalls, practical solutions, and future directions. *The American Naturalist*, *188*(4), 379-397.

Huhta, A. P., Tuomi, J., & Rautio, P.
(2000). Cost of apical dominance in two monocarpic herbs, Erysimum strictum and Rhinanthus minor. *Canadian Journal of Botany*, *78*(5), 591-599.

Lande, R.
(1982). A quantitative genetic theory of life history evolution. *Ecology*, *63*(3), 607-615.

Lowry, D. B., Rockwood, R. C., & Willis, J. H.
(2008). Ecological reproductive isolation of coast and inland races of Mimulus guttatus. *Evolution*, *62*(9), 2196-2214.

Lynch, M., & Walsh, B.
(1998). *Genetics and analysis of quantitative traits* (Vol. 1, p. 4). Sunderland, MA: Sinauer.

Orr, H. A.
(2005). The genetic theory of adaptation: a brief history. *Nature Reviews Genetics, 6*(2), 119-127.
Pennell, F. W.
(1947). Some hitherto undescribed Scrophulariaceae of the Pacific states. *Proceedings of the Academy of Natural Sciences of Philadelphia, 99*, 155-199.
Rathcke, B., & Lacey, E. P.
(1985). Phenological patterns of terrestrial plants. *Annual Review of Ecology and Systematics, 16*(1), 179-214.
Rieseberg, L. H., Archer, M. A., & Wayne, R. K.
(1999). Transgressive segregation, adaptation and speciation. *Heredity, 83*(4), 363-372.
Rieseberg, L. H., Widmer, A., Arntz, A. M., & Burke, B.
(2003). The genetic architecture necessary for transgressive segregation is common in both natural and domesticated populations. *Philosophical Transactions of the Royal Society of London B: Biological Sciences, 358*(1434), 1141-1147.
Rockman, M. V., & Kruglyak, L.
(2008). Breeding designs for recombinant inbred advanced intercross lines. *Genetics, 179*(2), 1069-1078.
Snow, A. A., & Whigham, D. F.
(1989). Costs of flower and fruit production in *Tipularia discolor* (Orchidaceae). *Ecology, 70*(5), 1286-1293.
Stearns, S. C.
(1976). Life-history tactics: a review of the ideas. *The Quarterly Review of Biology, 51*(1), 3-47.
Stebbins, G. L.
(1974). *Flowering plants: Evolution above the species level*. Cambridge, Mass: Belknap Press of Harvard University Press.
Stinchcombe, J. R., & Hoekstra, H. E.
(2008). Combining population genomics and quantitative genetics: finding the genes underlying ecologically important traits. *Heredity, 100*(2), 158-170.
Twyford, A. D., Streisfeld, M. A., Lowry, D. B., & Friedman, J.
(2015). Genomic studies on the nature of species: adaptation and speciation in *Mimulus*. *Molecular Ecology, 24*(11), 2601-2609.
van Kleunen, M.
(2007). Adaptive genetic differentiation in life-history traits between populations of *Mimulus guttatus* with annual and perennial life-cycles. *Evolutionary Ecology, 21*(2), 185-199.
Van Noordwijk, A. J., & de Jong, G.
(1986). Acquisition and allocation of resources: their influence on variation in life history tactics. *The American Naturalist, 128*(1), 137-142.
Vickery, R. K.
(1952). *A study of the genetic relationships in a sample of the Mimulus guttatus complex*. Dept. of Biological Sciences.
(1978). Case studies in the evolution of species complexes in *Mimulus*. *Evolutionary Biology* (pp. 405-507). Springer US.

Watson, M. A.
(1984). Developmental constraints: effect on population growth and patterns of resource allocation in a clonal plant. *The American Naturalist, 123*(3), 411-426.
Widmer, A., Lexer, C., & Cozzolino, S.
(2009). Evolution of reproductive isolation in plants. *Heredity, 102*(1), 31-38.
Zeng, Z. B., Houle, D., & Cockerham, C. C.
(1990). How informative is Wright's estimator of the number of genes affecting a quantitative character?. *Genetics, 126*(1), 235-247.