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Research

Replicate altitudinal clines reveal that evolutionary flexibility underlies adaptation to drought stress in annual *Mimulus guttatus*

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Summary

- Examining how morphology, life history and physiology vary along environmental clines can reveal functional insight into adaptations to climate and thus inform predictions about evolutionary responses to global change. Widespread species occurring over latitudinal and altitudinal gradients in seasonal water availability are excellent systems for investigating multivariate adaptation to drought stress.
- Under common garden conditions, we characterized variation in 27 traits for 52 annual populations of *Mimulus guttatus* sampled from 10 altitudinal transects. We also assessed variation in the critical photoperiod for flowering and surveyed neutral genetic markers to control for demography when analyzing clinal patterns.
- Many drought escape (e.g. flowering time) and drought avoidance (e.g. specific leaf area, succulence) traits exhibited geographic or climatic clines, which often remained significant after accounting for population structure. Critical photoperiod and flowering time in glasshouse conditions followed distinct clinal patterns, indicating different aspects of seasonal phenology confer adaptation to unique agents of selection. Although escape and avoidance traits were negatively correlated range-wide, populations from sites with short growing seasons produced both early flowering and dehydration avoidance phenotypes.
- Our results highlight how abundant genetic variation in the component traits that build multivariate adaptations to drought stress provides flexibility for intraspecific adaptation to diverse climates.

Introduction

Local adaptation is commonly observed among populations of geographically widespread plant species that inhabit diverse environments (Leimu & Fischer, 2008; Hereford, 2009). Understanding the functional and genetic mechanisms contributing to this process is essential, as this knowledge can assist in identifying factors that limit species ranges as well as aid in predicting and managing responses to environmental change (Anderson *et al.*, 2014). Clinal patterns of trait variation along geographic and environmental gradients have frequently been noted as initial evidence for differential selection among populations (Endler, 1986). In many such cases, the mean values of numerous traits exhibit correlated changes along geographic clines. For instance, high-elevation plant populations are adapted to be shorter (e.g. Moles *et al.*, 2009), more cold resistant (e.g. Zhen & Ungerer, 2008), and later flowering (e.g. Méndez-Vigo *et al.*, 2011; Montesinos-Navarro *et al.*, 2011) than low-elevation plants.

Interpreting how such multivariate responses have occurred historically is challenging, however, because determining which of the multifarious environmental factors, or agents of selection, have acted upon individual traits or suites of traits, the targets of selection, is rarely straightforward (MacColl, 2011; O'Brien *et al.*, 2011; Kooyers & Olsen, 2013). Collinear relationships among environmental factors can obscure whether multiple traits are responding to the same or unique agents of selection. Likewise, although correlated clines in multiple traits suggest an adaptive syndrome, these patterns may have arisen by independent directional selection on each trait, correlational selection, or a combination of direct and indirect selection due to genetic correlations among traits (Agrawal *et al.*, 2010). Finally, nonadaptive evolutionary processes can also yield clinal patterns. One means to disentangle this complexity is through surveying phenotypic and genetic variation from populations across a broad set of geographic transects or environmental gradients that impose similar but unique regimes of directional selection in different areas. This strategy reduces covariance between environmental variables, better enables independent evolutionary trajectories to be identified, and allows patterns of convergence to be tested more effectively (e.g. Oakeshott *et al.*, 1982; Huey *et al.*, 2000; Steiner *et al.*,

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2009). Here, we employ this approach to examine how a suite of life history and physiological traits has evolved in relation to climatic factors across the range of a widespread annual plant.

Local adaptation of plants to seasonal climates characterized by recurring drought stress frequently involves multivariate evolutionary responses (e.g. Aspinwall *et al.*, 2013; Lee & Mitchell-Olds, 2013). Suites of traits that constitute adaptations to drought stress are generally sufficiently distinct that they are categorized into three strategies: drought escape, dehydration avoidance, and dehydration tolerance (Ludlow, 1989). A drought escape strategy involves rapid growth and reproduction, allowing plants to complete their life cycle before drought onset. By contrast, avoidant plants resist dehydration in dry conditions through adaptations that improve water use efficiency (WUE). Many traits may contribute to dehydration avoidance, and this strategy has been associated with lower specific leaf area (SLA; Fonseca *et al.*, 2000), higher succulence (Eggli & Nyffeler, 2009), more trichomes (Ehleringer *et al.*, 1976), accentuated leaf lobing (Talbert & Holch, 1957), and altered stomatal size and density (Masle *et al.*, 2005). Finally, drought-tolerant plants can actually survive internal water deficits; however, we do not consider this strategy further as it is not as relevant in our annual plant. Although both drought escape and dehydration avoidance are predicted to evolve in areas of persistent drought stress, they are frequently viewed as alternative strategies or syndromes (Ackerly *et al.*, 2000). Indeed, empirical studies in multiple systems have found negative genetic correlations between traits involved in escape (e.g. flowering time, developmental rate) and avoidance (e.g. WUE) that constrain their joint evolution (Geber & Dawson, 1990, 1997; McKay *et al.*, 2003; Lovell *et al.*, 2013).

The common monkeyflower, *Mimulus guttatus*, is an excellent system for investigating mechanisms of local adaptation to climate due to its extensive distribution across diverse habitats throughout western North America (Grant, 1924; Vickery, 1978; Wu *et al.*, 2008). Two major *M. guttatus* ecotypes have been defined – annuals and perennials – based on habitat and phenotypic differences of such magnitude that some have recommended classifying them as distinct taxa (Vickery, 1978; Lowry *et al.*, 2008; Nesom, 2013). Perennial populations inhabit coastal or inland environments characterized by continuous water availability. By contrast, annual populations face seasonal droughts; the seepy hillside meadows, temporary streams, and cliff faces they inhabit have high soil moisture at the start of the spring growing season but dry out entirely as summer progresses.

Although the ecology and genetics of divergence between ecotypes has received considerable study (Hall & Willis, 2006; Lowry *et al.*, 2008; Hall *et al.*, 2010; Wu *et al.*, 2010; Friedman & Willis, 2013; Oneal *et al.*, 2014), how local adaptation allows populations of the annual ecotype to persist over a broad geographic range has received minimal attention. Across latitudes from southern California to Alaska and altitudes from sea level to *c.* 1700 m, annual populations encounter dramatic differences in the type and amount of precipitation received, mean and seasonal temperatures, and, consequently, the yearly timing of the growing season. For instance, the duration of the growing season varies along altitudinal gradients from as little as 6 wk up to as many as 5 months (Hall & Willis, 2006; Hall *et al.*, 2010). This environmental variation likely imposes strong selection for differentiation in life history and ecophysiological traits. Moreover, because the end of the growing season for annual *M. guttatus* depends on water availability, strategies to cope with drought stress are likely to be common in areas with low precipitation and/or short growing seasons (Galloway, 1995; Wu *et al.*, 2010).

Drought escape through shifts in the seasonal timing of flowering is likely an essential contributor to local adaptation among annual populations. Early flowering is a fundamental adaptation that distinguishes annual from perennial *M. guttatus* (Hall & Willis, 2006; van Kleunen, 2007), and developmental timing is also subject to temporally heterogeneous selection within at least one annual population (Mojica *et al.*, 2012). Changes in flowering time may occur by alteration in the length or speed of progress through vegetative phases, or by changes in how seasonal environmental signals (e.g. vernalization, photoperiod, temperature) gate or pace phenology to help ensure that reproduction occurs during the optimal time of year (Wilczek *et al.*, 2010). *Mimulus guttatus* is an obligate long-day plant that must experience a minimum day length for floral induction. Recent work on a modest number of lines demonstrated that both critical photoperiod requirement and flowering time in inductive conditions vary widely among annual *M. guttatus* populations (Friedman & Willis, 2013). However, the environmental correlates of this variation remain unexplored, and whether these two components of an integrated seasonal phenology evolve along unique or common trajectories is an open question. Likewise, the extent, distribution, and multivariate evolution of dehydration avoidance traits among annual populations lack systematic study. Previous studies of dehydration avoidance traits have solely focused on variation between ecotypes and taxa in the *M. guttatus* species complex (Wu *et al.*, 2010).

Here, we present a comprehensive investigation of patterns of phenotypic variation in a large battery of morphological, phenological and ecophysiological traits among annual *M. guttatus* populations sampled from 10 altitudinal gradients spanning a substantial portion of the species range. Through combining phenotypic information from a common garden experiment and a critical photoperiod survey with environmental and population genetic data in a regression-based modeling framework, we test for evidence consistent with local adaptation and examine patterns of covariation among traits. We specifically address three primary questions. Has differential selection among populations led to clinal divergence in drought escape and dehydration avoidance traits? Do critical photoperiod and flowering time under inductive conditions evolve as independent or correlated characters? Do drought escape and dehydration avoidance represent mutually exclusive strategies for mediating drought stress or can populations evolve both strategies? Our findings highlight how abundant genetic variation has allowed annual *M. guttatus* populations to flexibly evolve and thrive in diverse climates.

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Materials and Methods

Sampling

In May–June 2011, we collected *Mimulus guttatus* D.C. from 52 populations along 10 altitudinal transects, 3–10 populations per transect, in the western USA (Fig. 1, Supporting Information Table S1). Population locations were identified primarily from records of other recent collectors, from online herbarium records, or by searching prime habitat. The southernmost transect was located just north of the California/Mexico border and the northernmost transect was located in central Oregon. Our sampling greatly expands the numbers of high-elevation and Southern Californian annual populations evaluated relative to other recent work (Friedman & Willis, 2013; Oneal *et al.*, 2014; Friedman *et al.*, in press), allowing rigorous evaluation of geographic and environmental trends. Mature seed was collected if available. Otherwise, whole plants were collected, shipped to Duke University, and selfed.

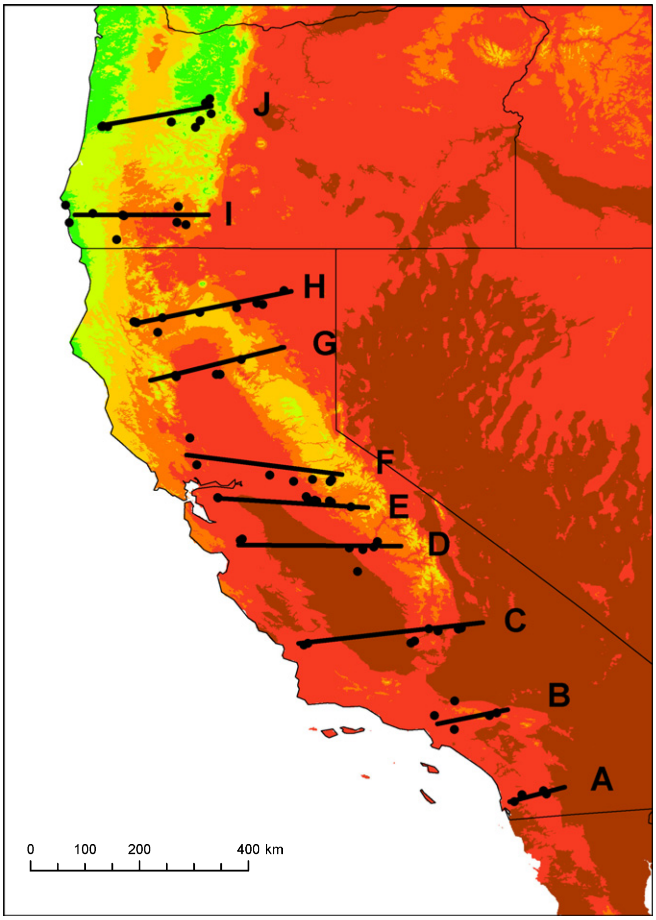

Fig. 1 *Mimulus guttatus* sampling locations across altitudinal gradients in Sierra Nevada and Cascades Mts, USA. Green to red gradient represents the annual aridity index (data extracted from CGIAR-CSI; Trabucco & Zomer, 2009) from mesic to xeric, respectively. Dots represent the location of each population. Each line and letter corresponds to an altitudinal transect.

Common garden experiment

Patterns of heritable phenotypic variation were characterized in a common garden experiment. For 2–10 maternal lines per population, we sowed seeds in three 2.5-inch square pots filled with saturated Fafard 4P soil (1728 pots total, 1012 adult plants survived, attrition largely due to inbreeding depression). After stratification (4°C, 10 d), pots were randomly sorted into 54 flats of 32 pots each and raised in the Duke University glasshouses (18 h 21°C : 6 h 18°C, day : night). Flat position was randomized every 7–10 d.

Flowering time was measured as the period from seedling emergence to the opening of the first flower. At flowering, we measured many aspects of vegetative and floral morphology. Floral traits – corolla length, corolla width, tube width, tube length, anther length and style length – were measured as in Fishman *et al.* (2002). We conducted a principal components analysis (PCA) on *z*-scores of flowering traits using the prcomp() call in R 3.0.1 (R Foundation for Statistical Computation, Vienna, Austria) and extracted PC1 (termed floral PC1 below; Table S2). Loading values indicate that floral PC1 corresponds to size (79.4% of variation). For shoot architecture, we measured plant height, stem diameter at the base of the first elongated internode, number of primary branches and length of longest branch. For leaf morphology, we measured leaf length, leaf width and petiole length for one second true leaf per plant. This leaf was also removed, weighed and photographed on graph paper. Leaf area and convex hull area were calculated with ImageJ 1.46 (National Institutes of Health, Bethesda, MD, USA). Then, the leaf's dry weight (DW) was recorded after drying at 50°C until no additional weight loss was measurable. We combined all vegetative traits into a PCA using prcomp() in R and extracted PC1 and PC2 (termed vegetative PC1 and PC2 below; Table S2). Loading values indicate that vegetative PC1 corresponded to overall size (42.5% of variation) and vegetative PC2 to branching (22.1% of variation). Ecophysiological metrics were also derived from raw measures. SLA was calculated as leaf area/DW. Succulence was calculated as (wet weight – DW)/leaf area (Reimann & Breckle, 1995). The lobing index was calculated as (convex hull area – leaf area)/convex hull area; higher values indicate greater lobing. We counted the number of glandular and nonglandular trichomes that extended beyond leaf margins under a dissecting microscope for a subset of individuals. Leaves surveyed for trichome counts were also submitted to the Keck Paleoenvironmental & Environmental Stable Isotope Laboratory (University of Kansas, KS, USA) for carbon isotope analysis. Data are presented as δ^{13}C relative to the Vienna-Pee Dee belemnite (VDPB) standard. Bud tissue from each individual was collected for genetic analysis.

Critical photoperiod survey

A survey for variation in the critical photoperiod required for flowering was conducted in growth chambers in the Duke University Phytotron. Plants were grown from selfed seed derived

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from lines used in the common garden. Photoperiod treatments were 8, 10, 11, 12, 13, 14 and 16 h light d^{-1}. Chambers were maintained at 20°C, 50% relative humidity and *c.* 625 umol m^{-2} s^{-1} photosynthetically active radiation. For each chamber, 2–4 maternal lines from 29 populations were each sown and stratified as above (224 pots per chamber). Plants were randomly sorted into seven flats of 32 pots per treatment. Flat position was randomized every 7–10 d for the length of the experiment (*c.* 140 d). To obtain an estimate of critical photoperiod, a logistical regression using the glm() command in R with a binomial error distribution was performed for each line using number of plants flowering vs not flowering as a response variable and photoperiod treatment as the independent variable. This model was then solved to estimate the photoperiod at which 50% of plants in a line would flower (ppd50). Lines that flowered in all treatments were assigned a ppd50 of 8 h ($n = 7$). For the 16 h treatment, abaxial casts of one second true leaf per plant were taken at flowering in polyvinylsiloxane dental impression material (Extrude medium; Kerr Manufacturing, Orange, CA, USA). Peels were then made with clear fingernail polish (L'Oreal Top Coat; L'Oreal, Paris, France). Stomatal densities were evaluated at ×400 magnification with a light microscope by averaging the stomata counts of three randomly selected fields of view per peel.

Data analysis

In order to quantify heritable patterns of range-wide trait differentiation, we used a restricted maximum-likelihood mixed modeling approach implemented with the lme4 R package (Bates *et al.*, 2011). Separate general linear mixed models were generated for each trait (flowering time, plant height at flowering, flower PC1, vegetative PC1, vegetative PC2, leaf lobing, SLA, stomatal density, succulence, total number of trichomes, and number of glandular trichomes). Transect, population (nested within transect) and maternal line (nested within population and transect) were included as random variables, and the residual variance was assigned as variance within maternal lines. To identify whether variation was associated with transect or population, we sequentially dropped each term from the model and conducted likelihood ratio tests on nested models. Because ppd50 is an aggregate measure obtained for each line, transect and population (nested within transect) but not maternal line were included as random effects in the mixed model for ppd50.

In order to examine relationships between phenotypic variation and geography, we first conducted mixed models in which either latitude or altitude was included as a fixed effect and population, maternal line (nested within population) and flat were included as random effects. We log-transformed count variables for which initial model residuals were non-normally distributed. Statistical significance of each fixed effect was determined by ANOVA using a Wald Chi-Square test with 1 df. We also evaluated full models including latitude, altitude and their interaction. We conducted additional transect-specific analyses to further explore latitude × altitude interaction effects identified for some traits.

Agents of selection

In order to identify potential agents of selection, we investigated associations between climate variables and phenotypic variation. Nineteen bioclimatic variables were obtained at 30 s resolution from the WORLDCLIM dataset (Hijmans *et al.*, 2005). Two additional variables, July potential evapotranspiration and aridity index, were obtained from the Consultative Group on International Agricultural Research – Consortium for Spatial Information (CGIAR-CSI; Trabucco & Zomer, 2009). Climate variable values were extracted for each population in ArcMap 9.3.1 (ESRI, Redlands, CA, USA). Because climate variables frequently covary, we conducted principal component analysis using *z*-scores of all 21 variables using prcomp() in R and extracted PC1 and PC2 values for each population (termed climate PC1 and PC2 below). Because preliminary data suggested that growing season length was important, we approximated growing season length using minimum winter temperature and relative precipitation in the driest month. This estimate accurately reflects within-cline differences in growing season but did not necessarily translate between clines (N. J. Kooyers, pers. obs.). To examine relationships between phenotypic and climatic variation, mixed models were conducted with a climate factor as a fixed effect and population, maternal line (nested within population) and flat as random effects. We also included seed source (selfed or field-collected) as a random variable within models. However, as it had no significant impact on model outcomes, we did not include this variable in the models for which we report results below. To determine whether relationships with environmental variables differed geographically, we conducted additional transect-specific analyses.

Population structure analysis

Clinal variation in trait means could result from selection or demographic processes. To distinguish between these possibilities, we characterized patterns of population structure for a subset of 28 representative populations. We genotyped 2–10 individuals per population (average 6.2; 173 total) for seven genetic markers (Table S3), including three microsatellites (AAT296, AAT217, and AAT278; Kelly & Willis, 1998) and four exon-primed intron containing markers (MgSTS571, MgSTS617, MgSTS474 and MgSTS278) frequently used in other population surveys on the *M. guttatus* species complex (e.g. Lowry *et al.*, 2008). Each individual came from a different maternal line. Genotypes were assayed by capillary electrophoresis (ABI 3130) and scored with GeneMapper v4.0 (Applied Biosystems, Foster City, CA, USA), followed by manual checks to ensure consistency. All loci had < 6% missing data.

Summary statistics – observed heterozygosity, expected heterozygosity, private alleles and deviations from Hardy–Weinberg equilibrium – were calculated using GenAlEx 6 (Peakall & Smouse, 2006). Pairwise Fst (Weir & Cockerham, 1984) was calculated between all population pairs in GenAlEx 6, and Jost's measure of absolute differentiation between populations (D_{est}) was calculated in SMODG (Crawford, 2010). We tested for a

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pattern of isolation-by-distance using a Mantel test conducted with the ade4 R package (Dray & Dufour, 2007). To characterize the partitioning of genetic variation within populations, among populations and among transects, we conducted analyses of molecular variance (AMOVAs) in Arlequin 3.5.1.2 (Excoffier & Lischer, 2010), specifying altitudinal transect as a grouping factor and using the conventional *F*-statistics option. Statistical significance was tested with 10 000 permutations. We visualized the optimal population structure without *a priori* geographical assumptions with the Bayesian clustering program STRUCTURE (Pritchard *et al.*, 2000). We conducted three runs for *K* values from 1 to 12 and examined average $\log_e$ likelihood values for each *K*. We identified the most likely *K* value using the criteria of Evanno *et al.* (2005). All runs had a burn-in period of 50 000 with a run-length of 250 000 and were run under a model allowing admixture and correlated allele frequencies. Additional models run with alternative parameters produced similar results.

In order to test whether patterns of phenotypic variation could be artifacts of population structure, we utilized an ANCOVA approach where we added neutral genetic variance as a fixed effect to the mixed models detailed above. We first conducted a principal coordinate analysis on our microsatellite data above using the adegenet R package. The PC1 and PC2 values for each individual were each used as fixed effects in each model (termed genetic PC1 and PC2 below; $n = 319$). All individuals in a line were assigned the same genetic PC values. If the effect of a climate variable remained significant after including both genetic PC effects, the correlation between the climate variable and trait is consistent with adaptive trait differentiation. However, if the correlation becomes nonsignificant, a role for neutral and demographic processes in producing the environment–trait correlation cannot be excluded.

Correlations between traits

In order to examine patterns of covariation between traits, we calculated Pearson correlation coefficients for pairwise combinations of traits at three levels: all individuals, maternal line averages and population averages. We evaluated significance assuming a *t*-distribution with $n-2$ df. Correlation tests with Spearman's *rho* statistic yielded similar results (data not shown). To test whether trait correlations are consistent range-wide, we subdivided the dataset by three geographic regions largely corresponding to genetic clusters identified with STRUCTURE and ran correlation tests on maternal line averages. In addition, to identify whether variation in trait correlations was associated with climatic variables, we calculated population-specific Pearson correlation coefficients and ran regressions with population correlation as a response variable and either latitude, altitude, aridity index, or climate PC1 as an independent variable. To test for alternative syndromes associated with dehydration avoidance and drought escape, we conducted an exploratory factor analysis using the pysch R package (Revelle, 2014). We incorporated dehydration avoidance (SLA, succulence, lobing) and drought escape traits (flowering time, vegetative PC1, height at flowering) into the analysis and assumed either two or three underlying factors.

Results

Geographic clines are common but latitudinal and altitudinal patterns are often incongruent

We observed substantial genetic variation among annual *Mimulus guttatus* populations in nearly every morphological, ecophysiological and phenological trait examined in our common garden experiment (Table 1). Mixed modeling analysis revealed significant relationships between latitude and several traits including height at flowering ($\chi^2 = 35.4$, $P < 0.001$), vegetative PC1 ($\chi^2 = 21.0$, $P < 0.001$, Fig. 2a), flowering time ($\chi^2 = 8.34$, $P = 0.004$, Fig. 2b), floral PC1($\chi^2 = 10.8$, $P = 0.001$), trichome number ($\chi^2 = 5.7$, $P = 0.017$, Fig. 2c) and lobing ($\chi^2 = 6.2$, $P = 0.013$). Plants from higher latitudes tended to be shorter, branch less, flower earlier, have larger flowers, make fewer trichomes and produce more highly lobed leaves than plants at lower latitudes. Notably, clinal patterns along altitudinal transects were not consistent with these latitudinal patterns (Fig. 2, Table S4). Only the relationship between altitude and height at flowering was marginally significant, but in contrast to the latitudinal trend, plants from higher altitudes tended to be taller ($\chi^2 = 3.07$, $P = 0.08$). These contrasting patterns suggest that environmental factors that differ in distribution along latitudinal and altitudinal gradients, rather than environmental variables that vary similarly along both geographic gradients, are likely the key agents of selection for many traits.

Strikingly, the one trait displaying a clear range-wide altitudinal cline was the critical photoperiod required for flowering, as estimated by ppd50 in our growth chamber survey ($\chi^2 = 13.8$, $P < 0.001$, Figs 2d, 3). Higher elevation plants required longer days to flower than lower elevation plants. A nonsignificant ppd50-latitude trend exists in the same direction ($\chi^2 = 1.57$, $P > 0.1$). By contrast, a range-wide altitudinal cline for flowering time under glasshouse conditions was not significant ($\chi^2 = 0.20$, $P > 0.1$), but the range-wide pattern obscures regional differences in phenological divergence. High-elevation populations from transects A–F (Southern Sierra Nevada Mts and Southern California; termed Sierras below) flowered later than low-elevation populations, but high-elevation populations from transects G–J (Northern Sierra Nevadas and Cascade Mts; termed Cascades below) flowered earlier than low-elevation populations (Table S5, Fig. 4). A significant latitude × altitude interaction effect for flowering time confirms the changing relationship between this trait and elevation across the range (Table 2). The incongruence of the geographic patterns for these two aspects of reproductive timing – critical photoperiod and time to flower in inductive conditions – reinforces the idea that these parameters are distinct component traits of an integrated seasonal phenology, each capable of independently diverging in response to separate agents of selection. The consistent observation of latitude × altitude interaction effects on putative drought escape traits and the absence of these effects on putative dehydration avoidance traits indicate that these two strategies have evolved in response to distinct environmental pressures (Table 2).

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Table 1 Summary statistics for functional traits for *Mimulus guttatus* measured in the common garden experiment and photoperiod survey

Trait	*n*	Mean	SD	Min	Max	Variance among transects (%)	Variance within transects among pops (%)	Variance within pops among lines (%)	Variance within lines (%)
Flowering time (d)	603	31.88	6.32	16.00	54.00	**17.15**	**46.40**	9.95	26.50
Flowering node	603	2.91	1.09	1	7	**11.73**	**18.93**	13.39	55.96
Plant height at flowering (mm)	603	462.37	203.08	53.00	1873.00	**2.35**	**21.72**	22.84	53.09
Vegetative PC1	603	0.09	1.93	−4.60	6.58	**3.25**	**13.03**	10.26	73.45
Vegetative PC2	603	0.01	1.42	−4.25	5.55	3.09	**9.64**	17.45	69.82
Floral PC1	603	0.01	2.05	−9.00	7.33	**19.61**	**24.15**	20.50	35.75
Stem diameter (mm)	603	2.02	0.65	0.55	4.21	**16.77**	**8.14**	10.83	64.26
Number of branches	603	6.38	2.40	0	19	**6.55**	**5.79**	12.44	75.22
Glandular trichomes	126	27.10	34.40	0.00	170.00	32.01	**4.67**	< 0.1	63.33
Total trichomes	126	63.60	66.30	0.00	350.00	4.49	**55.78**	7.42	32.32
Leaf area (mm^2)	654	352.40	216.84	15.42	1398.05	**10.46**	**10.42**	7.53	71.58
SLA (mm^2 g^{-1})	654	46.31	16.61	2.74	227.09	2.38	**39.27**	< 0.01	58.35
Lobing index	654	0.08	0.03	0.02	0.37	5.81	**17.87**	20.65	55.67
Stomatal density	178	6.88	3.19	2.00	18.67	< 0.1	**26.56**	19.96	53.48
Succulence (g mm^{-2})	586	0.19	0.09	−0.07	1.63	**4.14**	0.00	31.91	63.95
Carbon Isotope Ratio (δ^{13}C)	64	−32.60	1.03	−34.51	−30.08	< 0.1	< 0.1	50.5	49.5
ppd50	107	11.87	1.87	8.00	15.43	< 0.01	**58.71**	–	41.29

Bold values indicate statistical significance of likelihood ratio tests at $P < 0.05$. *n*, number of *M. guttatus* individuals used for each trait except for critical photoperiod (ppd50), where *n* = number of lines. SLA, specific leaf area.

Environmental correlations highlight potential climatic agents of selection

In order to identify selective pressures that may drive these geographic patterns, we extracted 21 climate variables for each population location and tested for associations with trait variation. We reduced the dimensionality of this highly correlated dataset by conducting a PCA. Climate PC1 explained 63.6% of the total variation, and loading scores indicate that it is directly correlated with annual temperature and inversely correlated with all precipitation indices (Table S2). Climate PC2 explained 24% of the total variation, and loading scores indicate that it is directly correlated with annual temperature range and seasonality (Table S2). Climate PC1 and latitude are tightly correlated ($r^2 = 0.59$, $P < 0.001$; Supporting Information Fig. S1), as are climate PC2 and altitude ($r^2 = 0.46$, $P < 0.001$).

Generally, many traits associated with latitude also had significant relationships with climate PC1, including vegetative PC1 (Figs 2a, S2a), flowering time (Figs 2b, S2b), trichome density (Figs 2c, S2c) and leaf lobing. However, some traits had significant relationships with climate PC1 that were not associated with latitude, including ppd50 ($\chi^2 = 10.0$, $P = 0.002$; Figs 2d, S2d), stomatal density ($\chi^2 = 3.7$, $P = 0.054$; Figs 2e, S2e) and succulence ($\chi^2 = 4.00$, $P = 0.05$, Figs 2f, S2f). Plants with higher climate PC1 values required longer days to promote floral induction, had higher stomatal densities and were less succulent. These additional relationships may emerge because using latitude alone as a predictive factor masks altitudinal differences in precipitation and temperature. No significant trait associations were detected for climate PC2 (Table S4).

Contrary to expectation, dehydration avoidance and drought escape strategies were not always associated with the areas of lowest precipitation. Plants native to areas of higher precipitation actually flowered earlier, had lower SLAs, exhibited more pronounced leaf lobing and had higher stomatal densities (Tables 3, S4). Some predicted patterns were observed, however. Plants from areas of lower precipitation areas were more succulent and had higher trichome densities (Table S4). Although these varied patterns may partly reflect evolutionary responses to other selection pressures, they likely also manifest due to the complex, changing relationship between water availability (whether as rainfall or snowmelt) and growing season length across the range. For instance, flowering time displays a consistent positive relationship with growing season length within regions, resolving the incongruent altitudinal clines in flowering time reported above, as the relationship between growing season length and altitude is positive in the Sierras and negative in the Cascades (Table S5).

Clinal patterns are consistent with selection despite pervasive population structure

We genotyped a subset of plants for seven markers to detect signals of population structure and control for demographic sources of clinal variation. Observed heterozygosity fell below expected heterozygosity for all markers and most populations (Tables S1, S3), as anticipated because most lines experienced one generation of selfing. Both F_{st} and D_{est} showed significant differentiation between almost all populations, providing evidence for population structure (Table S6). AMOVAs indicated that genetic variation is structured among transects (10.5%, $P < 0.001$) and among populations within transects (18.7%, $P < 0.001$). We

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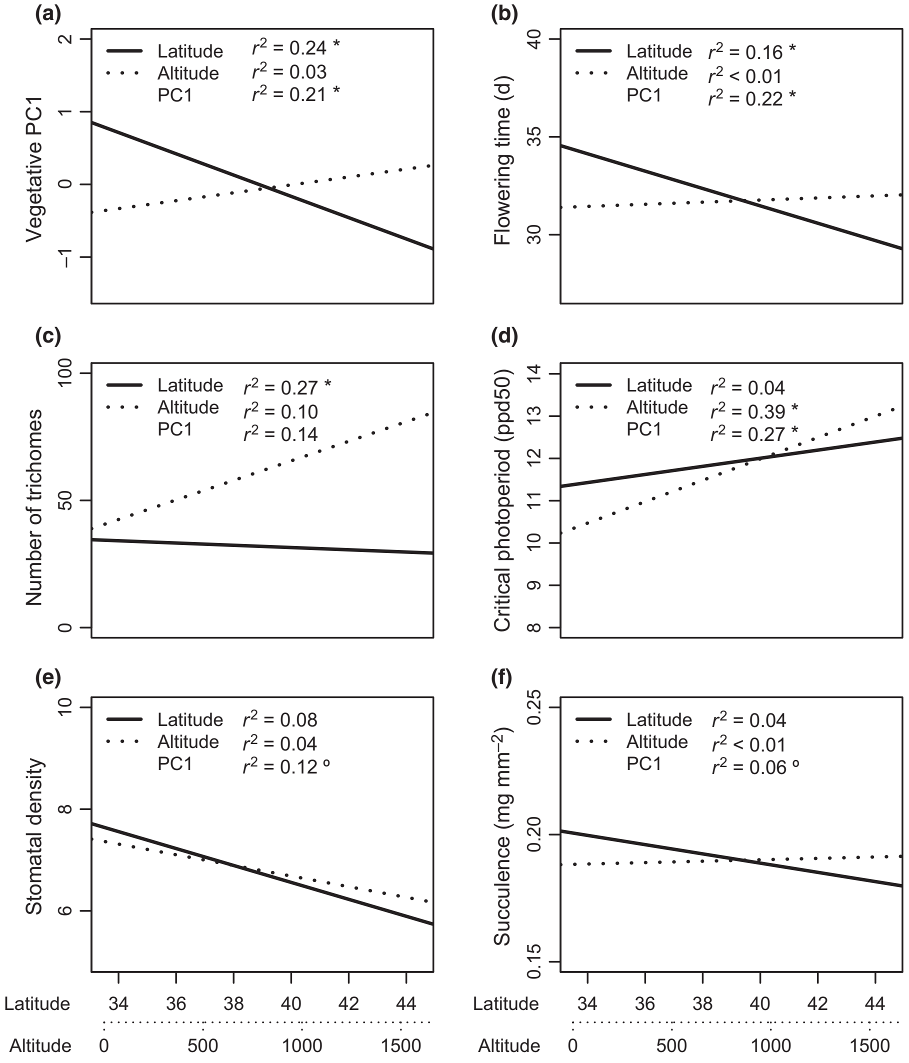

Fig. 2 Regressions of population means of (a) vegetative PC1, (b) flowering time, (c) trichome density, (d) critical photoperiod (ppd50), (e) stomatal density and (f) succulence against the latitude or altitude of each *Mimulus guttatus* population where plants were collected. In the legend, PC1 corresponds to regressions of each trait against climate PC1. Asterisks indicate statistical significance of correlations at $P < 0.05$, open circles indicates marginal significance at $P < 0.10$. Note that these correlations are different from the mixed models in text. Correlations in this figure are regressions of population means for traits against latitude or altitude, mixed models in the text examine associations between traits and altitude in latitude in a more powerful mixed model framework. For full mixed model results, see Supporting Information Table S4.

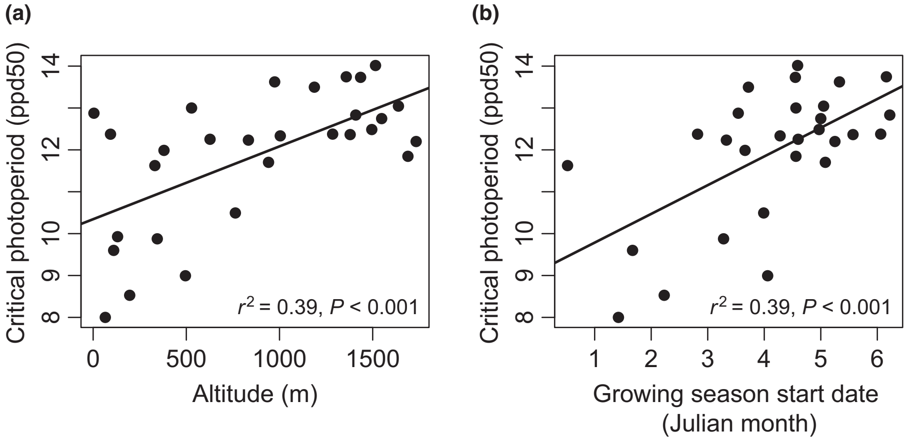

Fig. 3 Regression of critical photoperiod (ppd50) values against (a) altitude and (b) starting date of growing season for each *Mimulus guttatus* population. Starting date of the growing season was calculated as the date when the average minimum temperature was 4°C.

detected a pattern of isolation-by-distance (Mantel's $r = 0.39$, $P < 0.001$) but not isolation-by-altitude (Mantel's $r = -0.04$, $P = 0.71$). Analysis of number of ideal Hardy–Weinberg populations (K) with STRUCTURE found the greatest increase in ΔK at $K = 3$ with secondary increases at $K = 6$ (Figs 5, S3). At $K = 3$, each cluster corresponded to a geographic region: Southern California (transects A–C), Southern Sierra (transects D–F), and Northern Sierra/Cascade (transects G–J). At $K = 6$, each regional

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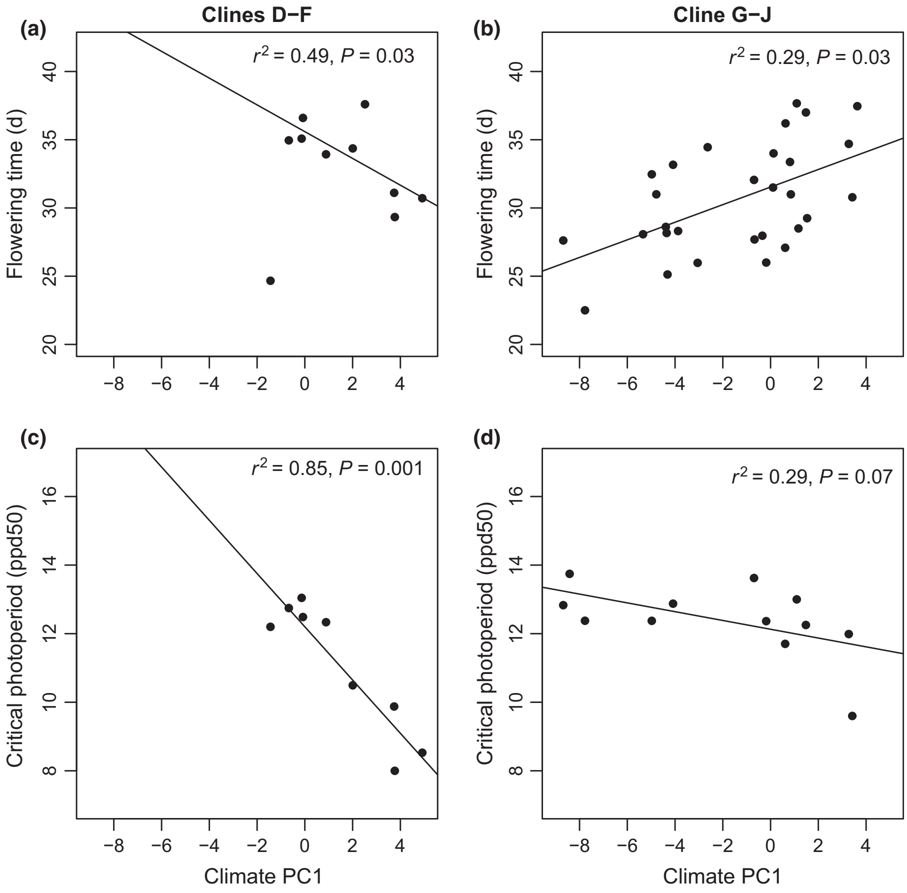

Fig. 4 Regressions of mean flowering time and ppd50 in (a, c) clines D–F and (b, d) clines G–J on climate PC1 for each *Mimulus guttatus* population. Increasing values of climate PC1 indicate increasing temperature and decreasing precipitation. Summary statistics for (a) do not include the outlier population BLD; the regression is not statistically significant when this population is included in the model.

Table 2 General linear mixed modeling results for the fixed effects of latitude, altitude and latitude × altitude on both drought escape and avoidance traits in *Mimulus guttatus*

Trait	Escape or avoidance?	Latitude χ^2 (*P*)	Altitude χ^2 (*P*)	Latitude × altitude χ^2 (*P*)
Flowering time	Escape	0.02 (0.88)	**3.70 (0.05)**	**3.84 (0.05)**
Flowering node	Escape	0.03 (0.85)	**6.36 (0.01)**	**5.95 (0.02)**
Height at flowering	Escape	2.42 (0.12)	**5.69 (0.02)**	**5.43 (0.02)**
Vegetative PC1	Escape	0.09 (0.76)	**9.35 (0.002)**	**8.99 (0.003)**
Succulence	Avoidance	0.05 (0.82)	1.95 (0.16)	1.95 (0.16)
SLA	Avoidance	0.91 (0.34)	1.42 (0.23)	1.33 (0.25)
Lobing index	Avoidance	1.61 (0.20)	0.12 (0.73)	0.24 (0.63)
Total trichomes	Avoidance	0.59 (0.44)	0.71 (0.40)	0.64 (0.42)
Stomatal density	Avoidance	0.01 (0.91)	1.18 (0.28)	1.26 (0.26)
δ^{13}C	Avoidance	0.25 (0.62)	$<$0.01 (0.99)	$<$0.01 (0.98)

Bold values indicate statistical significance of fixed effects at $P < 0.05$. SLA, specific leaf area.

cluster was further subdivided into two sections. These findings are consistent with results from a broader marker-based survey of the *M. guttatus* species complex (Oneal *et al.*, 2014) and recent genotyping-by-sequencing efforts (A. D. Twyford & J. Friedman, unpublished data).

In order to determine whether trait–environment associations persist after accounting for demography, we reduced the dimensionality of our genetic data by PCA and then conducted an ANCOVA for each trait using genetic PC1 (9.9% total variance explained) and PC2 (8.6%) as additional fixed factors. The genetic PCs recapitulate observed patterns of isolation-by-distance (Fig. S4). The addition of these covariates did not alter most climate PC1-trait relationships (i.e. height at flowering, floral PC1; Table 4) indicating that these patterns were products of selection. However, for some traits (i.e. flowering time, vegetative PC1) the addition of these covariates caused climate PC1–trait correlations to become marginal or insignificant. Because genomic variation is associated with climate in *M. guttatus* ($r^2 = 0.14$,

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$P < 0.001$ for genetic PC1–climate PC1 correlation; Oneal *et al.*, 2014), likely due to differential gene flow among populations with similar phenological adaptations, this is not unexpected. Nonetheless, for SLA, we found that adding genetic covariates strengthened a marginal positive relationship with climate PC1 to a significant one. These results likely indicate that despite high levels of population structure, climatic selection has driven phenotypic divergence among populations.

Dehydration avoidance and drought escape are not mutually exclusive

We observed significant pairwise correlations between many traits (Fig. 6), and the directions of these relationships were generally consistent irrespective of whether analyses were performed on all individuals, line means or population means (Table S7). The correlations were also generally consistent across geographic regions, and we detected little evidence for clinal relationships between correlation metrics and climate or geographic variables (Tables S8,S9).

Table 3 Result summary from models relating functional traits to climate variables typically associated with drought stress in *Mimulus guttatus*

	Annual precipitation	Precipitation in warmest month	Annual aridity	PC1
Flowering time	– **> 0.001**	– **> 0.001**	– **> 0.001**	**+** **> 0.001**
Total trichomes	– **0.016**	– **0.023**	– **0.022**	+ 0.055
SLA	– 0.073	– 0.120	– 0.071	+ 0.131
Lobing index	**+** **0.006**	+ 0.084	**+** **0.017**	– **0.039**
Stomatal density	+ 0.059	+ 0.090	+ 0.082	– 0.054
Succulence	– 0.069	– **0.024**	– **0.047**	**+** **0.045**
$\delta^{13}C$	– 0.213	– 0.289	– 0.223	+ 0.205

Plus and minus signs refer to the effect direction in each model, whereas numbers are the *P*-values for each model. Bold values indicate statistically significant associations at $P < 0.05$. Summary statistics were taken from mixed models with environmental factors as fixed effects and flat, population and maternal line (nested within population) as random effects. Trichome density was log-transformed before analyses. SLA, specific leaf area.

Considering trait correlation patterns within each drought strategy, traits traditionally associated with drought escape were highly correlated. Plants that flowered earlier generally were smaller and less branched (i.e. lower vegetative PC1 and higher vegetative PC2 values). By contrast, relationships among putative dehydration avoidance traits were less congruent. SLA was negatively correlated with succulence and carbon isotope ratio, but trichome number and stomatal density were not associated with any other avoidance traits. Thus, a dehydration avoidance 'syndrome' appears to involve the coordinated evolution of relatively few traits.

Considering trait correlation patterns between drought strategies, evidence for the predicted negative relationship between drought escape and dehydration avoidance syndromes is mixed. Flowering time was positively associated with succulence ($r = 0.194$; $P = 0.001$), stomatal density ($r = 0.273$; $P = 0.016$) and trichome density ($r = 0.311$; $P = 0.013$), as expected, although these patterns were not always consistent across grouping levels. To explore these relationships further, we ran an exploratory factor analysis using three escape traits (flowering time, vegetative PC1 and height at flowering) and three avoidance traits (succulence, SLA and lobing). If drought escape or dehydration avoidance syndromes exist and are negatively associated, then traits associated with escape and avoidance should correlate strongly but in opposite directions with the first factor. We do observe this predicted pattern in all analyses, independent of the number of assumed factors. The first factor axis has a high positive factor loading with flowering time, vegetative PC1, plant height and succulence, and a high negative loading with SLA (Table 5).

However, the relatively low values of the observed range-wide correlations suggest that drought escape and dehydration avoidance need not evolve as mutually exclusive ecological strategies. Indeed, contrary to expectation, plants that flowered earlier also had higher $\delta^{13}C$ values ($r = -0.39$, $P = 0.01$), suggesting that earlier flowering plants have higher WUE and that traits typically associated with dehydration avoidance may not always improve WUE. To more closely examine this departure from the strong genetic trade-off between early flowering and dehydration avoidance found in other systems, we examined trait correlations within populations in the Central Valley of California and the high elevations of the Cascades. These populations experience intense drought stress due to their comparatively short and dry growing seasons, and individuals from these populations were among the earliest flowering, most succulent, and lowest SLA-

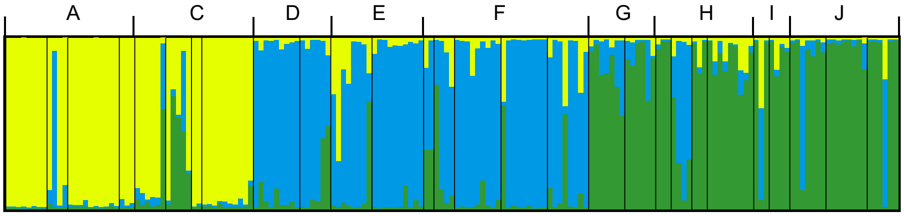

Fig. 5 Visual display of individual coefficients of membership sorted by transect based on multilocus genotypes using the program STRUCTURE. Analysis was constrained to the most likely number of populations ($K = 3$). Each vertical line represents a single *Mimulus guttatus* individual and color represents the portion of membership in each population cluster. Individuals were grouped by transect (A–J) and then by site within each transect.

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Table 4 Results summary from ANCOVA analyses

	Models without genetic covariates		Models with genetic covariates	
Trait	χ^2	*P*	χ^2	*P*
Flowering time	**4.03**	**0.045**	2.61	0.106
Height at flowering	**8.11**	**0.004**	**4.53**	**0.033**
Vegetative PC1	**4.16**	**0.042**	1.93	0.165
Vegetative PC2	0.07	0.785	1.32	0.250
Floral PC1	**6.82**	**0.009**	**10.78**	**0.001**
Stomatal density	0.74	0.390	2.03	0.154
SLA	3.49	0.062	**8.18**	**0.004**
Lobing index	0.20	0.658	0.33	0.563
Total trichomes	1.81	0.179	2.42	0.120

Bold indicates models that were significant at $P < 0.05$. Trichome density was log transformed. All models summarized in this table include only lines for which at least one *Mimulus guttatus* plant was genotyped. SLA, specific leaf area.

value individuals in the study. Strikingly, dehydration avoidance traits (succulence, SLA) and flowering time exhibited associations largely inconsistent with the expected trade-off. Flowering time was negatively correlated with succulence and positively correlated with SLA in the majority of Central Valley populations and high-elevation Cascade populations (Table S10).

Discussion

Abundant clinal variation in annual *M. guttatus*

Our common garden experiment and critical photoperiod survey revealed that ample genetic variation exists among annual populations of *Mimulus guttatus* for a broad suite of morphological, life history and ecophysiological traits. Furthermore, this phenotypic variation was often clinally distributed along geographic or environmental gradients, suggestive of adaptive divergence as a result of differential selection among sites. Seasonal water availability appears to be a chief selective pressure driving these patterns, as many drought escape and dehydration avoidance traits were associated with a gradient of heat and drought (climate PC1). For instance, consistent with previous findings comparing annual and perennial *M. guttatus* (Hall & Willis, 2006; Wu *et al.*, 2010), our results indicate that drought escape through faster development and initiation of flowering is an essential adaptation in annual *M. guttatus* that ensures reproduction is achieved before end-of-season droughts (Table 3).

A dehydration avoidance strategy also appears to contribute to drought stress adaptation among annual *M. guttatus* populations, although compared with drought escape, the patterns are less straightforward to interpret. Geographic trends differed and were weaker for putative drought avoidance traits, indicating that divergence has occurred in response to partly unique sets of environmental pressures (Table 2, Figs 2, S2). The correlations among dehydration avoidance traits were more diffuse as well, suggesting that phenotypic divergence has occurred in a largely uncoordinated manner (Fig. 6). Absence of a clear multivariate syndrome could indicate that changes in different traits can achieve substitutable gains in fitness under drought stress. Alternatively, how individual phenotypes are optimized with respect to other local environmental factors may vary among traits. For instance, stomatal density is often associated with differences in atmospheric composition (e.g. Woodward, 1987), as is trichome density with variation in herbivory (e.g. Holeski *et al.*, 2010).

Although succulence and trichome density increased with aridity as expected, range-wide trends for several other dehydration avoidance traits and flowering time were not in the predicted direction. These counterintuitive results may be explained by the complex relationship between precipitation and growing season across the range, particularly at high-elevation sites where most precipitation is initially received as snow and largely inaccessible until rapid late spring warming periods that are quickly followed by summer drought. Consequently, growing seasons at these sites are comparable in duration to the short springs characteristic of low-elevation populations in the Central Valley of California. Populations in both areas flower rapidly, have high stomatal densities and low SLAs, implying that selection has favored a 'live fast and die young' strategy in these locations (Fig. 4). This strategy likely incurs a trade-off between survival and fecundity, as plants that flower quickly also tend to produce smaller flowers (Fig. 6; Ivey & Carr, 2012; Mojica *et al.*, 2012).

We expect that the observed clines are largely shaped by natural selection, and our results partially corroborate this conclusion. Consistent with recent findings, our population genetic analysis revealed that annual populations of *M. guttatus* exhibit population structure consistent with isolation-by-distance, and neutral genetic variation is associated with temperature and precipitation (Oneal *et al.*, 2014). Despite this covariance of genomic variation and climate, clinal relationships between climate PC1 and several phenotypes (e.g. SLA) remain significant when controlling for population structure, strongly implicating differential natural selection among populations as the force driving these patterns. However, other trait–environment associations lose significance after controlling for population structure, and consequently a role for demographic processes in shaping patterns of divergence in these additional cases cannot be excluded.

Dissociable aspects of flowering track distinct features of the seasonal environment

Clinal variation in flowering time is widely observed across both latitudinal (e.g. Stinchcombe *et al.*, 2004; Montague *et al.*, 2008; Blackman *et al.*, 2011) and altitudinal gradients (Montesinos-Navarro *et al.*, 2011; Wolfe & Tonsor, 2014). These patterns are typically associated with mean annual temperature and growing season length, where higher latitude or altitude areas typically have shorter growing seasons abbreviated by cold or drought, favoring early flowering (Wilczek *et al.*, 2010). In principle, either reducing the critical photoperiod or shortening preflowering developmental phases could lead to flowering on an earlier calendar date in a seasonal environment (Roux *et al.*, 2006). Notably, however, we find that geographic patterns of variation

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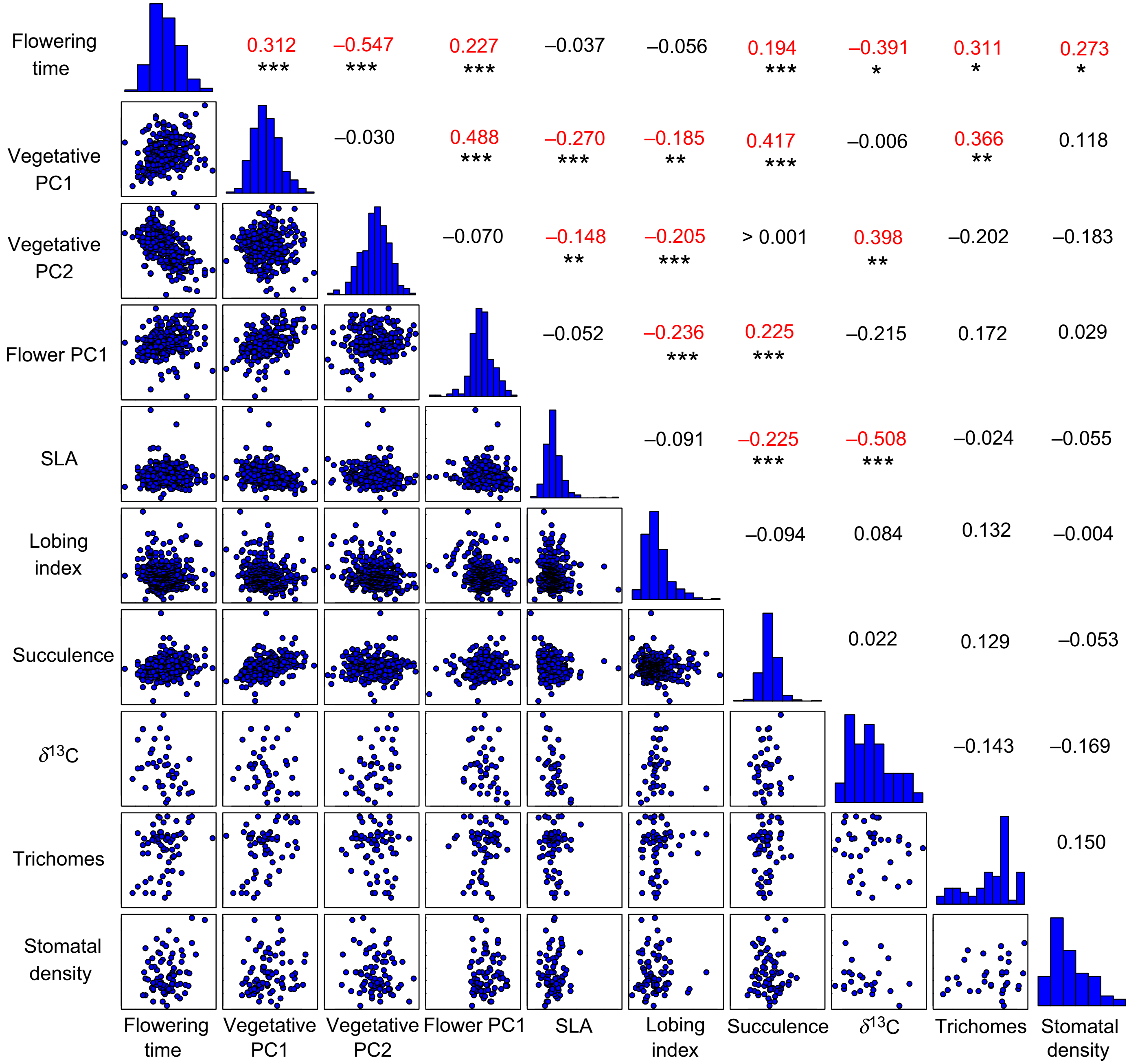

Fig. 6 Trellis plot depicting relationships between morphological, dehydration avoidance and drought escape traits. Scatterplots below diagonal are bivariate means for each *Mimulus guttatus* line. Plots along the diagonal are histograms for line means for each trait. Numbers above diagonal are Pearson correlation coefficients for each trait–trait association. Red values indicate statistically significant correlations. Asterisks are used to depict *P*-values associated with each bivariate correlation: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. SLA, specific leaf area.

in these two aspects of the seasonal timing of flowering are incongruent across the range of annual *M. guttatus*, indicating that these traits have evolved in response to different agents of selection and thus are not fully exchangeable means to the same evolutionary end.

Consistent with its function as an obligate requirement for floral induction, critical photoperiod primarily tracks the start of the growing season (Fig. 3). Critical photoperiod increases in correspondence with the later calendar dates at which growing degree-days begin accumulating at higher latitudes and altitudes, ensuring that floral initiation awaits the arrival of local spring and does not occur when plants are under snowpack. By contrast, flowering time under inductive conditions appears to track the duration of the growing season. Populations with longer periods of growth-permissive temperatures and seasonal water availability tend to be later flowering, a pattern likely favored by the increased fecundity attendant to flowering at a larger size. Thus, instead of consistency among latitudinal and altitudinal patterns, we found that the relationship between flowering time and altitude changed with latitude (Fig. 4).

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Table 5 Exploratory factor analysis identifying latent dehydration avoidance and drought escape variables in *Mimulus guttatus*

		Assuming two factors		Assuming three factors		
Variable	Traditional trait designation	Axis 1	Axis 2	Axis 1	Axis 2	Axis 3
Flowering time	Escape	0.29	0.43	0.24	0.1	0.51
Height at flowering	Escape	0.71	0.31	0.65	−0.02	0.34
Vegetative PC1	Escape	0.91	−0.13	0.9	−0.04	−0.07
SLA	Avoidance	−0.32	0.48	−0.4	−0.13	0.46
Lobing index	Avoidance	−0.08	−0.06	−0.02	0.93	0.01
Succulence	Avoidance	0.24	−0.02	0.24	0.01	0

Factor analyses were conducted using the fa() function in the pysch library. Each analysis was conducted using principal axis factoring with an oblique rotation. The root mean squared of residuals (RMSR) was 0.03 for the analysis assuming two factors and 0.01 for the analysis assuming three factors indicating there was appropriate fit for both models. Axis 1 in both factor analyses can be thought of as a drought strategy axis where higher axis values correspond to greater drought avoidance. That is, *Mimulus guttatus* plants with greater Axis 1 values are bigger, more succulent, have lower specific leaf area (SLA) and flower later than plants with lower values.

Our finding that two components of seasonal flowering are independently evolving targets of selection has additional implications. First, it demonstrates that a reaction norm parameter, either the critical photoperiod itself or an underlying liability trait, can change independently of the expressed value of that trait (Roff, 1996). Strikingly, geographically close (*c.* 5 miles) populations can have large differences in flowering time but have similar critical photoperiods (e.g. BLD: 24.7 d to flower, 13 h ppd50; 279: 35.6 d to flower, 13 h ppd50). Second, for this evolutionary flexibility to occur, ample genetic variation in each trait capable of supporting independent responses to selection must exist. Consequently, we predict that the genetic basis of divergence in these traits will largely involve unlinked loci rather than pleiotropic or tightly linked alleles.

Independent evolution of drought escape and dehydration avoidance strategies

Both drought escape and dehydration avoidance may serve as effective adaptations to water-limited environments. However, empirical studies have often found that plants which mount drought escape strategies are less capable of dehydration avoidance, and in some systems, these negative genetic correlations are attributable to antagonistic pleiotropy (Geber & Dawson, 1990, 1997; Dudley, 1996; McKay *et al.*, 2003; Ackerly, 2004; Lovell *et al.*, 2013). The range-wide pattern of divergence among annual *M. guttatus* populations resembles these results in that we found negative associations between escape (early flowering time, diminutive morphology) and avoidance (high succulence, trichome density, stomata density), and a clear drought strategy axis emerged from a factor analysis. However, within the most drought-stressed populations with the shortest growing seasons, similar correlations were not detected. Consequently, the overall trend likely reflects the varying demands of the selective environment across the landscape rather than genetic or physical constraints that limit the evolution of drought escape and drought avoidance traits in combination.

On a similar note, we find that flowering time is negatively correlated with carbon isotope ratio ($r = -0.39$, $P = 0.01$). This result contradicts the expectation that earlier flowering plants will have lower water use efficiencies, and it prompted us to search the literature for studies that measured both carbon isotope ratios (or WUE directly) and flowering time in a common garden. Of the 11 studies (10 species represented) we found with our nonexhaustive search, three studies reported a negative association between flowering time and either $\delta^{13}C$ or WUE (Farris & Lechowicz, 1990; Sherrard *et al.*, 2009; Wolfe & Tonsor, 2014). Six studies reported a positive association (Geber & Dawson, 1990; Craufurd *et al.*, 1991; Ehdaie *et al.*, 1991; Menedez & Hall, 1995; McKay *et al.*, 2003; Franks, 2011; Lovell *et al.*, 2013), one study reported that the association depended on environment (Ivey & Carr, 2012), and one study was inconclusive (Heschel & Riginos, 2005). One explanation for these diverse results may be that WUE measurements often depend on experimental conditions. Edwards *et al.* (2012) found a negative cross-environment correlation for WUE in *Brassica rapa* where plants with high WUE in well-watered conditions often had low WUE in water-limited environments. Our common garden was well-watered, which restricts the scope of inference possible; interestingly, Ivey & Carr (2012) found a positive genetic correlation between $\delta^{13}C$ and flowering in *M. guttatus* under a 'dry' treatment. Regardless, the inconsistent findings across studies suggest that our understanding of how drought-stress traits and fitness are related in natural populations is incomplete and deserves more comprehensive research.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Regression of climate PC1 values against latitude for each population.

Fig. S2 Regressions of population means of vegetative PC1, flowering time, trichome density, critical photoperiod (ppd50), stomatal density and succulence against the Climate PC1 of each population where plants were collected.

Fig. S3 Visual display of individual coefficients of membership sorted by transect based on multilocus genotypes using the program STRUCTURE.

Fig. S4 Regression of genetic PC1 and genetic PC2 with latitude.

Table S1 Summary of locations, traits, environmental correlates and genetic summary statistics for each population

Table S2 Summary of loading values and variance for climate, vegetative, floral and genetic PCA analyses

Table S3 Primers and summary statistics for each genetic marker

Table S4 Results of mixed models examining relationships between trait variation and climate variation

Table S5 Regression modeling results comparing flowering time against environmental variables separated by individual transects

Table S6 Summary of pairwise population differentiation using F_{st} and D_{est} statistics

Table S7 Summary of correlations between traits calculated using all individuals, line means and population means

Table S8 Summary of correlations between traits within Transects A–C, D–F and G–J

Table S9 Results from regressions between Pearson correlations coefficients describing trait correlations within populations and climate factors

Table S10 Trait correlations between flowering time and SLA, and between flowering time and succulence within populations

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