Title: Local adaptation to an altitudinal gradient: the interplay between constitutive trait variation and phenotypic plasticity in *Mimulus laciniatus*

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ABSTRACT

Organisms can adapt to environmental heterogeneity through two mechanisms: (1) constitutive expression of population genetic variation or (2) phenotypic plasticity. We investigated whether patterns of plastic and constitutively expressed traits along elevational and latitudinal clines in a North American endemic plant, *Mimulus laciniatus*, were consistent with local adaptation. We grew inbred lines of *M. laciniatus* from across the species' range to measure constitutive and plastic trait expression in several traits previously shown to be involved in adaptation to *M. laciniatus's* rocky outcrop microhabitat: flowering time, size-related traits, and leaf shape. We then tested for a relationship between variation in trait expression and population elevation and latitude, and examined correlation in trait expression between plastic and constitutive traits. We found that population variation in constitutive leaf lobing is associated with latitude, and identified multiple traits that show patterns consistent with local adaptation to elevation: critical photoperiod, flowering time, flower size, leaf lobing, and leaf lobing plasticity. These trends occur along multiple geographically independent altitudinal clines indicating that selection is a more likely cause of this pattern than gene flow among nearby populations with similar trait values. We did not find a correlation between constitutive and plastic leaf lobing at the individual genotype level, suggesting that while both traits appear to be adaptive, they operate under independent genetic controls. This indicates that both having more highly lobed leaves and greater leaf shape plasticity are likely adaptive at high elevation within *M. laciniatus.* Our data strongly suggest that traits known to be under divergent selection between *M. laciniatus* and close relative *Mimulus guttatus* are also under locally varying selection within *M. laciniatus.*

INTRODUCTION

Environmental heterogeneity can come in multiple forms, such as spatial and temporal variation in resource availability, and creates locally varying environments (Cortés and Wheeler 2018). This heterogeneity is a driver for numerous ecological and evolutionary processes, such as community composition, species richness, and local adaptation (Stein, Gerstner, and Kreft 2014; Yang et al. 2015; Forester et al. 2016). Where environments vary, populations with enough genetic variation have the opportunity to adapt to their local conditions via natural selection; this has been found to be true in a number of previous studies spanning multiple clades (Via and Conner 1995; Klich 2000; Hereford 2009; Nunez et al. 2021). Local adaptation occurs when an organism's trait expression optimizes fitness values for its local environment, and is thought to be achieved by low gene flow and/or strong selection between populations (Kawecki and Ebert 2004). Genetic variation in constitutive trait expression, here defined as the expression of a trait due to genetic differences in a common environment, has been the primary focus of studies in the local adaptation literature (Hereford 2009; Savolainen, Lascoux, and Merilä 2013). Phenotypic plasticity, or the ability of one genotype to express multiple phenotypes in response to the environment, has also been hypothesized to be locally adaptive, particularly in response to predictable environmental heterogeneity. However, local adaptation of plasticity has been more difficult to test in most systems (Thompson 1991; Via 1993; Gotthard and Nylin 1995; Via et al. 1995; Scheiner 2013). More research is needed in order to determine how these two methods of trait expression interact in response to environmental heterogeneity.

In a natural landscape, constitutive trait expression and phenotypic plasticity both contribute to an individual's lifetime fitness, and therefore both have the potential to play a role in local adaptation. It can be difficult to identify local adaptation of plasticity in natural populations, because plasticity must be measured using the same genotype in different environmental conditions. These constraints can be overcome with experimental designs using clonal replicates, or half-siblings in systems where inbreeding or cloning cannot be performed (Relyea 2002; Cooper et al. 2022). Plants are sessile and many of them can be inbred or replicated clonally making them ideal for investigating the relative contributions of constitutive and plastic trait expression to local adaptation. In this study we investigate whether patterns of population level variation in both constitutive and plastic trait expression are consistent with local adaptation in a native North American monkeyflower, *Mimulus laciniatus*.

Previous researchers interested in plasticity have postulated that plastic phenotypes are bound to be present in at least a few different environments (Sarkar 2004). As such, characterizing the plastic response of a species to environmentally relevant conditions can be important in understanding its life history. Experimentally, it can also be important to study plasticity in more than one trait across an organism's lifetime. Using these data, we can determine if there are any correlations between plastic expression of multiple traits, for which data are lacking in the plasticity literature (Nielsen and Papaj 2022). Similarly, we can use these data to comparatively examine strength of expression between plastic traits and constitutively expressed traits. This correlation of expression between plastic and constitutive traits is also understudied. By comparing the expression of plastic and constitutive traits, we can also investigate whether constitutive and plastic trait expression have independent genetic controls (Holeski, Chase-Alone, and Kelly 2010). This information is foundational in determining how plasticity is expressed from genotype to phenotype.

We expect phenotypic plasticity to evolve in response to temporal heterogeneity when presently sensed cues are reliable in predicting future conditions (Snell-Rood and Ehlman 2021). This plasticity is likely to be maintained if it confers a fitness advantage over non-plastic genotypes. For plasticity to respond to natural selection there must be genetic variation in phenotypic plasticity. Genetic variation in phenotypic plasticity is defined as a genotype by environment interaction, or GxE (Josephs 2018). Some traits which have a plastic response will not exhibit GxE and reaction norms for all genotypes will show similar slopes across environments. We expect to find GxE in traits where plasticity is locally adaptive.

Elevation and latitude each have the potential to exert spatially varying selection across populations. Environmental heterogeneity combined with low gene flow often causes local adaptation (Verheyen, Tüzün, and Stoks 2019). This scenario is thought to be particularly common in plants due to their sessile nature. Wos et. al (2022) found parallel local adaptation in *Arabidopsis arenosa* across four independent foothill-alpine clines. Similarly, local adaptation to elevation was found in response to drought stress in *M. guttatus* (Kooyers et al. 2015). Phenotypic plasticity is known to be involved in local adaptation in *Populus fremontii* populations across Arizona, where plasticity in traits such as bud set and plant height were found to vary along an elevational environmental gradient (Cooper et al. 2022). Local adaptation of phenotypic plasticity in a number of plant functional traits in *Phragmites australis* was found to

follow a latitudinal gradient in a study by Ren et al. (2020). However, not all variable traits investigated in plant species show local adaptation to environmental gradients, as seen in *Arabidopsis lyrata* floral traits as well as *Mimulus guttatus* trichome expression (Holeski, Chase-Alone, and Kelly 2010; Hämälä, Mattila, and Savolainen 2018). Therefore, more deliberate investigation must be done across a variety of species and traits in order to further understand locally adaptive trait expression.

One important environmental cue in plants is photoperiod, which temporally varies both predictably and unpredictably during the growing season for sessile alpine and subalpine organisms. Photoperiod varies predictably with shorter days occurring early in the year and the longest days occurring at the summer solstice. In this way, photoperiod is a reliable sensing cue, which is hypothesized to be important for the development of phenotypic plasticity (Snell-Rood and Ehlman 2021). Elevation and latitude are two environmental gradients that interact with photoperiod in a heterogeneous manner as a result of annually varying temperatures and snowpack. High latitude populations will always experience longer photoperiods during the spring and summer growing seasons than low latitude populations. Experienced photoperiod varies less predictably in mountainous environments (Figure 1a and 1b). High elevations accumulate more snowfall and have a later timing of snowmelt than low elevations, which means that for plants growing at the same latitude low elevation populations will germinate and develop under shorter photoperiods than those occurring at higher altitudes. However over time the amount of snowpack varies significantly meaning that the experienced photoperiod of a short-lived annual plant can fluctuate from year to year in alpine environments (Bormann et al. 2018). Therefore photoperiod may be an important cue that young seedlings utilize in alpine environments to determine time of year and their subsequent developmental trajectory.

Between closely related species in similar habitats, divergent selection can create and maintain differences in trait expression. A previous study by Ferris and Willis (2018) found that flowering time, plant height, and leaf shape were under divergent selection between *M. laciniatus* and its close relative *M. guttatus*. *M. laciniatus* has a more rapid flowering time, shorter height, and more lobed leaves than *M. guttatus* and these traits were selected for in its native granite outcrop habitat. We were interested in whether the same traits that are under divergent selection between these two closely related species could also be under locally varying selection within *M. laciniatus*. *M. laciniatus* is a Sierra Nevadan endemic plant that grows on

rocky granite outcrops from 1000-3300 meters in elevation (Fenster and Ritland 1994; DeMarche, Rice, and Sexton 2013). Unlike *M. guttatus* which displays rounded leaves, *M. laciniatus* has deeply lobed, narrow (laciniate) leaves as a defining characteristic of the species (Ferris et al. 2015). Depending upon elevation and the timing of snow melt, *M. laciniatus* populations experience different day lengths during the growing season, making this an interesting system to examine patterns of local adaptation in response to photoperiod fluctuation. *M. laciniatus* is a small, self-fertilizing annual plant, with low levels of gene flow expected between most populations (Ferris, Sexton, and Willis 2014; Sexton et al. 2016). For a plant species across an elevational gradient, flowering often occurs earlier in the year in low elevation plants than in high elevation plants (Neuffer and Hurka 1986; Lessard-Therrien, Davies, and Bolmgren 2014). In addition, some plant species have been found to adapt to elevational differences by growing larger and taller in low elevations and smaller and shorter at high elevations (Halbritter et al. 2018). Leaf shape, and in particular leaf dissection, has also been found to vary clinally across elevation and latitude within other plant species (Gurevitch 1988; Campitelli and Stinchcombe 2013; Wong et al. 2020). In many species it is unclear whether this trait variation and potential adaptation arises via constitutive expression, phenotypic plasticity, or a combination of both.

In this study we tested whether i) constitutive and plastic variation in leaf shape, flowering time, and size-related traits are consistent with local adaptation to elevation and latitude, and ii) constitutive and plastic trait expression correlate with one another in the cutleaf monkeyflower, *M. laciniatus*. We did this by growing genotypes from across *M. laciniatus'* elevational and latitudinal range in 1) a common garden in a long day environment to measure constitutive expression of traits, 2) a common garden of replicate genotypes in short days to measure plasticity, and 3) a third common garden experiment of replicate genotypes in long days to measure plasticity (Gunderson and Revell 2022). We used a linear mixed model approach to test whether constitutive and plastic trait expression correlated with either latitude or altitude. We also used a correlation matrix to measure relationships between constitutive and plastic trait expression at the individual genotype level. When looking for signatures of local adaptation, we expect to find a significant correlation between environmental gradients and variation in trait expression.

MATERIALS AND METHODS

Study system

We used inbred lines from natural populations of *M. laciniatus* across its geographic range to investigate phenotypic responses to photoperiod fluctuation. Twenty one populations of *M. laciniatus* descended from field-collected plants originating in different locations along the Sierra Nevada mountain range (California, USA) were used in this experiment (Table 1, Figure 2). These populations originate from six distinct watershed regions (Figure S1). Low levels of gene flow between populations have been observed in previous studies of *M. laciniatus* (Ferris, Sexton, and Willis 2014; Sexton et al. 2016). Therefore associations between phenotypic variation and elevation that are repeated across multiple watersheds may be more likely caused by natural selection than by gene flow.

Experimental design

To compare the variation in quantitative traits among *M. laciniatus* populations from across the species range, 3-10 unique inbred lines from each of 21 populations were grown in a common lab environment. Three genetic replicates of each genotype gave us a total of 1015 plants used to measure constitutive traits and 677 plants used to measure plasticity in this study. To measure genetic variation in constitutive trait expression in a single environment plants were grown at UC Davis in a Conviron walk-in chamber under 15 hour days at 21°C. To measure phenotypic plasticity in the same genotypes, plants were grown at two different photoperiods (11 hour & 15 hour days) in controlled environmental chambers at Tulane University. These long (15 hour) and short (11 hour) photoperiods represent the longest and shortest natural day lengths within *M. laciniatus'* native range.

Seeds were planted atop 3" pots filled with wetted Sun Gro Fafard 3B/Metro-Mix 830 soil. Pots were stratified in dark Percival chambers set at 4°C for 10 days and misted every other day. After 10 days, the pots were moved to a Conviron walk-in growth chamber set to 21°C. Pots were kept constantly wet on full flood benches and misted daily. After seedlings showed their first set of true leaves, flood benches were filled with water for 8 hours (during light hours) and empty for 16 hours daily.

Phenotypic measurements

To examine ecologically relevant phenotypic variation in *M. laciniatus* across populations, inbred lines, and photoperiods, we measured flowering time (days from germination to first flower), leaf lobing, and plant height, as well as floral traits as follows: corolla length, corolla width, longest stamen length, shortest stamen length, pistil length, and flower opening state (denoted as either pseudo-cleistogamous for a closed flower or chasmogamous for an open flower). *M. laciniatu*s exhibits pseudo-cleistogamy because while the size and shape of the outer corolla changes, the internal structure of the plant does not, as a true cleistogamous species would (Lord 1981). For plants that flowered, all phenotypic data were collected on the day of first flower. For plants that maintained their vegetative state, leaf lobing and plant height data were collected two months after germination. Leaf lobing data were collected by harvesting the most lobed leaf from the 2nd node of each plant. Leaves were taped to a sheet of white paper and scanned for digital convex hull analysis of leaf lobing in ImageJ (Ferris et al. 2015). Plant height was measured in millimeters (mm) to the apical meristem of each plant using a ruler, and all floral traits were measured in mm with calipers after removing the corolla from each flower using forceps. Herkogamy (stigma-anther separation) was calculated by subtracting the longest stamen length from the pistil length and acts as a metric of self-fertilization, where a smaller difference means a higher likelihood of selfing (Karron et al. 1997). Plasticity strength was calculated for each phenotype by taking the difference between mean phenotypic values of each line in each photoperiod (Massomo Pigliucci et al. 1996; Valladares, Sanchez-Gomez, and Zavala 2006). Slope of the reaction norm for each genotype was calculated by dividing plasticity strength by 4, the number of hours by which the two photoperiod treatments differ.

<u>Statistical analyses</u>

To examine patterns of trait variation consistent with local adaptation we tested for the relationship of constitutive trait expression against population elevation. We used a linear mixed effects model with the R package *nlme* to test the effect of population elevation and latitude on constitutive expression for all traits measured (Pinheiro, Bates, and R Core Team 2023). We then used an ANOVA to determine the significance of these relationships. This method was also used to examine the relationship of the strength of trait plasticity against population elevation and latitude.

Trait plasticity strength was calculated by subtracting mean short-day photoperiod trait values from mean long-day photoperiod trait values (Fraebel et al. 2020). To test for significance of plasticity in genotype, environment, and genotype by environment in all *M. laciniatus* traits across photoperiods, we used a linear model to test the effect of photoperiod on the difference between measured phenotypic value for each trait (R Core Team 2023). We then used an ANOVA to test for the significance of the fixed effect in the model. A Tukey post hoc test was used to determine which genotypes were significantly plastic in each trait.

To understand whether trait plasticity and trait constitutive values were developmentally correlated, and therefore unlikely to be under independent natural selection, we performed a correlation analysis. To do this, we created a correlation matrix using the R package *corrplot* which evaluated the correlation of trait expression between and among constitutive and plastic trait values (Wei and Simko 2021).

Graphs were created in R with the package *ggplot2* (Wickham 2016). Maps were created in R using the package *ggmap* as well as QGIS (Kahle and Wickham 2013; QGIS Development Team 2023).

RESULTS

<u>Substantial phenotypic plasticity in response to photoperiod</u>

To detect patterns of variation in phenotypic plasticity we grew populations from across *M. laciniatus's* range in both long and short days. We looked for significant differences in phenotype between the two photoperiod treatments at the genotype level in order to determine which traits had greater plastic responses. We also evaluated whether plasticity exists for each trait across all genotypes used in the study. Significant phenotypic plasticity was found in all traits studied (Figure 5, Figure 6, Table 4). Plant height showed the highest proportion of genotypes that exhibited significant plastic response to photoperiod change, with 70.6% of genetic lines responding with a significant plastic change in phenotype. Over 40% of all genotypes grew taller, flowered faster, and had larger flowers in long days. Additionally, a significant GxE interaction was detected in all traits studied (Table 4). This genetic variation in plastic response makes these traits the focus of our remaining analyses testing for patterns of local adaptation within *M. laciniatus.*

Environmental gradients influence both constitutive and plastic trait expression

To examine patterns consistent with local adaptation in *M. laciniatus* we measured ecologically relevant traits of populations from across the species range in a long day environment and tested for trait correlations with two environmental gradients: elevation and latitude. We found that the strength of constitutive trait expression significantly correlated with origin of population elevation in leaf shape (Figure 3c, 3i, Table 2). Constitutive leaf shape shows a significant positive association with population elevation, with leaves becoming more lobed at higher elevations. Two constitutive traits were marginally correlated with population elevation: flowering time and corolla width (Figure 3b, 3e). Populations from higher elevations flowered later and had larger flowers than low elevation populations.

Using a mixed model approach we found that the strength of phenotypic plasticity was significantly associated with population elevation in several traits. Critical photoperiod and leaf shape plasticity both show a positive association with population elevation (Figure 7b, 7d, Table 5). Critical photoperiod, the photoperiod threshold required to reach flowering, strongly differs based on population elevation. Higher elevation populations require a longer photoperiod to induce flowering than low elevation populations (Figure 7c). In addition, leaf shape plasticity is greater in high elevation populations when compared to low elevation populations (Figure 7d). Plasticity in a number of floral traits showed marginally significant trends ($0.1 > p > 0.05$) with elevation. These traits include both long and short stamen length, pistil length, and pseudo-cleistogamy, where plasticity is increased at higher elevations. Flowering time, plant height, leaf size, corolla length, corolla width, and herkogamy plasticity did not show any association with population elevation.

Using the same mixed model approach as for elevation, we looked for correlation of trait expression with population latitude. Out of all traits studied (constitutive and plastic), the only trait which showed any relationship with latitude was constitutive leaf lobing, which had a moderately significant association ($R^2 = 0.36$, $p = 0.0663$; Figure 4c). We found that leaves are likely to be more constitutively lobed at high latitudes. All other traits had no relationship with latitude (Figure 4, Figure 8, Table 3, Table 6).

Correlation of constitutive and plastic trait expression

To understand whether trait plasticity and trait constitutive values were developmentally correlated, and therefore unlikely to be under independent natural selection pressures, we performed a correlation analysis with all of our phenotypic data. We found that at the individual genotype level constitutive expression of traits did not greatly influence the strength of plastic trait expression (Figure 9). Within constitutive traits there were several that had stronger correlations ($r > +/-0.6$) with one another. For example leaf area, plant height and flowering time were all strongly correlated with each other as were corolla length, corolla width and stamen length. Within our significantly plastic traits all floral measurements were tightly correlated with each other: stamen length (long and short), pistil length, corolla length and corolla width ($r > +/-0.6$). For the majority of traits measured there was not a strong correlation between constitutive and plastic expression of the same phenotype ($r < +/-0.6$). For example, our results show that constitutive leaf shape expression of a genotype was only weakly correlated ($r = 0.11$) with the magnitude of leaf shape plasticity in that same genotype (Figure 9).

DISCUSSION

In this study we investigated whether quantitative traits that are involved in adaptation to *M. laciniatus*'s harsh granite outcrop environment - flowering time, leaf shape and flower size (Ferris and Willis 2018) - also appear to be under selection in response to environmental variation across the species' range. We were also interested in genetic variation in both the constitutive and plastic expression of these traits. To test for patterns consistent with local adaptation to elevation and latitude we grew replicates of genotypes from across *M. laciniatus*'s geographic range under both long and short day lengths. Plasticity and GxE were detected in response to photoperiod in all traits that we measured. For plasticity to be locally adaptive there must be GxE in the traits' expression and genetic variation in plasticity associated with variation in the environment. We found that patterns of genetic variation in the constitutive expression of flowering time and flower size were moderately associated with elevation making them interesting traits to investigate in future studies about adaptation across *M. laciniatus's* geographic range. In our study both constitutive and plastic leaf shape expression were significantly associated with elevation, strongly suggesting that this trait is involved in local adaptation within *M. laciniatus*. Critical photoperiod follows this same association with elevation, suggesting it may be locally adaptive as well. Furthermore we found that leaf lobing

expression in long days was positively associated with latitude as well as elevation indicating that increased lobing may be adaptive in colder environments.

Local adaptation to elevation in *M. laciniatus* occurs in both constitutive and plastic traits

Reciprocal transplant experiments have found selection for early flowering time, more highly lobed leaves, and larger flowers in *M. laciniatus*'s harsh granite outcrop habitat (Ferris and Willis 2018; Tataru, Wheeler, and Ferris 2023). However, it is not known whether these same traits are involved in local adaptation across *M. laciniatus*'s range. One environmental variable that is likely to drive population level adaptation in this system is variation in elevation across the Sierra Nevada, CA. Using a long day common garden we found that constitutive leaf lobing expression was significantly associated with population elevation.

Populations from higher elevations had more highly lobed leaves than populations from lower elevations; this was true across the species range and multiple watersheds indicating that selection is more likely to be driving this trend than gene flow among nearby populations (Figure 3). This pattern indicates that more lobing may be adaptive at higher elevations. We also saw a marginally significant positive trend between leaf lobing and latitude which further suggests that it is adaptive to have more highly lobed leaves in Northern populations. It is hypothesized that lobed leaves could be involved in cold tolerance, which is an environmental condition shared by high elevations and high latitudes (Nobel 2009; Bhattarai et al. 2022). These types of environmental conditions may serve as increased leaf lobing signals controlled by the CUC genes (Chitwood and Sinha 2016). The patterns of leaf shape variation with both elevation and latitude would be consistent with more lobed leaves having higher cold tolerance. This would support theoretical work from the physiological literature that hypothesizes that because lobed leaves have a thinner boundary layer they equilibrate more efficiently to air temperature through convection and therefore should not drop below ambient temperature on clear nights when leaves radiate heat to the sky. This could prevent lobed leaves from incurring frost damage when ambient temperature is near, but still above freezing (Nobel 2009; Bhattarai et al. 2022). Further functional physiological work will be necessary to rigorously test this hypothesis.

Leaf shape and flowering time were previously found to be under divergent selection between *M. laciniatus* and and its close relative *M. guttatus's* native habitats (Ferris and Willis 2018). *M. guttatus* occupies more continuously moist seep and stream habitats but often occurs

within a meter of *M. laciniatus*'s granite outcrops. A significant driver of divergent adaptation between these two species microhabitats appears to be water availability, although other factors like light intensity and temperature also vary between the two (Ferris et al 2014; Tataru et al 2023). Granite outcrop habitat is likely to have increased temporal heterogeneity of resources like light, water, and temperature than *M. guttatus* meadow seep habitat, meaning that leaf lobing could serve as an adaptation to this heterogeneity. This indicates that leaf lobing in particular confers a stronger adaptive advantage to the unique challenges encountered annually in *M. lacninatus*'s granite outcrop environment. Leaf shape, size, thickness, and number have all been studied previously in various plant species and identified as locally adaptive to similarly harsh marginal habitats (Nagy 1997; Dudley 1996; Parker, Rodriguez, and Loik 2003; Ferris et al. 2015). In one study on annual plant species *Gilia capitata*, individuals with less leaf lobing were selected for at both coastal and inland environments, which indicates that leaf lobing may not be a beneficial trait in those types of environments (Nagy 1997).

Flowering time and corolla width also show marginal evidence of local adaptation to elevation in *M. laciniatus* as trends of greater flowering time and wider corollas are present in higher elevation populations across the geographic range of *M. laciniatus.* This difference in flowering time and flower size may be influenced by increased water availability at high elevations from greater snowmelt. Bigger, later flowering plants such as *M. guttatus* usually occur in more continuously moist environments (Ferris and Willis 2018). Increased flowering time and larger flowers at high elevations in *M. laciniatus* may be a product of adaptation to this greater water availability since later flowering, larger plants are more fecund than small early flowering plants. A large body of work exists in local adaptation of flowering time phenology and flower size, indicating that these traits often experience selection in nature in order to optimize fitness (Lowry and Willis 2010; Wang et al. 2014; Dickman et al. 2019; Leinonen et al. 2020). In general, if snow levels are lower and melt occurs earlier at low elevation, water resources will run out faster. Therefore low elevation plants need to flower sooner and faster in order to reproduce at low elevations compared to high (Fox 1990).

<u>*M. laciniatus* exhibits extensive plasticity and GxE induced by photoperiod</u>

Phenotypic plasticity is expected to evolve when future environmental conditions will vary, and presently sensed cues are reliable in predicting those future conditions (Snell-Rood and Ehlman

2021). We therefore may expect that plasticity evolved in response to *M. laciniatus*' variable environment, and was subsequently selected against in populations which have more stable, temporally homogeneous environments from year to year. Degree of plasticity varies within all traits examined at the genotype level. Traits such as plant height appear to show a strong plastic response, but less GxE than in other traits (Figure 6). It is also possible that plant height may be more biologically constrained in short days than other traits, making this more universal plastic response apparent due to genetic limitation (Auld, Agrawal, and Relyea 2010). A reason for this biological constraint could be due to the fact that traits such as plant height tend to be controlled by the same pathways as flowering, which is connected to light sensing (M. Pigliucci and Schlichting 1996; Mockler et al. 2003). This is well documented in the *Arabidopsis* system, in which much is known about genetic controls linking flowering and plant height, such as the *terminal flower* gene as well as *FLOWERING LOCUS C* (Alvarez et al. 1992; Salehi et al. 2005). These loci act antagonistically to genetic pathways that regulate a vegetative-to-reproductive switch in resource allocation. Our finding of similar morphology in *Mimulus* is an exciting opportunity for further research into the genetic mechanisms and regulatory pathways controlling these traits in the *Mimulus* system.

We identified significant plasticity and GxE in all traits when comparing all genotypes against each other. Identifying GxE within plastically expressed traits means a genomic control modulates plastic trait expression, which means selection can act on these different reaction norms to select for the most beneficial level of plasticity expression. In this way we expected to find more GxE in traits that may be locally adaptive, as selection can favor different levels of plasticity at different locales. However, (Saltz et al. 2018) state that cryptic genetic variation will produce GxE when populations are compared across experimental environments that are novel for some populations but familiar to others. When this happens, populations experiencing familiar environmental conditions will show lower GxE than populations experiencing those conditions for the first time. This hypothesis agrees with our data, as short days are not novel for low elevations but are likely novel to high elevations. Therefore it is not surprising that we detected significant GxE in all traits between our two common gardens differing in photoperiod. Because of this reason, we cannot reliably use GxE as a metric for local adaptation in this study.

<u>Population elevation has a stronger influence in local adaptation than latitude</u>

Not all traits studied exhibited a plastic response that correlated with the origin of population elevation. However, for critical photoperiod and leaf shape plasticity, population elevation influences the strength of plasticity. As elevation increases, proportion of plants reaching flowering under short days decreases (i.e. the critical photoperiod increases as elevation increases). This is a pattern indicative of local adaptation at lower elevations in order to flower under shorter day lengths that lower elevation populations experience while they are growing in the early spring. These results are supported by Friedman & Willis (2013) which similarly found that flowering in *M. laciniatus* is triggered by a critical photoperiod that differs based on population elevation with higher elevation populations requiring a longer photoperiod to induce flowering. However, our results on critical photoperiod flowering conflict with findings from Leinonen et al. (2020), in which they found that mid-elevation populations required the longest photoperiod to reach flowering. In addition, Leinonen et al. (2020) found no significant correlation of population elevation with other *M. laciniatus* phenological traits in response to photoperiod. This conflict is not terribly surprising as these previous studies examined only a few populations of *M. laciniatus* (<6 populations) compared to our comprehensive sampling of the species range (21 populations total). *M. guttatus* is also known to have locally adapted to photoperiod, one such example being the geothermally adapted *M. guttatus* of Yellowstone National Park, WY. These plants have adapted to flower under short winter days when geothermal vents make the surrounding environment wet and warm, suitable for plant growth and reproduction (Lekberg et al. 2012).

Photoperiod is a reliable sensing cue across generations to indicate time of year. Photoperiodism, also known as daylight sensing, has been documented in many plant species as a crucial cue for seasonal change (Davis 2002; Mockler et al. 2003; MacQueen et al. 2021). Plants use photoperiod as a means to determine time of year and appropriately allocate resources accordingly to vegetative or reproductive states (Keller and Körner 2003). Across time, selection favors annual plants that set seed by the end of suitable growing conditions. High elevation populations in other species have been found to be locally adapted to longer photoperiods similar to our findings, such as in *Populus* and red spruce tree species (Pauley and Perry 1954; Butnor et al. 2019) as well as more herbaceous alpine plant species such as *Arabidopsis arenosa (Wos et al. 2022)*. High-elevation adaptation to photoperiod has also been identified in non-plant species, such as pitcher plant mosquitoes and burying beetles (Bradshaw and Lounibos 1977; Tsai et al.

2020). A trait that allows for sensing time of year, such as photoperiodism would be beneficial for *M. laciniatus*, as it provides information to these plants on how much time there is left to grow in the season before senescence historically occurs. This in turn can inform how and when resources are directed during a plant's life span (i.e. to growth or reproduction). However, photoperiodism may not be beneficial to plants experiencing novel photoperiods as the cue becomes unreliable, such as when our high elevation plants never reached flowering under short days. In addition, even though short photoperiod leaves are less lobed, they are larger on average than long photoperiod leaves. This indicates that for the genotypes that didn't flower in a short photoperiod, the resources were instead being shunted into vegetative tissue, which could be a resource conservation measure (Freschet et al. 2010; Reich 2014).

Similar to our critical photoperiod result, the strength of constitutive leaf lobing and leaf shape plasticity increases as elevation increases. Constitutive leaf lobing is also the only trait to show any association with latitude - a marginally positive association. Leaf lobing may be more important for plants that live in environments with high light exposure, hot or dry climates, or be important for freeze tolerance. Functional hypotheses regarding this correlation include the impact leaf shape has on either water retention or temperature regulation. A lobed leaf has fewer minor veins than a rounded leaf, therefore less water pressure is needed to prevent wilting of leaf tissue (Yapp 1912; Nicotra et al. 2011). A more lobed leaf also has a thinner boundary layer, which is a static layer of air directly covering the leaf's surface. A thinner boundary layer increases the efficiency of heat exchange between a leaf and the air around it. However, lobing may also offer a benefit of freeze tolerance, in which efficient heat exchange maintains lobed leaf temperatures at air temperature rather than falling below ambient temperature on cold clear nights when leaves radiate heat to the sky. Both of these traits unique to lobed leaves may make them adaptive in colder, hotter, or drier environments (Nobel 2009). A study using *Solanum lycopersicum* found that shade-avoidant plants had more complex leaf structure in both lobing and serration, indicating that high light can also drive a more complex leaf shape (Chitwood et al. 2015). In our system leaf shape appears to be more important for adaptation to elevation than leaf area, as evidenced by our own data (Figure 2, 7) as well as a study by Dickman et al. (2019) which found that specific leaf area in *M. laciniatus* did not differ based on elevation in a pre- and post-drought resurrection study.

Within the *M. guttatus* species complex, lobed leaf shape has been found to correlate with dry, rocky environments (Ferris et al. 2015) and to be under selection in *M. laciniatus*' native habitat (Ferris and Willis 2018). Here we found that *M. laciniatus* populations from high elevation and northern elevations have the most highly lobed leaves and the greatest leaf shape plasticity. This suggests that in our system lobed leaves may have a cold tolerance function. If leaf shape plasticity is maladaptive or costly to maintain at low elevations, the trait may have been selected against and is therefore no longer present in low elevation populations (Hendry 2016). High elevation environments tend to have fluctuating environmental conditions while lower elevations are more stable. Leaf shape and leaf plasticity may be locally adaptive at high elevations due to lobed leaves aiding adaptation to these harsh, fluctuating environmental conditions (Chevin and Lande 2011).

M. laciniatus is an example of parallel evolution in plasticity

In traits that show parallel signatures of local adaptation, evolution has repeatedly selected for specific phenotypes in particular environments (in this case, elevations and by proxy photoperiods). An exciting finding of this study is discovering potential parallel evolution of a phenotypically plastic trait, leaf lobing, and its constitutive counterpart. We have shown that while these traits seem to both be important in adaptation to high elevation environments, our correlation matrix analysis indicates that they could be under separate genetic controls and therefore independent selection.

Because *M. laciniatus* is a small, self-fertilizing annual plant, gene flow is expected to be low in most, if not all, populations (Ferris, Sexton, and Willis 2014). In *M. laciniatus*, gene flow is known to be driven by elevation (isolation by climate environment) rather than purely distance between populations (DeMarche, Rice, and Sexton 2013; Sexton et al. 2016). This isolation by environment can be caused by either gene flow between populations of similar elevation, or by selection driving repeated independent adaptation to elevation at the genetic level. Greater genetic isolation than expected between some populations in Sexton et al.'s (2016) study seems to suggest that climate mediated selection could be a driver of local adaptation to elevation in this species. While further research is necessary to understand the origin of isolation by environment in *M. laciniatus*, it has been shown that populations have historically adapted to elevation (Sexton, Strauss, and Rice 2011). While Sexton et al (2011) focused on germination

and flowering time divergence between populations, the trends support our findings that elevation is a driver of between-population divergence in *M. laciniatus*. The similar patterns of leaf shape plasticity that we find across spatially separate altitudinal clines imply parallel evolution of phenotypic plasticity, which further supports its role in local adaptation. In high-elevation populations, increases of leaf shape plasticity, critical photoperiod, and constitutive leaf lobing are corroborated across the geographic distribution of *M. laciniatus*. Parallel evolution of a similar phenotype in a similar environment is evidence that selection is causing the repeated phenotypic pattern rather than neutral demographic processes (Schluter et al. 2004). Therefore the parallel clines that we find in these three traits are strongly suggestive that leaf shape, leaf shape plasticity, and critical photoperiod are under selection across an elevational gradient in *M. laciniatus*.

<u>Constitutive and plastic trait expression seem genetically independent</u>

For all traits measured, constitutive trait expression has very minimal influence on the degree of plastic trait expression (Figure 9). Notably in leaf lobing, which both constitutively and plastically follow similar locally adaptive relationships with elevation, these traits still show little influence on the others' expression. In other words, at the individual genotype level there was little correlation between how lobed a leaf was in a long day environment and how plastic that genotype's leaf shape expression was between day lengths. The independence of expression in these two traits implies that there is likely a separate genetic basis behind the expression of each. This type of independence between constitutive and plastic trait expression has been theorized in a handful of studies (Ehrenreich and Pfennig 2016; Johansson et al. 2013), and identified in others (Lind et al. 2011; Snell-Rood et al. 2011; Tétard-Jones, Kertesz, and Preziosi 2011). While Snell-Rood et al. (2011) focus on an extreme form of plasticity, polyphenism, their study aimed to investigate their developmental divergence hypothesis first discussed in the literature with regards to plasticity by West-Eberhard (1989). Snell-Rood et al. (2011) found divergence in patterns of gene expression between different morphs in two species of horned beetle, *Onthophagus taurus* and *Onthophagus nigriventris*. Plasticity-biased genes were found to be more divergent than those with shared patterns of expression between beetle morphs. Tétard-Jones et al. (2011) also found independent plasticity QTL located separately from main effect

QTL in barley growth, *Hordeum vulgare*, adding more evidence that plasticity is controlled by separate genetic loci than constitutive trait expression loci.

In a trait that is up- or down-regulated by common developmental pathways, expression of phenotypic plasticity is not linked to specific environmental cues and is more likely to be linked with constitutive trait expression (Snell-Rood et al. 2011). However, traits may also be controlled by environment-specific gene regulation, which adjusts expression in these common developmental pathways and can contribute to phenotypic plasticity. In other terms, additional genes may be triggered to cause differences in leaf shape in different environments. These adjustments reduce pleiotropic constraints and in this way can facilitate rapid adaptation to different environments (West-Eberhard 1989, 2005). Ehrenreich and Pfennig (2016) provide a useful model to compare regions of genomic differentiation between a canalized constitutive trait expressed in some genotypes and a phenotypically plastic trait expressed in other genotypes. In this model, a canalized constitutive genotype is crossed with a phenotypically plastic genotype and inbred to create recombinant offspring. These offspring can then be surveyed for constitutive or plastic phenotype and genetically mapped to determine loci associated with lack of plasticity in a particular trait. For *M. laciniatus*, this direction of research should aid in determining the genomic regions of differentiation between constitutive and plastic trait expression.

Conclusion

Our study investigated how constitutive and plastic trait variation can contribute to local adaptation in heterogeneous environments. The parallel evolution of leaf shape, critical photoperiod, and leaf shape plasticity across 21 *M. laciniatus* populations spanning multiple watersheds is consistent with a signature of local adaptation (James et al. 2023). The results of this study raise a number of questions for future research. First, what genes underlie the plastic responses observed due to photoperiod? Are the genes that control for a plastic response in changing photoperiod the same that control constitutive trait expression, and does this hold true for all traits studied? Second, what is the function of leaf lobing in the field setting? While many functions are hypothesized above, there is currently a lack of data to validate these claims. In addition, by further studying the strength of selection on plastic traits it would be possible to investigate costs associated with plasticity expression in mismatched environments. Our data supports the idea that highly lobed leaf shape is locally adaptive in high elevation and northern

populations of *M. laciniatus.* In terms of local adaptation between *Mimulus* species we see evidence that ephemeral water availability in *M. laciniatus's* dry rocky habitat may be driving the evolution of lobed leaf shape (Tataru et al 2023; Ferris and Willis 2018). However high elevation highly lobed populations of *M. laciniatus* tend to have higher water availability than low elevation populations with less lobing, implying that the selective force causing local adaptation in leaf shape within this species may be different than the force driving divergent evolution between species. Future experimental studies will be able to confirm or deny this hypothesis.

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DATA AVAILABILITY

Data and code are available from the Dryad Digital Repository.
https://datadryad.org/stash/share/xyNBULQagWnkmI3oAbWKPEIJcZPvxR-1xg3zxwGEnKI

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FIGURES

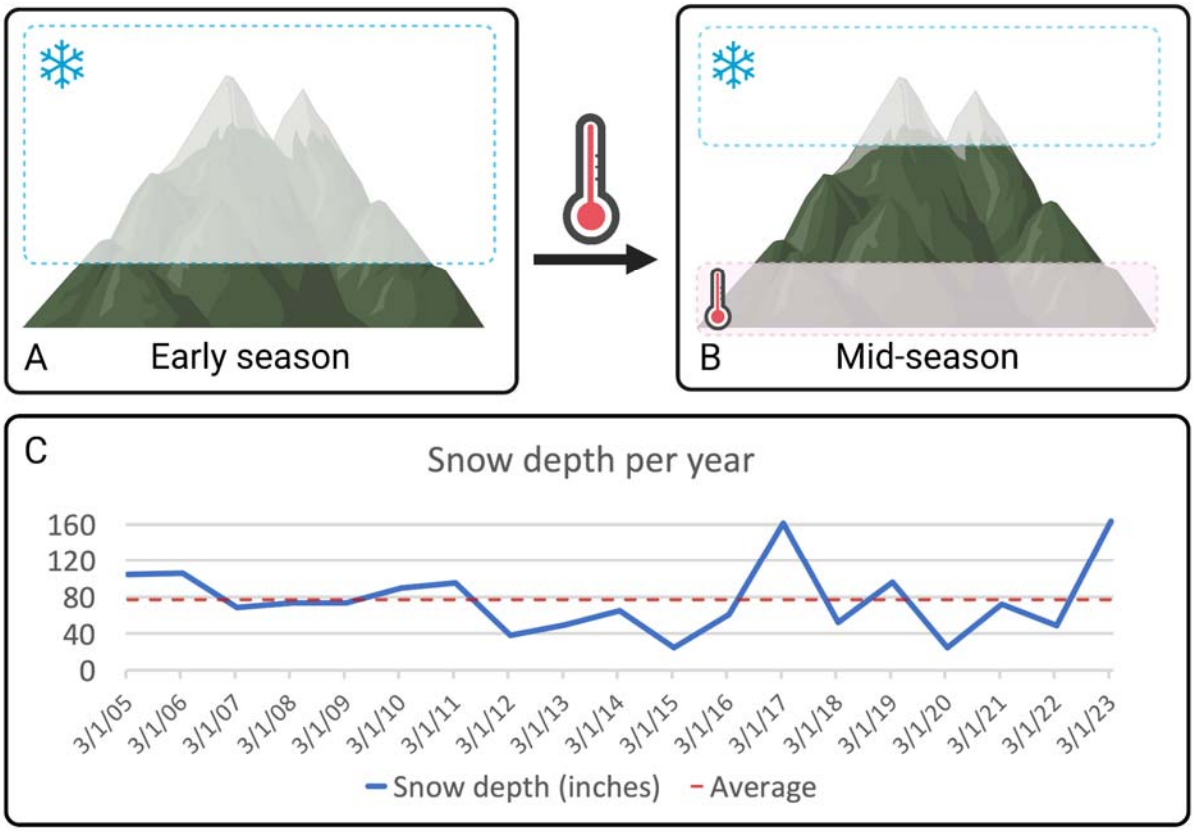

Figure 1. a) Early in the growing season, only lower elevation populations have warmed up enough to experience complete snowpack melt. These populations begin to grow earlier in the spring, and therefore experience a shorter photoperiod. b) By early to mid summer, all snowpack has melted from higher elevation sites, therefore high elevation populations grow during longer photoperiods. c) Snowpack level changes from year to year, and the pace of warming may be slower or faster. This creates fluctuation in experienced photoperiod over time as well as over elevation. Snow depth data collected at Dana Meadows in Yosemite National Park (California Data Exchange Center 2023). Created with BioRender.com.

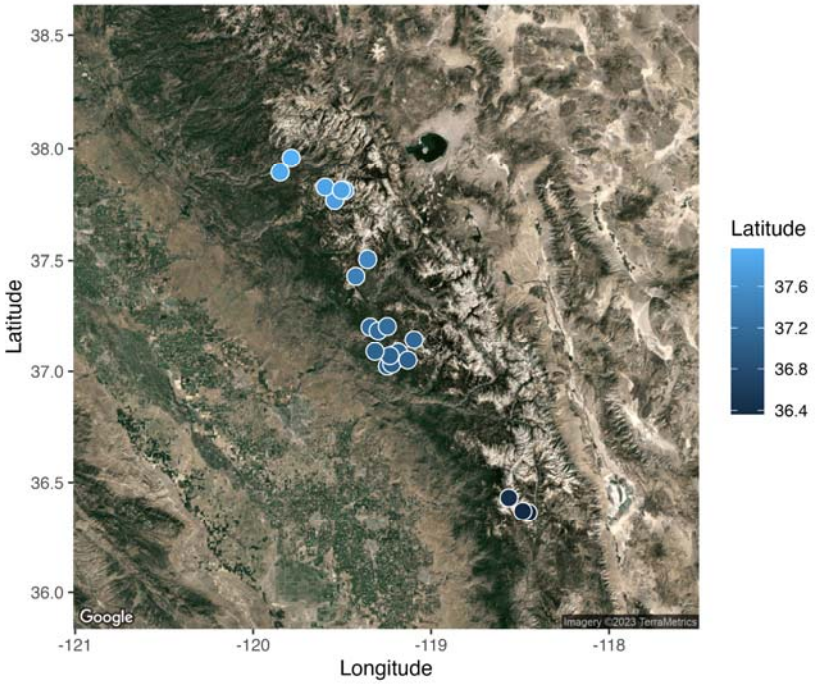

Figure 2. A map of the Sierra Nevada mountain range with each population mapped to its original collection location. Populations are colored based on latitude.

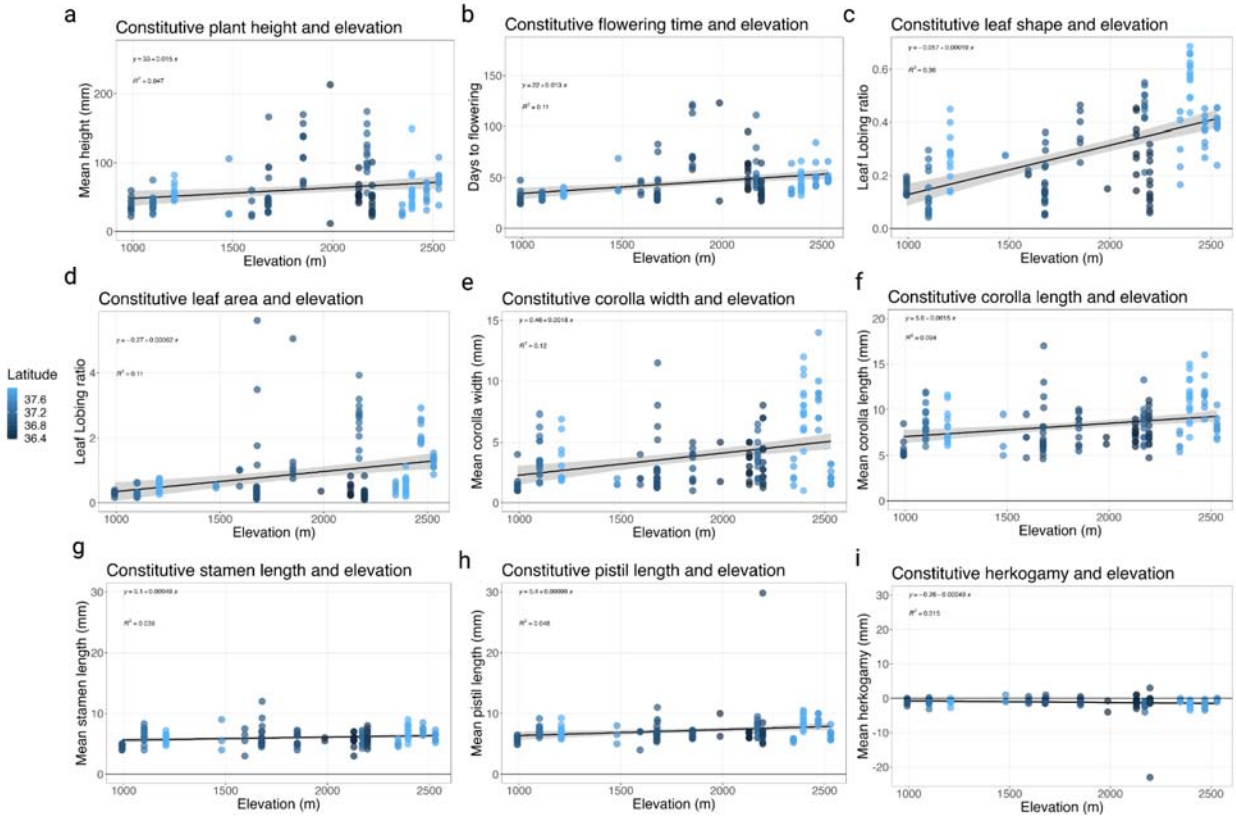

Figure 3. Constitutive trait mean expression per genotype plotted against elevation of origin for each population. See Table 2 for statistical analysis of correlation.

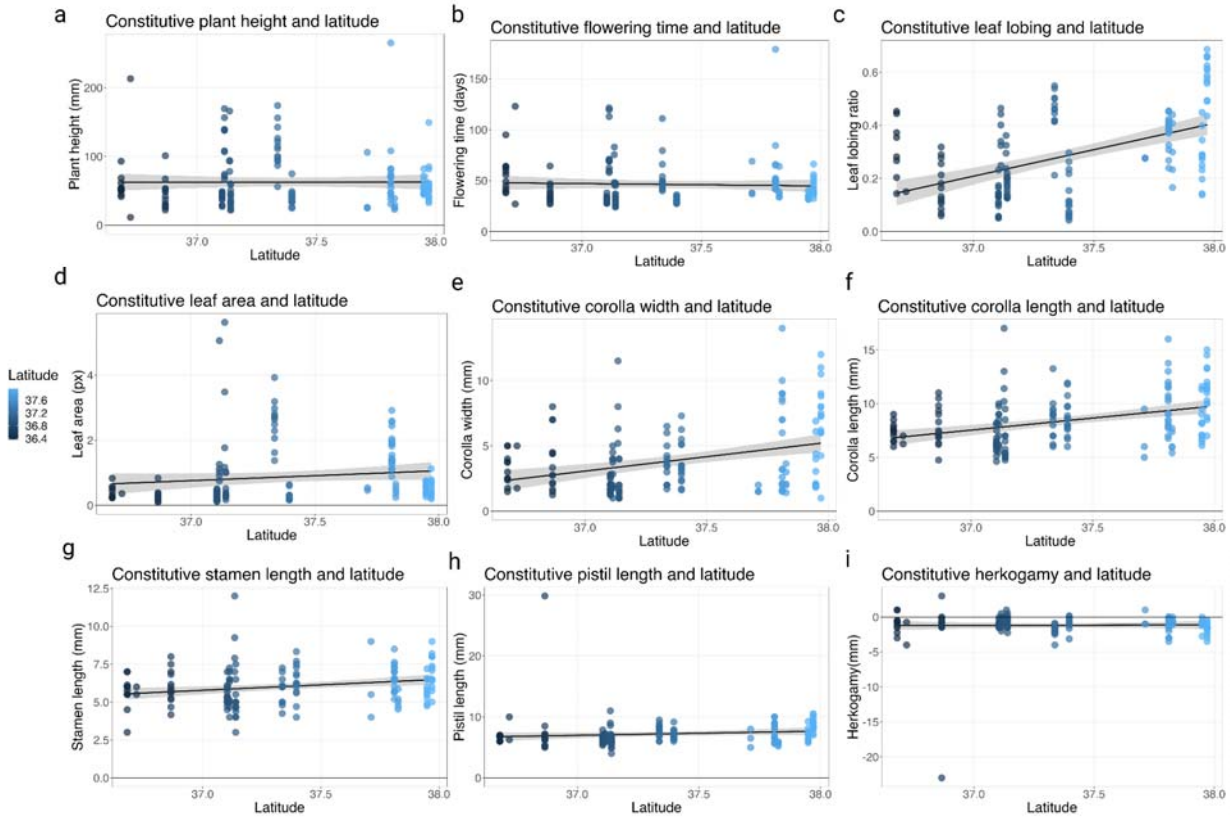

Figure 4. Constitutive trait mean expression per genotype plotted against latitude of origin for each population. See Table 3 for statistical analysis of correlation.

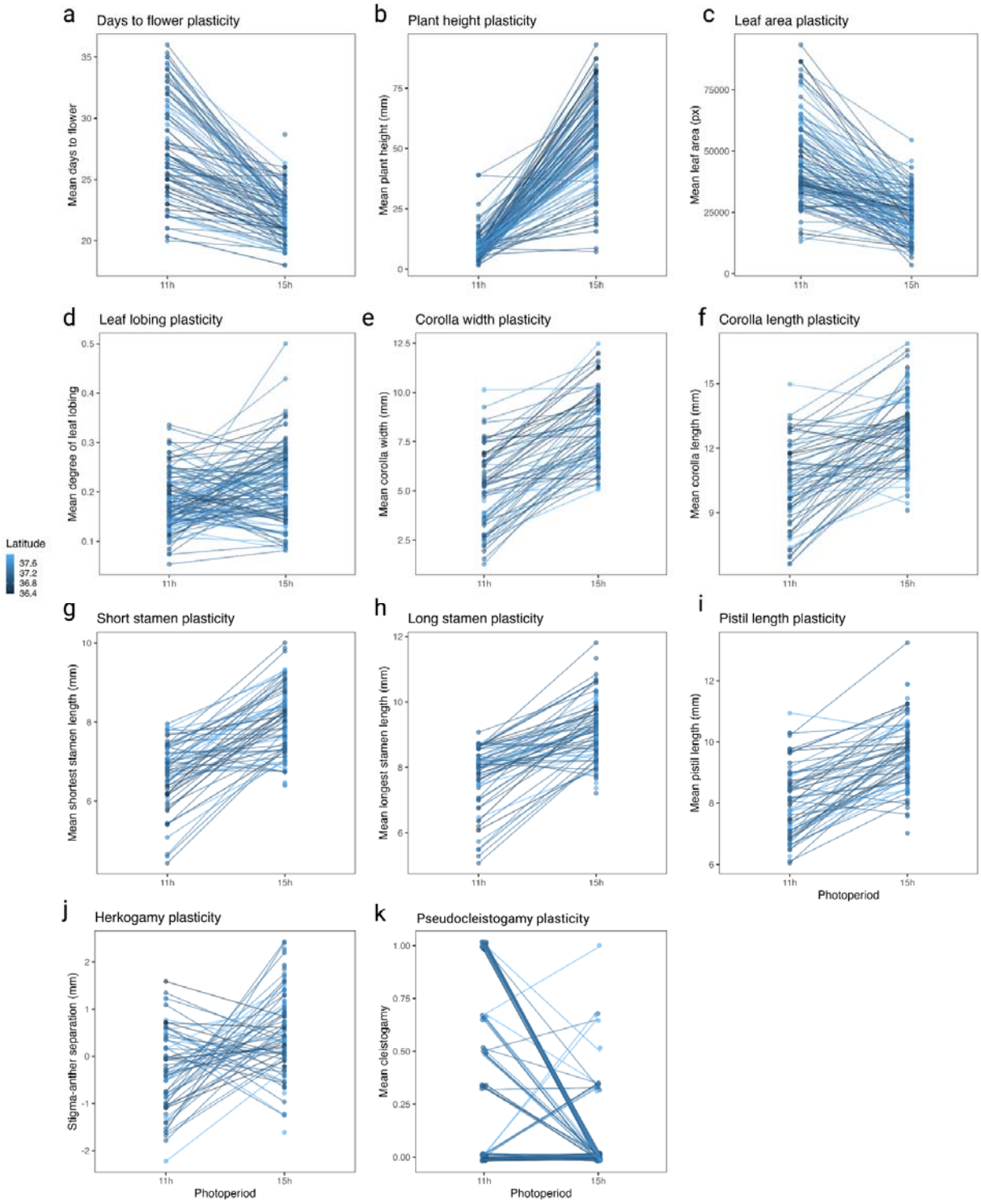

Figure 5. Reaction norms for all traits studied. Reaction norms faceted by population can be found in Figure S2. See Table 4 for genotype by environment significance per trait.

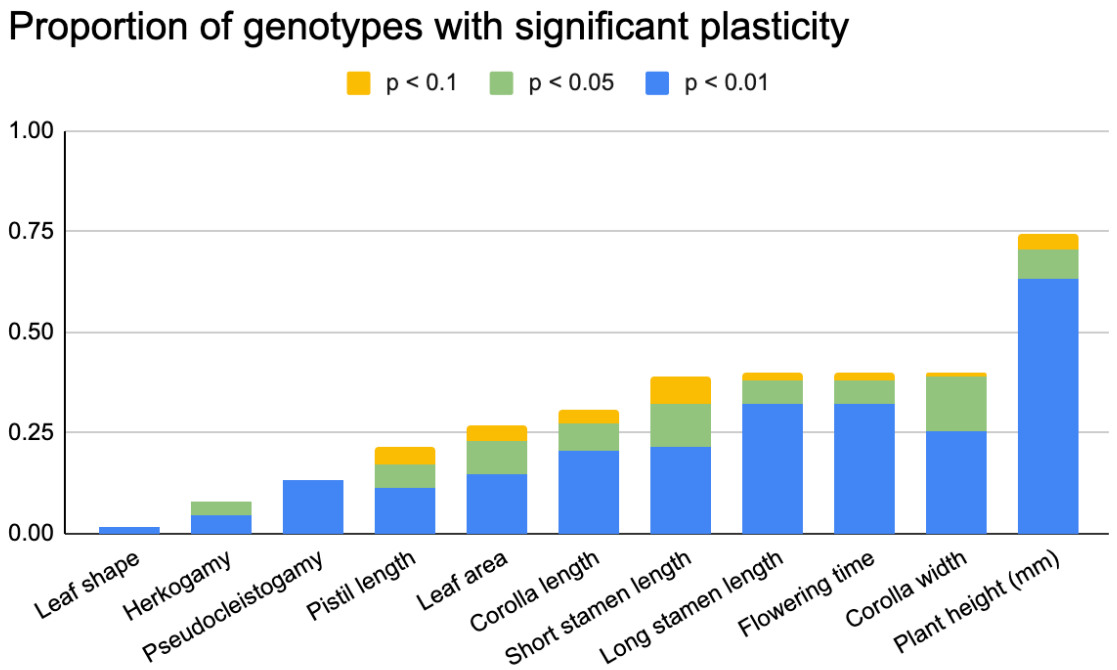

Figure 6. The proportion of genotypes that had significant plasticity per trait.

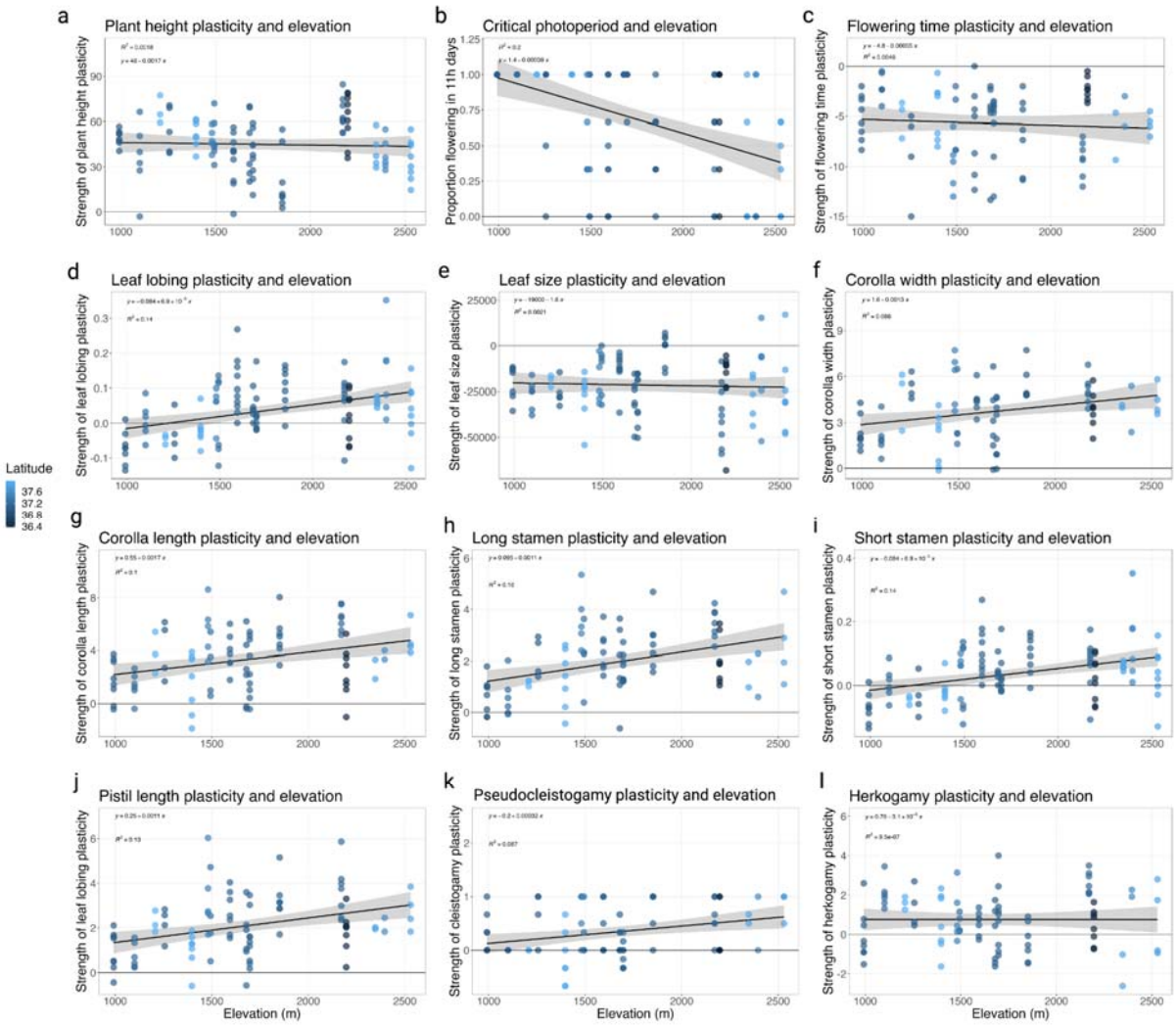

Figure 7. Trait plasticity mean expression per genotype plotted against elevation of origin for each population. See Table 5 for statistical analysis of correlation.

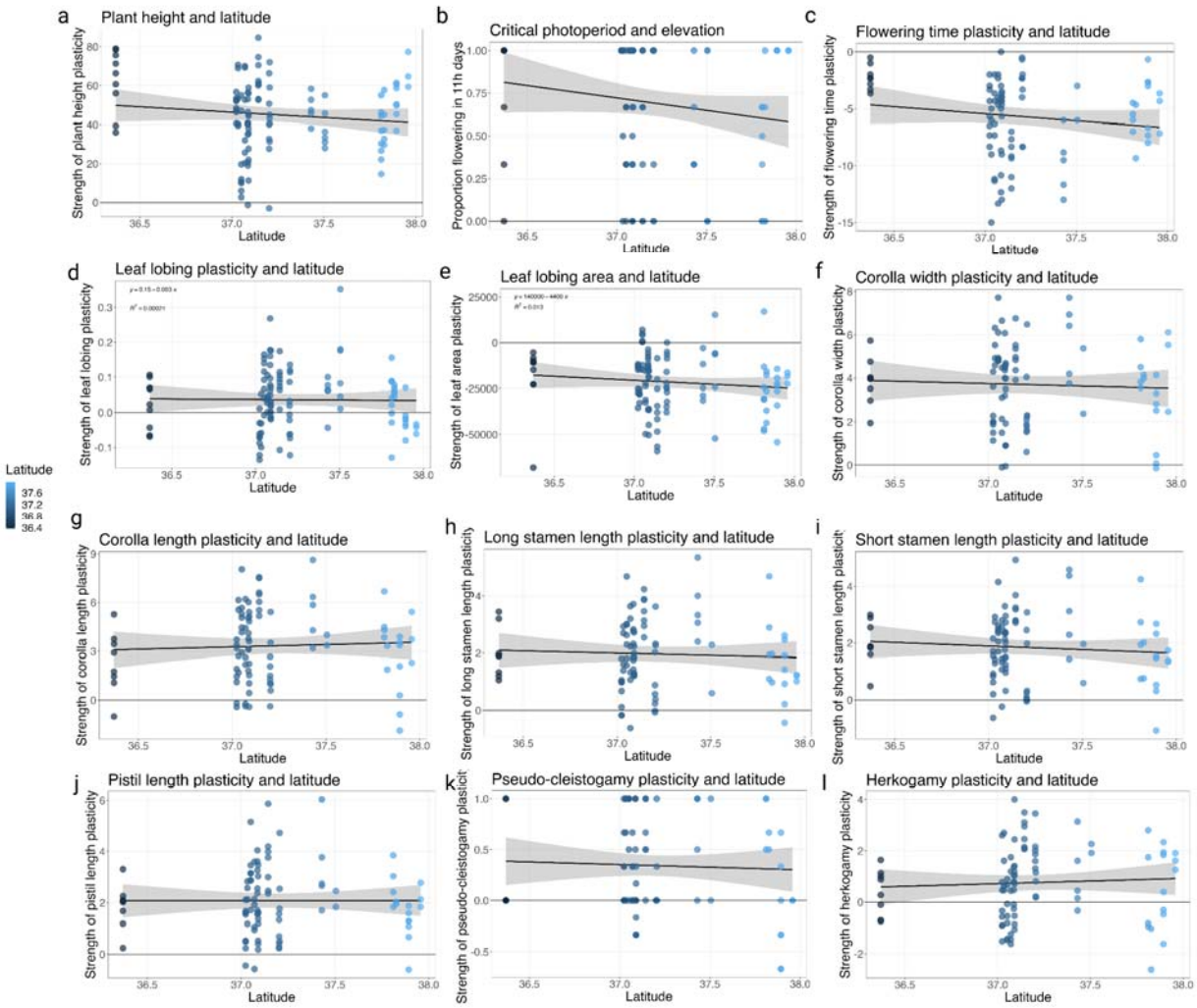

Figure 8. Trait plasticity mean expression per genotype plotted against latitude of origin for each population. See Table 6 for statistical analysis of correlation.

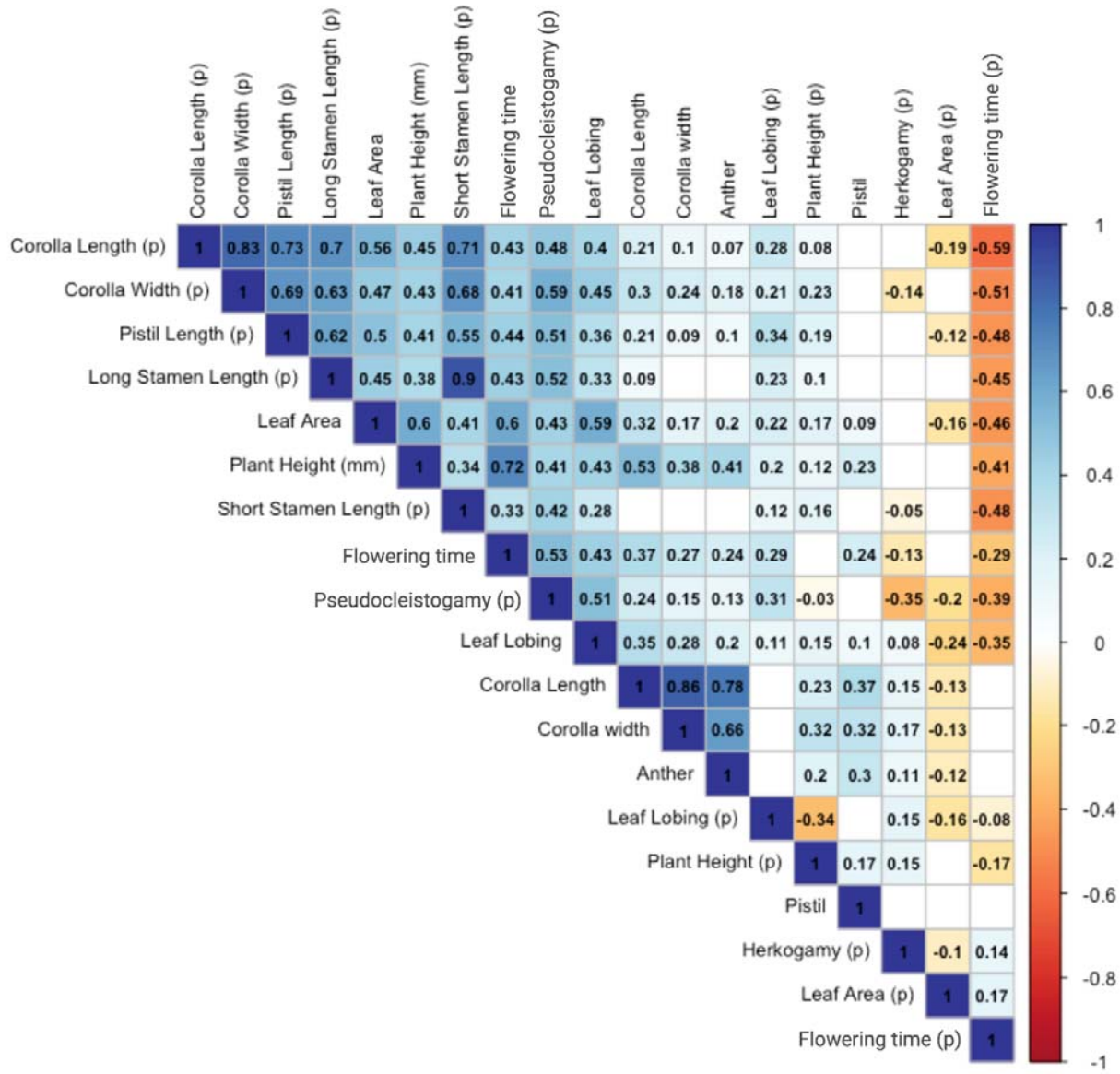

Figure 9. Correlation matrix of constitutive trait expression and plastic trait expression. Plastic trait expression is denoted by (p). Blank squares had an insignificant correlation ($p > 0.05$) when tested with a t-test.

TABLES

Population information

Population name	GPS coordinates (Decimal Degrees)	Location description	Elevation (m)
BCL	37.2049, -119.246	Big Creek, Fresno County, CA	1493.45
BML	37.090509, -119.3150	Mill Run Lane, Shaver Lake, CA	1696.74
CECL	37.300143, -119.4835	Central Camp Rd, Madera County, CA	1274.67
CRV	37.812585, -119.50447	South of Olmstead Point parking area, Yosemite National Park, CA	2577.44
DNK	37.0511, -119.13102	Dinky Creek Rd, Sierra National Forest, CA	1851.57
GB	37.0708, -119.23165	Grand Bluff off Hwy 168, Sierra National Forest, CA	1679.37
HEH	37.89314, -119.84853	Evergreen Rd, Yosemite National Park, CA	1396.83
HHR	37.957206, -119.7860	Hetch Hetchy Reservoir trail, Yosemite National Park, CA	1207.56
HUL	37.14372, -119.09606	Hwy 168 before south turn off to Huntington Lake Rd, Fresno County, CA	2170.68
LIB	36.36958, -118.48498	Generals Hwy east of Little Baldy, Sequoia National Park, CA	2196.28
OPN	37.8107, -119.4852	Olmstead Point, Yosemite National Park, CA	2529.72
PETL	37.03346, -119.22122	Peterson Rd, Fresno County, CA	1256.63
QAL	36.7203, -118.02306	By Quail Flat off Generals Hwy, Tulare County, CA	1986.99
SBL	37.02384, -119.2455	Sandy Bluffs, Hwy 168, Sierra National Forest, CA	991.77
SHL	37.08682, -119.18388	Hwy 168, Shaver Lake, CA	1594.33
ST	37.76637, -119.54167	Snow Trail near falls, Yosemite National Park, CA	1869.95
TRT	37.42990, -119.42321	Hwy 41 below Turtleback Dome, Yosemite National Park, CA	1480.65
WCL	37.20227, -119.34225	Willow Creek by Bass Lake Rd, Fresno County, CA	1100.27
WLF	37.50492, -119.35631	Tioga Rd east of White Wolf, Yosemite National Park, CA	2395.31
WUV	36.681, -118.88028	Along road to Wuksachi Village, Sequoia National Park, CA	2129.638
YCN	37.8264, -119.5957	Yosemite Creek on Tioga Rd, Yosemite National Park, CA	2346.85

Table 1. Geographic data on each *M. laciniatus* population used in the study.

Constitutive trait relationship with elevation

Trait	F-value	P-value	Direction of correlation
Leaf shape	**12.276596**	**0.003*** **	**+**
Flowering time	4.179683	0.056*	-
Corolla width (mm)	4.086909	0.058*	+
Pistil length (mm)	2.57640	0.126	
Leaf area (px)	2.4211552	0.137	
Corolla length (mm)	2.27840	0.149	
Herkogamy (mm)	1.8451199	0.191	
Plant height (mm)	0.397350	0.536	
Stamen length (mm)	0.34039	0.5668	

Table 2. Significance of correlation between constitutive trait expression and population elevation. An ANOVA was used to generate these statistical comparisons. For all traits, numDF = 1, denDF = 18. *** = $p < 0.01$; ** = $p < 0.05$; * = $p < 0.1$.

<u>Constitutive trait relationship with latitude</u>

Trait	F-value	p-value	Direction of correlation
Leaf lobing	4.017275	0.0663*	+
Corolla length	1.837361	0.1983	
Flowering time	1.753481	0.2083	
Herkogamy	1.535635	0.2372	
Corolla width	1.0265023	0.3295	

Anther	0.7934602	0.3892	
Plant height	0.6417213	0.4375	
Stigma	0.0470382	0.8317	
Leaf area	0.0344051	0.8557	

Table 3. Significance of correlation between constitutive trait expression and population latitude. An ANOVA was used to generate these statistical comparisons. For all traits, numDF = 1, denDF = 13. *** = p < 0.01; ** = p < 0.05; * = p < 0.1.

GxE

Trait	G	E	GxE
Plant height	df = 112 F = 4.0948 p < 0.001	df = 1 F = 2251.7352 p < 0.001	df = 109 F = 3.4516 p < 0.001
Flowering time	df = 112 F = 8.47 p < 0.001	df = 1 F = 809.2933 p < 0.001	df = 88 F = 3.8501 p < 0.001
Leaf lobing	df = 112 F = 3.3609 p < 0.001	df = 1 F = 49.5744 p < 0.001	df = 108 F = 2.0924 p < 0.001
Leaf area	df = 112 F = 5.6028 p < 0.001	df = 1 F = 600.5746 p < 0.001	df = 108 F = 2.8207 p < 0.001
Corolla length	df = 112 F = 5.7080 p < 0.001	df = 1 F = 506.5164 p < 0.001	df = 88 F = 2.8753 P < 0.001
Corolla width	df = 112 F = 6.7879 p < 0.001	df = 1 F = 699.9515 p < 0.001	df = 88 F = 2.2367 p < 0.001
Short stamen length	df = 112 F = 4.433 p < 0.001	df = 1 F = 530.3004 p < 0.001	df = 88 F = 2.8457 P < 0.001
Long stamen length	df = 112 F = 4.9323 p < 0.001	df = 1 F = 477.1790 p < 0.001	df = 88 F = 2.4494 p < 0.001
Pistil length	df = 112	df = 1	df = 88

	F = 4.5903 p < 0.001	F = 409.9971 p < 0.001	F = 1.8443 p < 0.001
Herkogamy	df = 112 F = 1.8216 p < 0.001	df = 1 F = 104.4350 p < 0.001	df = 88 F = 3.1339 p < 0.001
Cleistogamy	df = 112 F = 2.7126 p < 0.001	df = 1 F = 138.1892 p < 0.001	df = 87 F = 3.5217 p < 0.001

Table 4. Significance of genotype (G), environment (E), and genotype by environment (GxE) interactions on plasticity. An ANOVA was used to generate these statistical comparisons. df = degrees of freedom, F = F-value, p = p-value

<u>Plasticity strength relationship with elevation</u>

Trait	F-value	P-value	Direction of correlation
Critical photoperiod	**13.44429**	**0.003*** **	**+**
Leaf shape plasticity	**8.790705**	**0.010*** **	**+**
Short stamen length plasticity	4.068938	0.063*	+
Pseudo-cleistogamy plasticity	3.933972	0.0673*	+
Pistil length plasticity	3.836726	0.070*	+
Long stamen length plasticity	3.798262	0.072*	+
Corolla length plasticity	3.7866308	0.117	
Corolla width plasticity	1.746458	0.208	
Plant height plasticity	0.365843	0.555	
Herkogamy plasticity	0.1446548	0.709	

Flowering time plasticity	0.115048	0.740	
Leaf area plasticity	0.044490	0.836	

Table 5. Significance of correlation between strength of plasticity and population elevation. An ANOVA was used to generate these statistical comparisons. For all traits, numDF = 1, denDF = 14. *** = $p < 0.01$; ** = $p < 0.05$; * = $p < 0.1$.

<u>Plasticity strength relationship with latitude</u>

Trait	F-value	p-value
Critical photoperiod	0.651162	0.4342
Leaf area	0.3967746	0.5397
Flowering time	0.3773531	0.5496
Short stamen	0.293991	0.5968
Long stamen	0.217931	0.6483
Leaf lobing	0.173843	0.6835
Corolla length	0.0889135	0.7703
Plant height	0.0513789	0.8242
Cleistogamy	0.030234	0.8646
Pistil	0.00739	0.9328
Herkogamy	0.00497844	0.9448
Corolla width	0.0027088	0.9593

Table 6. Significance of correlation between plastic trait expression and population latitude. An ANOVA was used to generate these statistical comparisons. For all traits, numDF = 1, denDF = 13. *** = $p < 0.01$; ** = $p < 0.05$; * = $p < 0.1$.

SUPPORTING INFORMATION

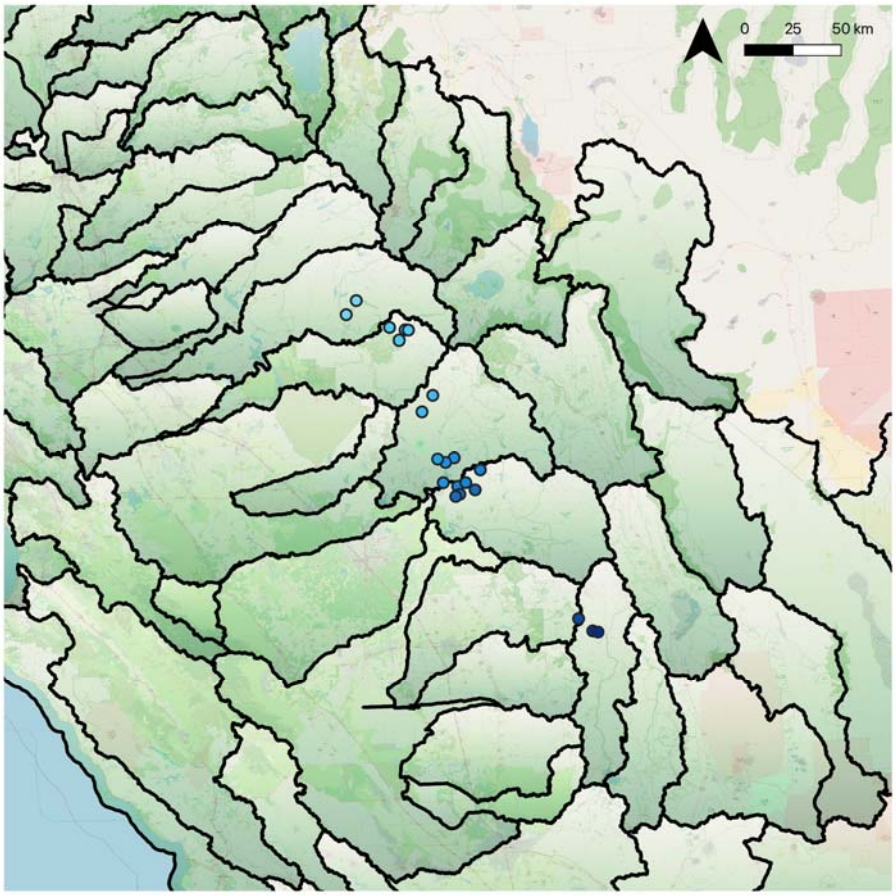

Figure S1. A map of study populations and watershed boundaries in California.

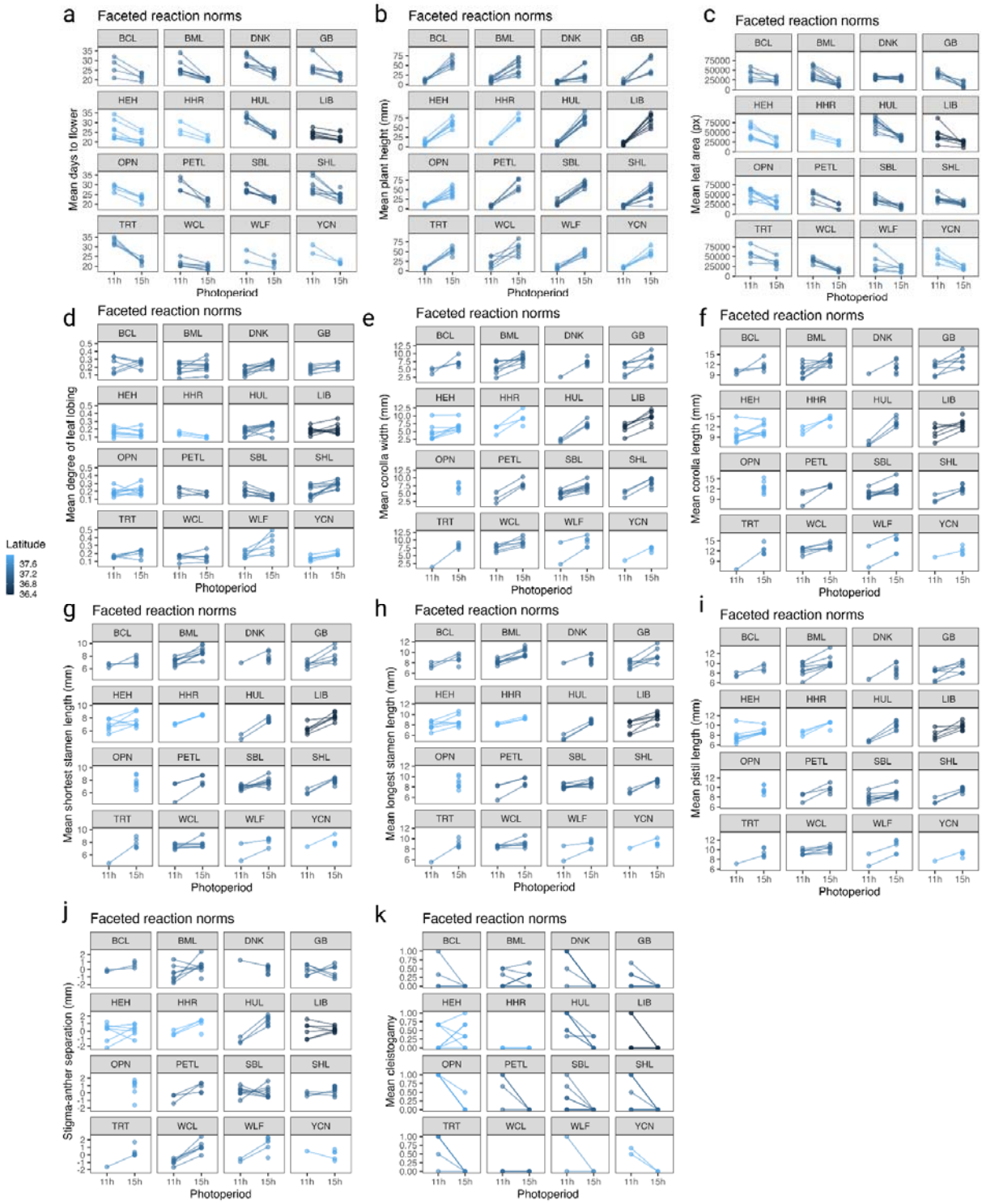

Figure S2. Reaction norms for each trait, faceted by population.