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PHENOTYPIC VARIATION IN SEEP MONKEYFLOWERS ALONG A FINE-SCALE SOIL MOISTURE GRADIENT

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ABSTRACT

Spatial and temporal variation in water availability is known to play a large role in shaping plant phenotypes within species. For ecologically and phenotypically diverse species complexes such as seep monkeyflowers (*Erythranthe guttata* (DC.) G.L.Nesom complex, Phrymaceae), it is not always clear how acclimation to local soil moisture environments contributes to morphological, physiological and phenological variation. Here we investigate phenotypic variation and plasticity in annual and perennial seep monkeyflowers where they co-occur within meters of each other on the Central Coast of California. Although the annual form tends to occur in drier rock outcrops and the perennial form occurs in adjacent perennial seeps, there appears to be a gradient of indistinguishable phenotypes between these microhabitats. We first tested and confirmed that key morphological, physiological, and phenological life history traits varied with soil moisture along the natural gradient from rock outcrops to seeps and with experimental water additions in the rock outcrops. To determine the role of plasticity in maintaining trait differences in the field, we collected seeds from individuals at the extremes of the soil moisture gradient to grow under common conditions. In the common garden, petal size and flowering time differences were partially maintained, while vegetative and physiological trait differences were not maintained. This indicates that much of the phenotypic variation between microhabitats is environmentally induced, although other factors may be at play. These results improve our understanding of how plants respond to fine-scale variability in soil moisture and suggest that plasticity plays a key role in the phenotypic diversity observed in seep monkeyflowers.

Key Words: drought escape, life history strategy, microhabitat, *Mimulus guttatus* sensu lato, phenotypic variation, plasticity, sympatric taxa.

Phenotypic variation within plant species in the California Floristic Province is in part due to the region's immense environmental heterogeneity. Numerous studies examining phenotypic variation across California's large and fine-scale environmental gradients have long provided key insights on how plants both adapt and acclimate to local conditions (Clausen 1940, 1948; Linhart 1974; Nagy and Rice 1997; Lowry 2008; Gould et al. 2013). Nevertheless, for many species and species complexes it remains unclear what environmental and genetic factors contribute to phenotypic variation, which often involves shifts in suites of traits.

Spatial and temporal variation in soil moisture is thought to be a major driver of phenotypic differences in plants (Geber and Dawson 1990; Ackerly et al. 2000; Kooyers 2015). Water availability varies dramatically along a longitudinal gradient in the California Floristic Province, from the more stable mesic conditions on the coast to extreme seasonality and desert conditions further inland (Lancaster and Kay 2013; Baldwin 2014). Plants can optimize growth and reproduction in these different environments with specific combinations of morphological, physiological and phenological traits (Bradshaw 1965; Hamann et al. 2018). For example, annuals adapted to the long dry season of California's interior often have drought escape traits—reduced vegetative growth, rapid metabolism, and early flowering—allowing them to quickly

complete their life cycle when water is available (Kooyers 2015). Drought-adapted annuals may also exhibit drought-resistant or water-conserving traits such as small, thick leaves and higher water use efficiency (WUE) (Welles and Funk 2021). In contrast, herbaceous perennials in mesic coastal sites have largely drought-intolerant traits—higher vegetative allocation, larger leaves, slower metabolism, and delayed flowering (Ayodele Cole 1967; Wu et al. 2010). In California, this inland-annual and coastal-perennial biogeographic pattern for herbaceous plants can be found within a single genus (e.g., *Abronia* Juss. (Nyctaginaceae) and *Dithyrea* Harv. (Brassicaceae), and within species complexes, e.g., *Erythranthe guttata* sensu lato (DC.) G.L.Nesom (Phrymaceae) and *Eschscholzia californica* Cham. (Papaveraceae) (Wilson 1972; Lowry 2008; Baldwin et al. 2012; Still and Potter 2013). Intra-specific phenotypic variation related to water availability can also be found at small spatial scales, with some excellent examples along vernal pools, on different soil types, and on different slope aspects (Linhart 1974; Lambrecht 2013; Dittmar and Schemske 2018). Generally, comparative work within ecologically and phenotypically diverse species sheds light on how acclimation and adaptation to contrasting soil moisture regimes contribute to phenotypic divergences (Clausen 1940; Lambrecht and Dawson 2007; Kim and Donohue 2013; Pearse et al. 2019).

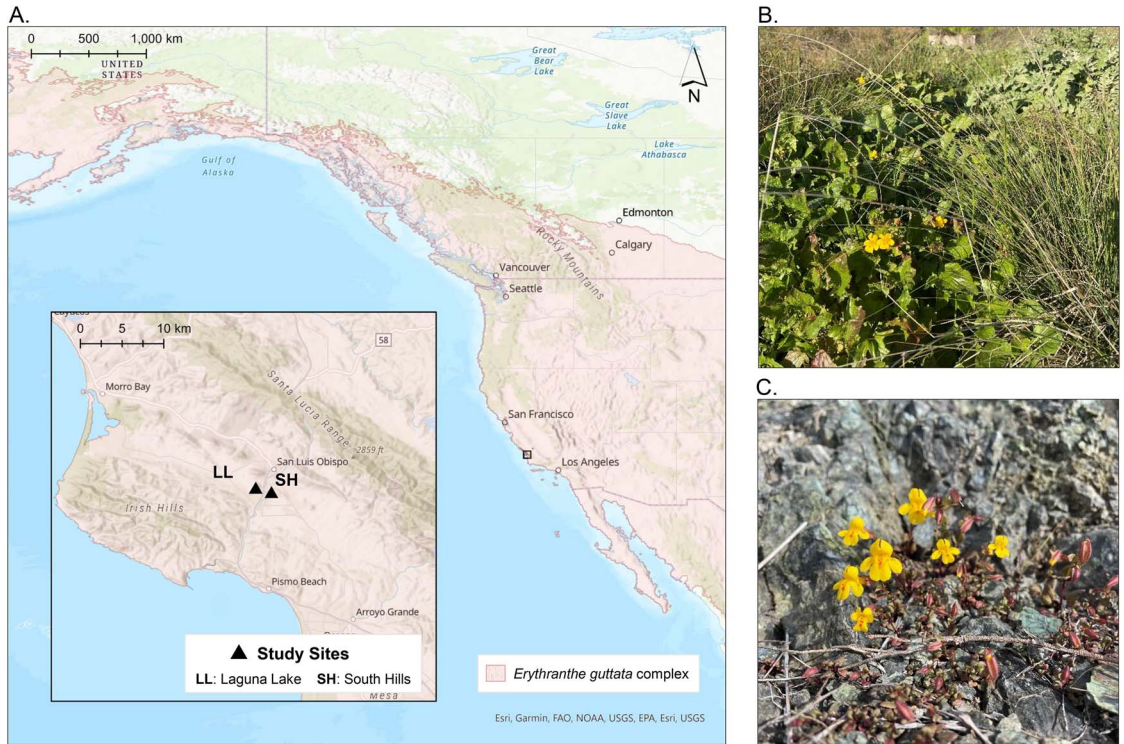


FIG. 1. (A) The estimated native range of seep monkeyflowers—*Erythranthe guttata* complex (Maitner et. al 2018) Inset: The two study sites where the perennial and annual seep monkeyflowers co-occur in the Central Coast ranges of San Luis Obispo County, California. Map created with Arc GIS Pro (ESRI 2011, ArcGIS Desktop: Release 10, Redlands, CA). (B) Perennial seep monkeyflowers '*Erythranthe guttata*' (DC.) G.L.Nesom in a seep microhabitat. (C) Annual seep monkeyflowers '*Erythranthe serpentinicola*' D.J.Keil in a rock outcrop microhabitat.

Seep monkeyflowers (*Erythranthe guttata* complex) are a model system for studying phenotypic and genetic variation related to water availability (Wu et al. 2010; Ivey and Carr 2012; Mantel and Sweigart 2019). They are part of an ecologically diverse species complex occurring throughout the deserts, mountains, and coasts of western North America (Fig. 1A). Seep monkeyflowers have often been split into several ecotypes or species largely based on the morphological and phenological differences between annual and perennial forms (Grant 1924; Nesom 2012). However, widespread population genetic surveys throughout their range have found little evidence to support a taxonomic split between annual and perennial seep monkeyflowers, likely because of strong homogenizing gene flow where these populations overlap (Oneal et al. 2014; Twyford and Friedman 2015). The perennials occur along consistently wet streams and seeps and produce large flowers, leaves, and matting stolons and they flower throughout the summer. The annuals grow in ephemerally moist habitats and have a short lifespan, diminutive size, and early flowering time. Although this life history divergence may be environmentally induced in certain populations depending on seasonal water availability (Vickery 1959; Dole 1992), research has found that reproductively isolated coastal-perennial and

inland-annual seep monkeyflower populations show locally adaptive morphological and phenological trait differences associated with a chromosome inversion polymorphism (Lowry et al. 2008; Lowry and Willis 2010). However, less is known about whether these trait differences have a genetic basis at finer scales where annual and perennial seep monkeyflower populations co-occur and likely interbreed.

In two uncommon cases in the Central Coast Ranges of San Luis Obispo County, California, annual and perennial seep monkeyflowers grow within meters of each other along serpentine seeps. While the annuals occur largely in the summer drying rock outcrops and the perennials in the perennial seeps, a gradient of intermediate phenotypes occur between these microhabitats (Zell unpublished data). One explanation for this pattern is that the annuals and perennials are two locally adapted ecotypes (Lowry 2008) or species (Nesom 2012; Keil 2020), with incomplete reproductive barriers in intermediate habitats resulting in a hybrid zone. An alternative explanation is that there is little to no genetic differentiation between the annuals and perennials, and the observed life history divergence is primarily explained by a plastic response to different soil moisture regimes. If this were the case, the key morphological, physiological and phenological traits responsible for

different life history strategies would co-vary with and respond plastically to soil moisture. This system provides an excellent opportunity to uncover the relationship between phenotypic variation and water availability in a species that is known to both vary plastically and to locally adapt to different soil moisture conditions (Galloway 1995; van Kleunen 2007; Lowry and Willis 2010).

In this study, we examined phenotypic trait variation and plasticity among co-occurring seep monkeyflowers that appear to have a life history divergence correlated with soil moisture on the Central Coast of California. We first quantified whether morphological, physiological, and phenological traits co-vary with soil moisture in the field and with supplemental water treatments in the field. We expected to find a higher incidence of drought-escape or drought-resistant traits in plants in the drier soils in the rock outcrops. Morphological traits such as stolon production, floral size, and vegetative size are expected to decrease with decreasing water availability due to resource limitation or to minimize water loss over larger surface areas (Lambrecht and Dawson 2007). We measured two traits related to plant physiology: specific leaf area (SLA) — the ratio between leaf area and leaf dry mass, which tends to correlate positively with metabolic rates (Poorter et al. 2009; Gonzalez-Paleo and Ravetta 2018)— and stomatal density, which tends to correlate positively with stomatal conductance (Carlson et al. 2016). Since thinner leaves (higher SLA) and increased stomatal density are associated with rapid resource metabolism, plants following a drought-escape strategy may have higher SLA and stomatal density (Wu et al. 2010; Kooyers 2015). However, plants following a drought-resistant strategy may display the opposite, with lower SLA and stomatal density—traits that are associated with higher WUE (Kooyers 2015). The phenological trait that we measured was flowering time. We expected plants in drier soils to flower earlier because early flowering is a drought escape strategy that ensures reproduction in water limited habitats (Wu et al. 2010).

We also examined whether phenotypic trait differences observed in the field between contrasting environments are maintained under more stable moisture conditions in a common garden using first generation field-collected seeds from the extremes of the soil moisture gradient: that is, from seep and rock outcrop microhabitats. Since quantitative traits such as size, flowering time, SLA, and stomatal density are known to vary plastically with water availability (Bradshaw 1965; Sultan 2000; Bongers et al. 2017), individuals sourced from seep and rock outcrop habitats grown under common conditions are expected to have reduced trait differences. However, certain trait differences could be partially or wholly maintained in a common environment, which would be consistent with genetic differentiation in contrasting moisture environments or maternal effects.

MATERIALS AND METHODS

Study System

The perennial seep monkeyflower in this study is currently recognized as *Erythranthe guttata* (DC.) G.L.Nesom (Fig. 1B), a stoloniferous perennial herb with a native range spanning from Alaska to northern Mexico (Vallejo-Marin et al. 2021). The annual seep monkeyflower in this study was recently described as a distinct species, *Erythranthe serpentinicola* D.J.Keil (Fig. 1C), with only three confirmed populations in the coast ranges of west-central San Luis Obispo County, California (Keil 2020). It is a spring ephemeral that specializes on shallow serpentine rock outcrops in close proximity to perennial seeps (Keil 2020). Due to their divergent life history strategies, *E. guttata* and *E. serpentinicola* should be distinguishable based on their overall size and stolon growth (Nesom 2012; Friedman et al. 2015). However, size and stolon growth also vary with different soil moisture regimes in seep monkeyflowers (Mantel and Sweigart 2019; Wu et al. 2010), which may explain why these putative species appear to be indistinguishable in intermediate habitats between rock outcrops and seeps (Zell unpublished data). Because of the indistinct phenotypic boundaries at these sites and the unconfirmed taxonomic split between annuals and perennials in this species complex, for the purposes of this study, we use the common name ‘seep monkeyflower’ to refer to both *E. guttata* and *E. serpentinicola*.

This research took place in the City Open Spaces of San Luis Obispo at two sites (Fig. 1A), each containing both perennial serpentine seeps and adjacent serpentine rock outcrops: Laguna Lake Park (35.26663, -120.68254), and South Hills (35.262134, -120.665198). Serpentine rock outcrops are known for having a low capacity to hold moisture and low vegetation cover which increases evaporation from the soil (Cacho and Strauss 2014; Harrison et al. 2015). Serpentine seeps, on the other hand, often flow year-round just below the ground surface, providing a moist microhabitat throughout long summer droughts in California (Harrison et al. 2000; LeCroy et al. 2021). Therefore, a steep soil moisture gradient likely occurs from the serpentine seeps to adjacent rock outcrops, particularly during spring and early summer when seep monkeyflowers are flowering and setting seed.

Experimental Design

The two sympatric study sites, Laguna Lake (LL) and South Hills (SH), consisted of roughly 10,000 m² areas containing both annual and perennial seep monkeyflowers, differentiated when possible based on stolon presence or absence. Voucher specimens for annuals and perennials from each site were deposited at the Hoover Herbarium (OBI) at California Polytechnic State University, San Luis Obispo. The annuals tended to occur in dense, homogenous patches at one extreme

in the rock outcrops and the perennials in infrequent matting clusters at the other extreme in the seeps, with a gradient of indifferentiable phenotypes between these microhabitats. Due to the patchy distribution and indistinct phenotypic boundaries between populations, 2 × 1-meter plots were established in the densest patches of seep monkeyflowers in three different microhabitats: rock outcrop, intermediate and seep. LL had five rock outcrop, two intermediate, and three seep plots. SH had two rock outcrop, one intermediate, and two seep plots. Differences in water availability between these three microhabitat categories were confirmed with soil moisture measurements (volumetric water content, VWC) taken at approximately peak flowering time in all 15 plots in the study using a soil moisture probe (TEROS 10, METEK group, Pullman, WA). Environmental variables that may influence water availability—soil depth, vegetation cover, and surface temperature—were also measured in each plot (Appendix S1). On average, rock outcrops had the lowest soil moisture levels (2.7 ± 0.7 SE VWC), intermediate plots were outside of seeps and had intermediate soil moisture levels (25.3 ± 0.4 VWC), and seep plots were often completely inundated and had the highest soil moisture levels (39.3 ± 0.5 VWC).

Microhabitat plots were established during early to peak flowering in March 2021. In June 2021, seeds were collected from each plot and grown in the common garden the following year (January–June 2022).

Field Surveys

The two main objectives of the field surveys were to determine whether morphological, physiological, and phenological traits co-varied with the soil moisture gradient and to assess the extent of trait divergence between seep monkeyflowers found in the most extreme microhabitats: rock outcrop versus seep. When a given plot was approximately at peak flowering, we collected morphological and physiological trait data from three individuals with fully expanded flowers closest to randomly generated numbers along a 200 cm transect placed approximately through the center of the plot. We measured soil moisture at the base of each of the three individuals at a depth of 1 cm using a soil moisture probe (for a similar approach, see Ferris et al. 2014). If the soil was less than 1 cm deep, we measured soil moisture within 5 cm of the selected individual where we could fully insert the probe 1 cm into the soil.

Watering Addition Experiment

We performed a water addition experiment in the LL rock outcrop population to determine whether increasing water availability would induce trait shifts. In January 2022, two weeks after the first winter rain event, ten paired 22 × 22 cm plots were established along a southwest-facing serpentine hillside roughly 20 meters north of the field survey plots described above. Paired plots were approximately 60 cm apart, and roughly at the same elevation, slope, and aspect; each plot contained at least 50 seep monkeyflower seedlings.

One plot from each pair was randomly assigned with a watering treatment: approximately two liters of water were added using a watering can every two days until all the plants in the plots senesced, from January 6 to April 3. The dry-control plots did not receive any supplementary water and were subject to naturally drying conditions in the rock outcrop habitat. During the three month duration of the experiment, light rain, and mist resulted in roughly three inches of precipitation each month (Weatherspark 2022). Eight of the ten dry-control plots did not have any individuals survive to flowering, as seedling mortality was high following a heat wave in mid-February. In plots with individuals that survived to flowering (two control and ten watered plots), we recorded morphological and physiological trait data for two haphazardly chosen flowering individuals within the plot. For the eight dry-control plots with no survival, we haphazardly sampled any flowering individuals located within a one-meter radius of the control plot, but still approximately 60 cm outside of the watered plot. In addition, we measured soil moisture at the base of each focal individual following the same methods described above. All trait and soil moisture sampling took place between mid-February and early March, approximately during peak flowering time for the LL rock outcrop population. For each plot we then calculated the average trait and soil moisture value which was used in downstream analyses.

Common Garden

The objective of the common garden experiment was to determine whether phenotypic differences between the seep and rock outcrop individuals in the field were maintained under common environmental conditions. For this study, we used field-collected seeds from three or four haphazardly selected individuals from seep and rock outcrop plots from both study sites. We did not collect seeds from intermediate plots, to maximize the likelihood of detecting trait differences in the common garden. Ultimately, we collected 12 seep and 12 rock outcrop maternal seed families from both LL and SH. In late November 2021, seeds were sown in four-inch pots (two replicate pots per family) with moistened potting soil (Black Gold Organic, Sun Gro Horticulture, Agawam, MA), randomly sorted into trays, and stratified in the dark at 4°C for seven days (for similar approach see Wu et al. 2010). After stratification, trays were brought to a lathhouse on the Cal Poly, San Luis Obispo Campus, which had temperature and day length conditions similar to those experienced by seeds in the field located only ~4 km away. For the duration of the experiment, soil was kept continuously and moderately moist by watering each tray regularly. All pots were thinned to one focal individual per pot within one week of germination. For each focal plant, the date of germination and first flower were recorded, and all other traits were measured at the date of the first fully expanded flower using the methods described below.

Trait Measurements

We measured morphological, physiological, and phenological traits that are known to vary in seep monkeyflowers under different soil moisture regimes (e.g., Wu et al. 2010; Friedman et al. 2015; Mantel and Sweigart 2019).

Morphology. We measured seven morphological traits: stolon number and six traits related to plant size. Stolons were defined as any horizontal stem branching from the basal nodes on the main vertical stem of the plant. All size traits were measured to the nearest millimeter. Vegetative traits included stem width and leaf length. Stem width was measured at the second internode. Leaf length was measured from the widest point between the apex to the base of an individual leaf collected from the second node of each plant. Floral traits included corolla tube length, corolla width, calyx length, and pedicel length. In the field, a single fully expanded flower from each individual was haphazardly chosen for all floral measurements. In the common garden, the first fully expanded flower was measured for each individual. The maximum width across the bottom lobe constituted corolla width and the distance from the end of the pedicel to the fusing point of the top and bottom lobes constituted corolla tube length. Calyx length was measured from the end of the pedicel to the top (longest) lobe of the calyx. Pedicel length was measured from the base of the pedicel to the receptacle.

Physiology. We measured two traits associated with plant physiology: specific leaf area (SLA) and stomatal density. We measured SLA (ratio of leaf area to leaf dry mass) by removing a leaf from the second node of each individual, measuring leaf blade area with the LeafByte Application (Getman-Pickering et al. 2020), drying the same leaf for over a week at approximately 40°C, and weighing it to the nearest milligram. We measured stomatal density by painting clear nail polish (Top Coat Sealer, Probelle, Dania Beach, FL) on the abaxial leaf surface and then peeling the polish off and viewing it under a light microscope at 100x magnification. We haphazardly selected three fields of view on the same peel and recorded the average number of stomata from the three views.

Phenology. Phenological measurements of flowering time were specific to the field or common garden. In the field, we assessed flowering time by counting the number of flowers within each plot every 2–4 days throughout the flowering period of both species (February–July 2022). From these data we estimated the days to peak flowering for each plot as the time between January 1, 2022 and peak flowering (the date with the maximum number of flowers). In the common garden, flowering time was estimated as the number of days from germination to first flower for each individual.

Statistical Analyses

All analyses were performed using R statistical software (version 4.1.2, R Foundation for Statistical

Computing, Vienna, Austria) and associated data and code are available on Dryad (doi:10.5061/dryad.18931zd1z).

Traits and soil moisture in the field. To quantify how morphological, physiological, and phenological traits vary along a continuous soil moisture gradient in the field, we used separate linear mixed effects models for each of the ten continuous traits (*lmer* function, *lme4* R package v1.1-30). Soil moisture was included as a fixed effect predictor, while trait was treated as the response variable, and plot nested within site was included as a random effect. The experimental unit of observation was an individual (three individuals per plot). We natural log-transformed (*log* function, *base* R v3.6.2) response variables that had non-normally distributed residuals. Statistical significance of soil moisture as a predictor for each trait was evaluated with an ANOVA (*anova* function, *car* R package v3.1-0). To account for the possibility of false discovery from multiple test comparisons, we present corrected P-values using a Benjamini-Hochberg correction for ten tests (*p.adjust* function, *stats* R package v3.6.2).

Traits and supplementary water in the field. To ensure that the watering treatment effectively increased soil moisture during peak flowering, we conducted a paired t-test to compare soil moisture in dry-control to watered plots (*t.test* function, *stats* R package v3.6.2). To determine whether the rock outcrop annuals are capable of plastically responding to an increase in soil moisture, we conducted eight additional paired t-tests to compare the trait values between watered and dry-control paired plots for each trait separately. We present corrected P-values using a Benjamini-Hochberg correction for nine tests (*p.adjust* function, *stats* R package v3.6.2). To evaluate the extent of plasticity for each trait, we calculated the percent difference between paired watered and dry-control plots for each trait ($[(\text{watered} - \text{dry-control}) / (\text{watered} + \text{dry-control}) / 2] \times 100$) and then averaged it across all watered and dry-control plots.

Traits in the field versus the common garden. To determine whether rock outcrop and seep individuals differed in morphological, physiological, and phenological traits in the field and in the common garden, we used separate linear mixed-effects models for each of the nine traits in both the field and common garden (18 models total). We natural log-transformed response variables that had non-normally distributed residuals. Microhabitat type (2 levels: rock outcrop, seep), site (2 levels: LL, SH) and their interaction were included as fixed effects. For all of the field models except for ‘peak plot flowering time,’ plot was included as a random effect, since trait data was collected from three individuals sampled in each plot. For the common garden models, maternal line nested within plot was included as a random effect since we collected seed from multiple individuals in each plot and two replicates were grown from each individual whose seeds were collected. For both the field and common garden

TABLE 1. Linear Mixed effects Models Examining Whether Soil Moisture (Volumetric Water Content, VWC) Predicted Plant Traits (10 Separate Models). Significant relationships ($\alpha = 0.05$) between soil moisture and traits following Benjamini-Hochberg correction for multiple tests ($n = 9$) are indicated in bold font.

Response variable	F	P	R ²
Stolon number	57.313	<0.001	0.630
log Stem width (mm)	65.814	<0.001	0.736
log Leaf length (mm)	69.316	<0.001	0.771
Corolla width (mm)	33.113	<0.001	0.505
Corolla tube length (mm)	56.214	<0.001	0.682
log Calyx length (mm)	55.114	<0.001	0.671
log Pedicel length (mm)	31.513	<0.001	0.433
log SLA (cm ² /g)	25.513	<0.001	0.371
log Stomatal density	3.414	0.085	0.117
log Peak flowering each plot (days)	4.913	0.050	0.260

model results, we present the corrected P-values using a Benjamini-Hochberg correction for 10 tests (*p.adjust* function, *stats* R package v3.6.2).

RESULTS

Trait Variation in the Field

Traits co-vary with soil moisture in the field. All traits in the field except stomatal density significantly co-varied with soil moisture (Table 1, Fig. 2). Stolon number, vegetative size (stem and leaf) and floral size (corolla, calyx, and pedicel) increased with increasing soil moisture. These findings indicate that individuals in drier soils were more likely to have drought-escape traits—that is, no stolon production and small vegetative and floral size traits. Individuals in wetter soils were more likely to have drought-intolerant traits, including stolon production and large vegetative and floral size traits. For physiological traits, SLA decreased with increasing soil moisture, meaning that individuals in drier soils had higher SLA (thinner leaves) relative to individuals in wetter soils. For phenology, there was a weak positive relationship between average days to peak flowering and average soil moisture for each plot. This means that plots in drier soils tended to reach peak flowering earlier than plots with wetter soils.

Traits vary with supplementary water in the field. The watering addition treatment in the rock outcrop habitat significantly increased soil moisture in the watered plots compared to the dry-control plots at the time of trait data collection (Table 2). None of the individuals in either treatment plot produced stolons. However, we found significant differences in morphological size traits and in the SLA between individuals in watered versus dry-control plots (Table 2, Fig. 3). Looking at the average percent difference between paired watered and dry-control plots (Table 2), vegetative size traits were over 50% larger in the watered plots. Floral size traits, on the other hand, were only 20-30% larger. SLA was more than 75% lower in the

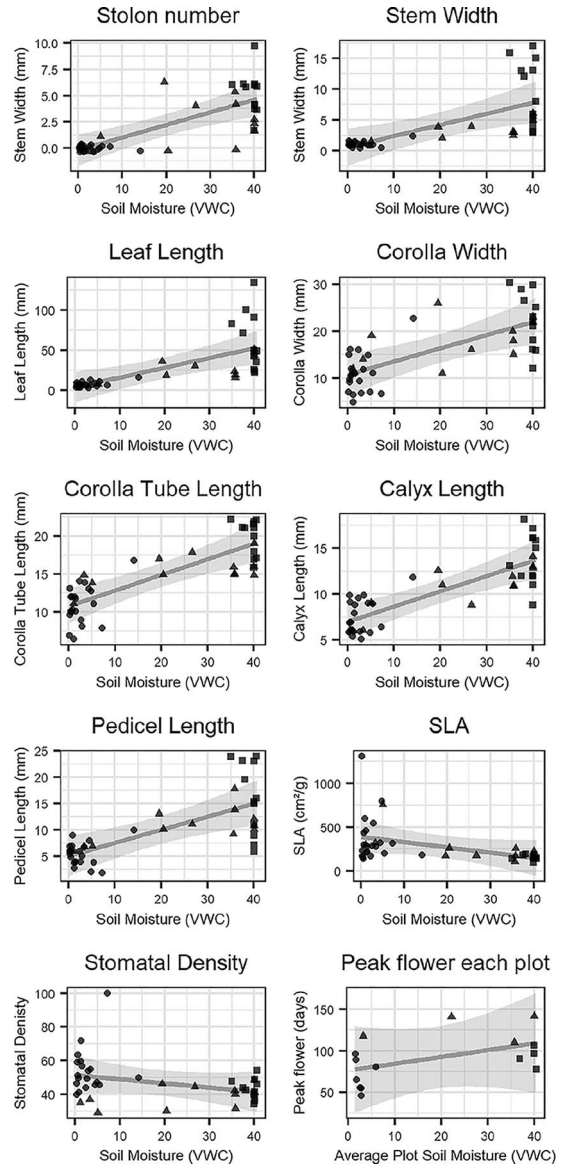


FIG. 2. Visualization of trait variation along a natural soil moisture gradient: scatterplots for each trait with linear regression line (grey line) and 95% confidence interval (grey shading) included. Symbols represent individuals from rock outcrop (circles), intermediate (triangles), and seep (square) plots. See Table 1 for linear regression statistics.

watered plots, while stomatal density showed less than a 5% difference between paired plots. Although flowering time was documented, we were unable to compare peak flowering time between watered and dry-control plots because most dry-control plots did not flower.

Field trait differences between individuals in contrasting microhabitats. There were significant differences in all measured traits between rock outcrop and seep individuals at the two study sites, LL, and SH

TABLE 2. Watering Addition Experiment, Paired T-tests. For each of the eight traits and soil moisture, the mean percent difference between paired watered and dry-control plots ($[(\text{watered} - \text{dry-control})/(\text{watered} + \text{dry-control})/2] \times 100$) is included. Significant differences ($\alpha = 0.05$) between watered and dry-control paired plots following Benjamini-Hochberg correction for multiple tests ($n = 10$) are indicated in bold font.

Response variable	Mean % pairwise difference (± 1 SE)	t	P
Stem width (mm)	52.5 \pm 11.4	-3.79	0.011
Leaf length (mm)	53.3 \pm 9.0	-3.99	0.011
Corolla width (mm)	20.5 \pm 7.4	-2.79	0.029
Corolla tube length (mm)	17.6 \pm 4.1	-3.19	0.017
Calyx length (mm)	22.8 \pm 6.2	-3.19	0.017
Pedicle length (mm)	29.5 \pm 8.4	-3.59	0.012
SLA (cm^2/g)	89.7 \pm 8.2	6.45	0.007
Stomatal density	3.0 \pm 8.5	0.44	0.738
Soil moisture (VWC)	111.3 \pm 16.8	-4.78	0.007

(Table 3A, Fig. 4A). As expected, none of the rock-outcrop individuals produced any stolons, while the seep individuals produced many stolons. Rock outcrop individuals were much smaller in vegetative and floral sizes, and they had a higher SLA (thinner leaves) and a higher stomatal density compared to seep individuals. Flowering time was significantly different between microhabitats, with rock outcrop plots reaching peak flowering earlier than seep plots (Fig. 5A). There was no significant interaction between ‘microhabitat’ and ‘site’ for any of the field models, meaning that there were similar trends at both sites.

Trait Variation in the Common Garden

Common garden trait differences between offspring from contrasting microhabitats. In the common garden, the trait differences between individuals sourced from rock outcrops and seeps were only partially maintained (Table 3B, Fig. 4B). There were no significant differences in stolon number, vegetative size traits, and SLA detected between individuals sourced from rock outcrop and seep plots. Differences in corolla size and flowering time were maintained in the common garden, although they were not as drastic as the differences observed in the field (Appendix S2). Stomatal density differed between individuals sourced from rock outcrop and seep plots, however, this was site dependent (Table 3B, interaction between site and species $P < 0.05$). At LL, stomatal density was highest for rock outcrop sourced individuals whereas at SH, stomatal density was lowest for rock outcrop sourced individuals.

DISCUSSION

Here we demonstrated that the morphological, physiological, and phenological traits of seep monkeyflowers co-vary with soil moisture along a fine-scale soil moisture gradient. From our supplemental watering experiment, we found evidence that soil moisture can

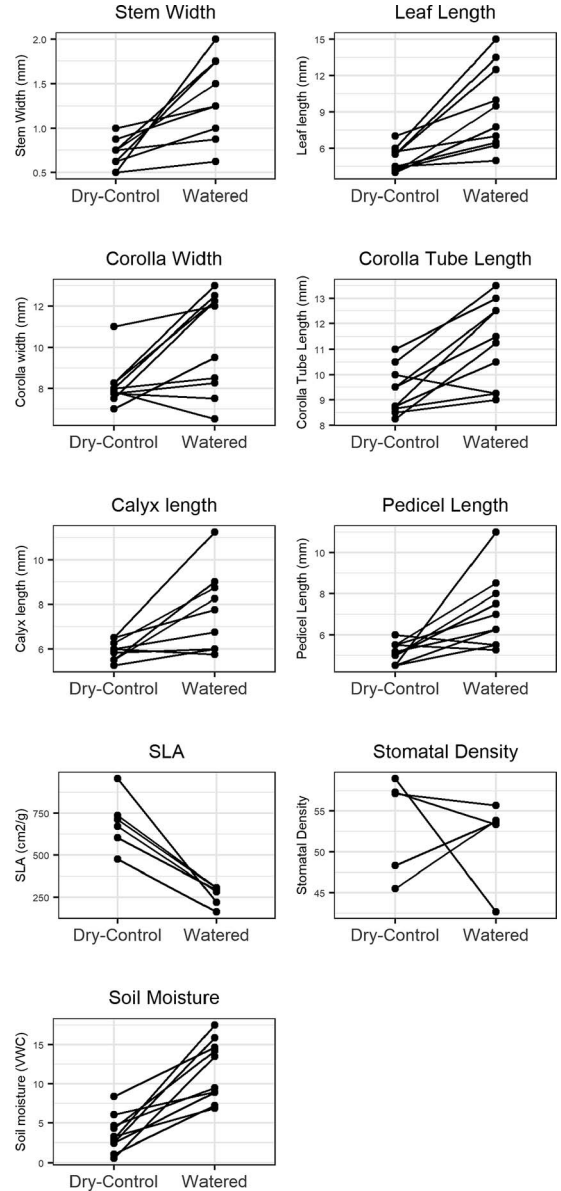


FIG. 3. Visualization of water addition experiment results: average percent difference between traits measured from watered and dry-control paired plots. See Table 2 for t-test statistics.

environmentally induce trait differences in rock outcrop populations. Under common garden conditions, trait differences observed in the field between the two most extreme microhabitats were either not detected or were notably reduced, depending on the trait. This finding is evidence that plastic responses to contrasting soil moisture habitats play a large role in shaping trait variation in this system. However, since certain trait differences were partially maintained in the common garden, future work will investigate the levels of genetic differentiation associated with trait variation between

TABLE 3. (A) Field and (B) Common Garden Linear Mixed Effects Models Examining Whether Microhabitat (2 levels: rock outcrop, seep), Site (2 levels: LL, SH), and Their Interaction Predict Plant Traits. Significant P-values ($\alpha = 0.05$) following Benjamini-Hochberg correction for multiple tests ($n = 10$) are indicated in bold font.

Response Variable	Microhabitat		Site		Microhabitat \times Site		P
	t	P	t	P	t	P	
A. Field							
Stolon number	8.0	< 0.001	0.0	1.000	0.0	1.000	
log Stem width (mm)	-5.7	0.003	2.2	0.158	-1.6	0.201	
log Leaf length (mm)	-5.5	0.003	3.1	0.076	-3.1	0.076	
Corolla width (mm)	-3.4	0.004	3.6	0.011	-2.7	0.076	
Corolla tube length (mm)	-5.8	0.003	2.0	0.158	-1.9	0.201	
log Calyx length (mm)	-3.0	0.021	1.1	0.453	-1.3	0.267	
log Pedicel length (mm)	-3.5	0.014	1.9	0.158	-0.4	0.697	
log SLA (cm ² /g)	2.9	0.013	0.4	0.823	1.8	0.201	
log Stomatal density	3.2	0.020	0.4	0.823	-1.6	0.201	
log Peak plot flower (days)	-3.0	0.027	0.2	0.880	1.8	0.201	
B. Common Garden							
Stolon number	-1.5	0.167	-0.9	0.397	1.2	0.324	
log Stem width (mm)	0.3	0.865	-0.4	0.8502	0.2	0.878	
log Leaf length (mm)	0.1	0.917	0.2	0.9319	0.5	0.818	
Corolla width (mm)	-4.2	0.001	-0.1	0.9319	2.5	0.075	
Corolla tube length (mm)	-3.5	0.003	0.5	0.8502	2.1	0.107	
log Calyx length (mm)	-2.3	0.121	-0.8	0.8502	1.8	0.210	
log Pedicel length (mm)	1.6	0.161	2.7	0.0802	-0.4	0.818	
log SLA (cm ² /g)	1.5	0.177	1.4	0.5241	-1.7	0.201	
log Stomatal density	-2.6	0.031	-2.4	0.101	3.1	0.034	
Germination-flower (days)	-3.8	0.002	-0.5	0.850	1.6	0.210	

seep monkeyflowers occurring in these contrasting microhabitats.

Our findings that plants in drier soils did not produce stolons, were smaller overall, had higher SLA, and flowered earlier are largely consistent with studies on annual seep monkeyflowers, and matches predictions of the drought escape hypothesis (e.g., Wu et al. 2010; Mantel and Sweigart 2019). Reduced stolon production and size in drier soils may be the result of a plastic drought-induced developmental response related to resource limitation (van Kleunen 2007; Lambrecht et al. 2017). Temporarily moist environments may also impose selection for rapid allocation of resources to reproduction rather than vegetative growth such as stolon production (van Kleunen 2007; Friedman et al. 2015). Drought conditions may also directly select for smaller size, a drought avoidance trait, since larger flowers and leaves have a higher surface area and lose more water through transpiration (Lambrecht 2013; Kuppler et al. 2021). Recent phylogenetic analyses on members of the *Erythranthe guttata* complex found evidence for parallel evolution of smaller narrow and lobed leaves in *Erythranthe laciniata* (A.Gray) G.L.Nesom, *Erythranthe filicifolia* (Sexton, K.G.Ferris and Schoenig) G.L.Nesom, and *Erythranthe nudata* (Curran ex Greene) G.L.Nesom, which may be adaptive for the drier rock outcrop habitats in which they occur (Ferris et al. 2015). Our finding of higher SLA in drier soils is also consistent with a drought escape response found in seep monkeyflowers (Wu et al. 2010). Rapid metabolism and growth in water-limited environments may result in thinner leaves and a high SLA (Gonzalez-Paleo and

Ravetta 2018). Studies on seep monkeyflowers (Mantel and Sweigart 2019) and in other herbaceous species (Gonzalez-Paleo and Ravetta 2018) have found that SLA tends to be higher in short-lived annuals compared to herbaceous perennials. Early flowering time is also associated with the drought escape strategy (Wu et al. 2010; Kooyers 2017) and may be due either to plasticity or to genetic differentiation (Wu et al. 2010; Ryan and Cleland 2021). Comparative work on coastal and inland *E. guttata* has found evidence for heritable flowering time differences (Lowry et al. 2008). In addition, finer scaled studies on annual and perennial members of the *Erythranthe guttata* complex have found evidence that water limitation imposes selection for early flowering (Ferris and Willis 2018; Mantel and Sweigart 2019). Our finding that stomatal density does not vary with soil moisture, however, is not consistent with previous work that found that annual seep monkeyflowers from drier habitats had lower stomatal density and were more drought tolerant than perennial seep monkeyflowers from wetter habitats (Wu et al. 2010; Mantel and Sweigart 2019). However, these studies were conducted in greenhouse settings, not along a natural soil moisture gradient.

From the water addition experiment, we found that traits respond plastically to greater soil moisture within the rock outcrop habitat. Individuals in watered plots were larger and had a lower SLA than those in control plots, consistent with the patterns observed along a natural soil moisture gradient in the field. However, these differences between watered and dry-control individuals were less than half that observed in naturally

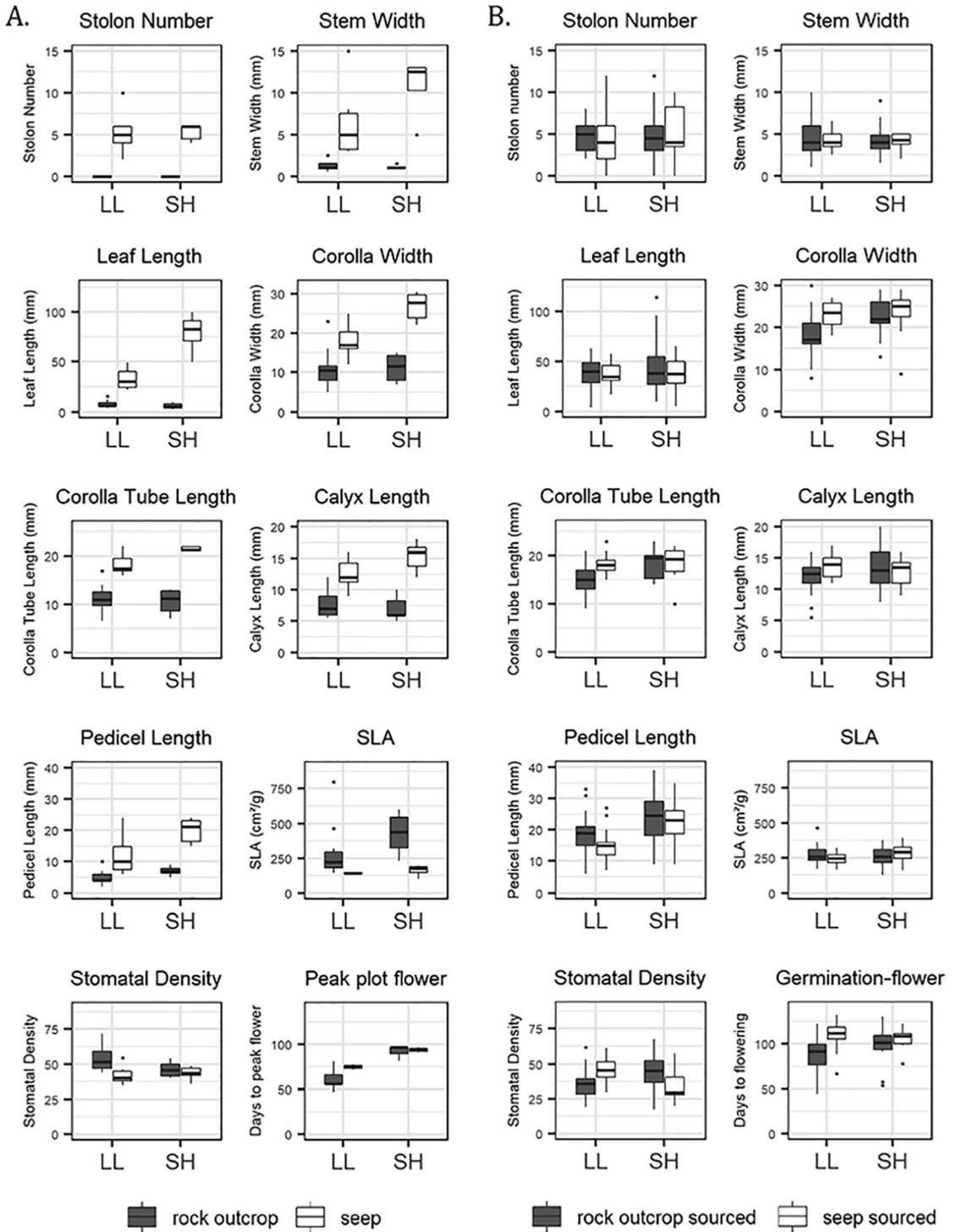


FIG. 4. (A) Field and (B) Common garden boxplots of the raw trait data visualizing differences between microhabitats (grey and white fill) and site (X-axis). Thick horizontal line represents the median of all values. See Table 3 for linear mixed effect model statistics.

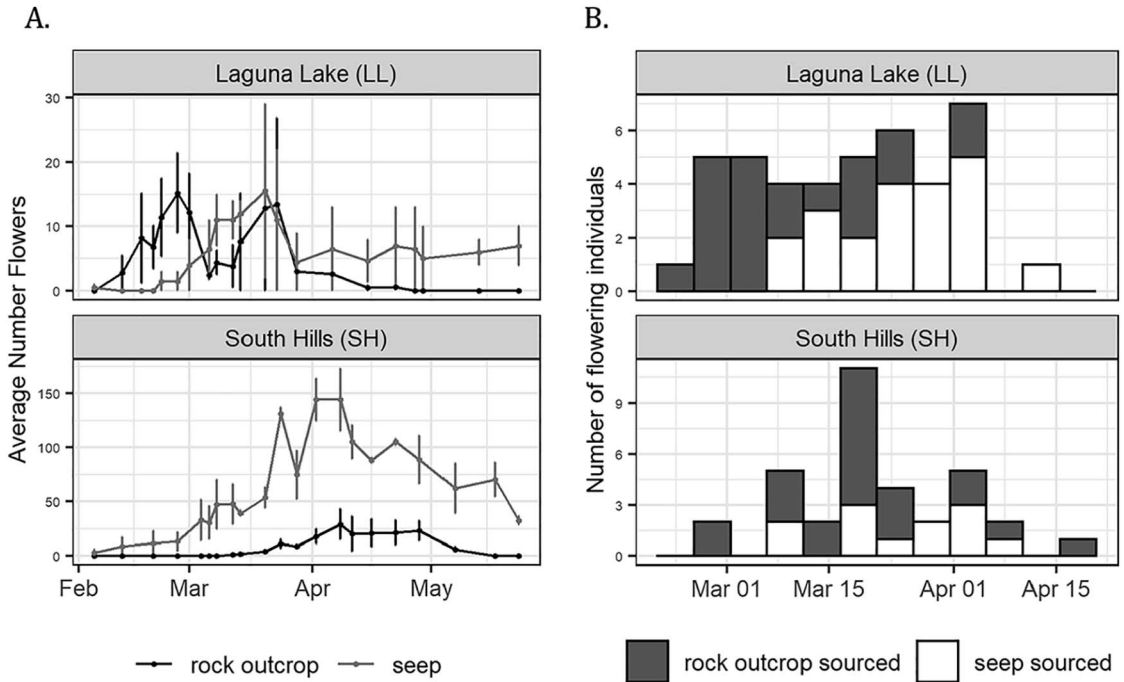


FIG. 5. (A) Field phenology visualization. Flowering time differences (average number of flowers per plot over time) between microhabitats at each site. Vertical lines represent one standard error. (B) Common garden phenology visualization. Flowering time differences (frequency of first flowering individuals over time) between microhabitats sourced from each site.

occurring rock outcrop and seep individuals (Table 2, Appendix S2). This discrepancy may be in part because our water addition experiment was not able to subject rock outcrop populations to seep-like soil moisture levels. Seep plots were 39 VWC on average at peak flower, while experimentally watered rock outcrop plots were just 12 VWC on average at peak flower. We were also unable to account for genetic variation between individuals in paired plots and environmental differences between microhabitats such as soil depth, soil composition and competition. Comparing the traits of cloned individuals planted in different microhabitats in the field would be another effective way to evaluate the relationship between trait plasticity and soil moisture.

In the common garden, there was no significant difference in stolon number between individuals sourced from the rock outcrop and seep plots, even though a partial flowering time divergence was maintained. This result was unexpected because seep monkeyflower stolon production is known to be highly correlated with flowering time—researchers have found more than one QTL that affects both traits (Lowry and Willis 2010; Friedman et al. 2015). We only subjected our common garden plants to permanently wet conditions, simulating a seep-like environment. Because there may be genetic variation for plasticity in the rock outcrop and seep individuals, simulating rock outcrop conditions with a dry down treatment in our common garden may have resulted in more stark differences in vegetative traits

between the individuals from the rock outcrop and seep plots (Murren et al. 2006; van Kleunen 2007). We also only counted stolons at anthesis and could have missed a divergence between more developed forms. Measuring the lengths of the stolons and tracking stolon growth and production over time would provide more information on the differences in vegetative allocation between the rock outcrop and seep sourced individuals.

One intriguing finding from our common garden and watering addition experiment was that there were differences in the extent of plasticity between vegetative and floral traits (Table 2, Appendix S2). A possible explanation for this result is that leaves and stems have longer periods of meristematic activity relative to flowers, so their development is more likely to be shaped by environmental conditions over time (Stebbins 1950; Bradshaw 1965). Clausen et al. (1940, 1948) found supporting evidence for this hypothesis with their common garden and reciprocal transplant studies on *Achillea millefolium* L. (Asteraceae) and *Potentilla glandulosa* Lindl. (Rosaceae), where plasticity varied with different traits along an elevation gradient. Another hypothesis is that floral and vegetative traits are genetically uncoupled due to differences in selective pressures acting independently on traits (Berg 1960; Armbruster et al. 1999). Plasticity in vegetative traits may be favorable under fluctuating and unpredictable soil moisture conditions, while plasticity in floral traits may be unfavorable under pollinator-mediated selection. Further

manipulative and observational studies over multiple generations are necessary to investigate how pollinator or herbivore-mediated selection under different drought treatments influence floral and vegetative trait plasticity.

The divergence in peak flowering time between microhabitats in the field appears to be mostly driven by the early flowering time of rock outcrop plots at the LL site (Figs. 4 and 5). The maintained differences in flower size and flowering time in the common garden also appear to be notable in individuals grown from seeds sourced from LL (Figs. 4B and 5B). This is preliminary evidence that there could be variation in gene flow between the sites in our study, potentially leading to higher levels of genetic differentiation between microhabitats at the LL site relative to the SH site. A divergence in flowering time between rock outcrop and seep populations, likely the result of both plasticity and selection in different soil moisture habitats, could be enough to promote temporal and pollinator-mediated reproductive isolating barriers. Previous research on an annual herb, *Leptosiphon parviflorus* Benth., found that both selection and plasticity under different soil moisture regimes drive flowering time differences and likely contribute to adaptive differentiation across distances less than 100 meters (Dittmar and Schemske 2018). Future reciprocal transplants and population genomic analyses are necessary to test whether divergent selection and temporal isolation contribute to local adaptation along the fine-scale soil moisture gradient in this system, particularly at the LL site, where we found preliminary evidence for heritable flowering time differences. Additional research is necessary to uncover whether homogenizing gene flow between microhabitats, particularly at the SH site, where there is more of an overlap in flowering time, is responsible for the lack of trait differentiation under common garden conditions. Such investigations are important for better understanding how the environment facilitates the formation and maintenance of species boundaries.

CONCLUSION

Despite occurring within meters of each other with ample opportunity for intraspecific gene flow, the seep monkeyflowers in this study occupy extremely variable soil moisture conditions— from rock cracks to completely inundated soils. At these extremes, the monkeyflowers exhibit strikingly different life history strategies, or morphological, phenological and physiological traits. Our findings suggest that these trait differences are primarily related with trait plasticity and potential selection under different soil moisture regimes. Overall, these results provide important functional insights into how plants tolerate contrasting soil moisture conditions and help uncover the ecological and evolutionary processes responsible for phenotypic variation.

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