The role of cold cues at different life stages on germination and flowering phenology

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PREMISE OF THE STUDY: The timing of major phenological transitions is critical to lifetime fitness, and life history theory predicts differences for annual and perennial plants. To correctly time these transitions, many plants rely on environmental cues such as exposure to extended periods of cold, which may occur at different stages throughout their lifetime.

METHODS: We studied the role of cold at different life stages, by jointly exposing seed (stratification) and rosettes (vernalization) to cold. We used 23 populations of *Mimulus guttatus*, which vary from annuals to perennials, and investigated how cold at one or both stages affected germination, flowering, growth, and biomass.

KEY RESULTS: We found that stratification and vernalization interact to affect life cycle transitions, and that cold at either stage could synchronize flowering phenology. For perennials, either stratification or vernalization is necessary for maximum flowering. We also found that germination timing covaried with later traits. Moreover, plants from environments with dissimilar climates displayed different phenological responses to stratification or vernalization.

CONCLUSIONS: In general, cold is more important for seed germination in annuals and plants from environments with warm temperatures and variable precipitation. In contrast, cold is more important for flowering in perennials: it accelerates flowering in plants from lower precipitation environments, and it increases flowering proportion in plants from cooler, more stable precipitation environments. We discuss our findings in the context of the variable environments plants experience within a population and the variation encountered across the biogeographic native range of the species.

KEY WORDS   climate; *Erythranthe guttata*; flowering time; genetic variation; germination; life history; local adaptation; *Mimulus guttatus*; stratification; vernalization.

Variation in phenological responses among plants provide some of the most compelling evidence for adaptive differences that reflect the seasonality of a plant's environment. All aspects of phenology can be under selection, including the timing of seed germination (Kalisz, 1986; Baskin and Baskin, 1998), vegetative growth and heteroblasty (Diggle, 1999; Sola and Ehrlén, 2007), flowering (Franks et al., 2007), and fruit set (Thompson and Willson, 1979; Stiles, 1980). However, selection certainly does not act on each stage independently, because the timing of one life history transition can affect the environment experienced by all downstream stages (Vuorisalo and Mutikainen, 1999; Donohue et al., 2010). For example, the timing of seed germination can affect fecundity (Kalisz, 1986; Galen and Stanton, 1991; Purrington and Schmitt, 1998) and flowering time (Donohue, 2002), which subsequently affects the maternal environment of seed maturation (de Casas et al., 2012),

which then feeds back into the degree of seed dormancy (Roach and Wulff, 1987; Herman and Sultan, 2011; Kendall and Penfield, 2012; Postma and Ågren, 2016).

At the crux of life history theory is that fitness is maximized when the schedule of different stages is optimal with respect to an organism's growth, mortality, and fecundity (Roff, 2002). The schedule and survival of different stages of a plant (e.g., seedling, juvenile, adult phases) forms the foundation of explanations for the existence of a diversity of life history strategies (Cole, 1954; Charnov and Schaffer, 1973; Fox, 1993), and theory shows that selection on phenology is expected to be different for annuals and perennials. Annual, semelparous plants initiate flowering only once and complete their life cycle in one year. Iteroparous perennials, however, cycle repeatedly through vegetative and flowering phases in synchrony to the changing seasons, and may delay or abstain from

flowering in their first year to acquire sufficient biomass to allow them to sustain the perennial life cycle. In addition to selection on reproductive timing, the timing of seed germination is critical as an organism transitions from its least to its most vulnerable stages, because mortality at the seedling stage is expected to be much higher than at later stages (Harper, 1977).

Although the ultimate driver of phenological patterns is likely selection on survival and fitness, understanding the proximate mechanisms that plants use to correctly time life stage events can tell us about how selection acts on phenology (Forrest and Miller-Rushing, 2010). Because of the reliability of day length and seasonal temperature regimes, many plants have evolved to use these predictable cues to time major life history events to ensure they occur at appropriate times (Rathcke and Lacey, 1985; Donohue et al., 2010; Huijser and Schmid, 2011; Andrés and Coupland, 2012; Footitt et al., 2013). In many temperate environments, a requirement for stratification evolves as a mechanism to ensure spring germination where seed dormancy is broken by exposure to cold (Vleeshouwers et al., 1995; Finch-Savage and Leubner-Metzger, 2006). Similarly, some temperate plants must undergo exposure to the prolonged cold of winter to be competent to flower in the spring (vernalization) (Chouard, 1960; Kim et al., 2009). The cycles of the seasons, and the integration of life stage transitions, means that selection for stratification and/or vernalization should act jointly.

The majority of the mechanistic research on stratification and vernalization has used *Arabidopsis thaliana* as a model, which is a short-lived obligately annual plant. This work has established that seed dormancy varies geographically, with high dormancy associated with regions with long dry summers, and low dormancy associated with short wet summers (Boyd et al., 2007; Montesinos-Navarro et al., 2012). Similarly, *A. thaliana* varies in its requirement for vernalization to flower, depending on environmental conditions of the source populations (Stinchcombe et al., 2004; Stinchcombe et al., 2005). Although traditionally most of the research on stratification and vernalization mechanisms proceeded separately (but see Nordborg and Bergelson, 1999), recent work investigating climatic and biogeographic associations with dormancy and flowering has established patterns of covariation among life cycle traits (Debieu et al., 2013; Vidigal et al., 2016; Hämälä et al., 2017; Marcer et al., 2017). In general, this work has established that seed dormancy and flowering time are negatively correlated, indicating coevolution, and that this covariation follows a latitudinal and temperature gradient. With this pattern established in a short-lived annual plant, we wanted to investigate a species with more complex life cycles, including perennial overwintering strategies, to identify how cold temperatures at different stages affects life cycle transitions (Figure 1 in Friedman and Rubin, 2015).

In this study, we use *Mimulus guttatus* (Phrymaceae), which contains distinct annual and perennial ecotypes, to test for differences among life history groups from a range of environments in their responses to stratification and vernalization. The annual populations thrive in a climate characterized by wet conditions in autumn, winter, and spring, followed by summer drought. The perennial populations are protected from the summer drought by growing in soils that remain wet year round because of their proximity to springs or rivers. Earlier studies have shown that *M. guttatus* seed germinate under a wide range of day and night temperature combinations in the presence or absence of light, and that a diurnal shift in temperature leads to higher germination (Vickery, 1967; Vickery, 1983). Importantly, field observations show that at least some annual and perennial
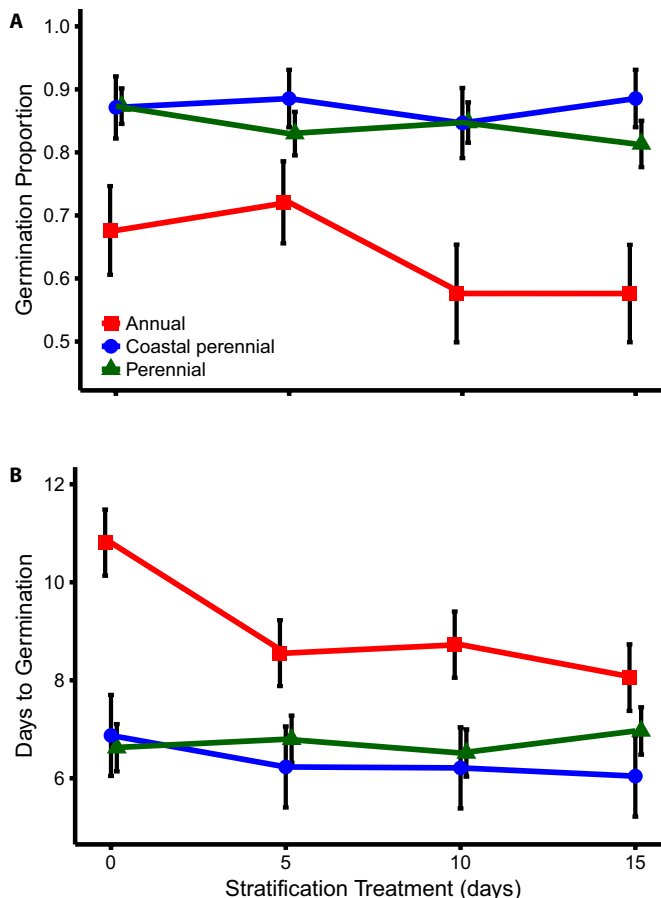

FIGURE 1. Relationship between (A) germination proportion and (B) days to germination across stratification treatments for *Mimulus guttatus* ecotypes. The four stratification treatments are shown on the *x*-axis. Red squares and lines indicate annuals, blue circles indicate coastal perennials, and green triangles indicate perennials. Plotted values are adjusted to account for other variables in the model (see Table 1 for the complete list of all model terms).

populations germinate in both autumn and spring (Waser et al., 1982; Ivey and Carr, 2012; personal observation: MJR), although to our knowledge, experiments testing for stratification requirements have never been investigated in this species, nor has the ecological significance of germination timing been explored. As a long-day plant, there are no rapid-cycling autumn flowering plants (i.e., all plants experience cold at either seed or rosette stages). Previous research has demonstrated variability in requirements for vernalization in *Mimulus guttatus* (Friedman and Willis, 2013; Preston et al., 2016) including variation within populations (JF: personal observation). The observed variation in germination and vernalization, combined with the variation in life cycle, provides a unique opportunity to test fundamental features of phenological responses in plants. To examine the interactions between the timing of cold on two different life stages among plants with different life cycles, we use a fully factorial design with various levels of seed stratification, followed by two levels of vernalization across a collection of 23 *M. guttatus* populations. The key insight provided by this study is the contingency between the response to cold at different life stages, and how this differs for annual and perennial plants from a broad geographic range.

METHODS

Study System

Mimulus guttatus (DC.; also known as *Erythranthe guttata* (Fisch. ex DC.) G.L.Nesom) is a predominantly outcrossing species distributed over a broad geographic range across western North America. To investigate variation between populations and ecotypes, we included 23 populations, comprising annual (A; $n = 7$), perennial (P; $n = 12$), and divergent coastal perennial (CP; $n = 4$) that have been recognized as distinct genetic or morphological groups (Pennell, 1947; Lowry et al., 2008; Twyford and Friedman, 2015; Appendices S1 and S2 (see the Supplemental Data with this article). To capture some of the structure of the variation, we included 2–3 full-sib families from each population (specifically 19 populations contained three families, and the remaining four populations had two families). From each family we used 40 seeds (split into four treatments: see below).

To reduce maternal effects in early life stages, we generated seed for our experiment in uniform conditions. We grew field-collected seed from each population in a walk-in growth chamber (Environmental Growth Chambers, Chagrin Falls, Ohio, USA) at 21°C in a 16-h photoperiod (16L/8D). These plants were used to produce the seed for the current experiment. We created outbred full-sib seed by intercrossing unrelated random pairs of plants from within each of the 23 populations. We maintained plants in the growth chamber until the seeds were almost dehiscing. We then collected seed and kept it in coin envelopes at room temperature for six months to reduce primary seed dormancy and remove any after-ripening requirement, following the methodology used by Vickery (1967).

Experimental Treatments

To test the effect of cold stratification on germination, we used four stratification treatments (0, 5, 10, or 15 days). We selected these lengths to encompass the range of stratification duration typically used in research on this species (e.g., Sweigart et al., 2008; Fishman et al., 2014; Ferris et al., 2015). We imposed the stratification treatments by placing the planted seed into a random position in a single walk-in growth chamber at 4°C with no light (0L/24D) for either 5, 10, or 15 days. The seed for the 0-days stratification treatment were planted and left in the dark at 21°C for 24 h. For each treatment, we planted 10 replicate seed per full-sib family. Specifically, we pipetted a single seed into the center of a 2-inch net pot, which had been filled with Fafard 4P Growing Mix with a top layer of soil sieved with a 7-mm sieve to standardize the environment. We stagger planted treatments, so that all pots finished their stratification treatment on the same day. After stratification, we moved pots to create 10 randomized blocks comprising one pot per family per stratification treatment. We moved all pots ($n = 2600$) to flood benches in the Syracuse University greenhouse with the following conditions: day/night temperature 21°C/18°C and a 10.5-hour photoperiod (10.5L/13.5D). We bottom-watered pots and misted twice daily; and checked for germination, scoring daily for the appearance of cotyledons between 10:00 AM-12:00 PM. We excluded seed that took longer than 23 days to germinate from the study, because this extended into the next treatment (see below) and altered their germination environment.

To test whether cold at different life stages interact on traits expressed later in life, we imposed a vernalization treatment on seedlings. After 24 days in the greenhouse, we randomly selected five of the 10 blocks and moved the plants back into the growth chamber for 28 days at 4°C with an 8-h photoperiod (8L/16D). This duration has been shown to be effective at vernalizing *M. guttatus* (Preston et al., 2016). The remaining five blocks experienced a no-vernalization treatment, and remained in the greenhouse, at which point the photoperiod was changed to long days (16L/8D). After 28 days, the vernalized plants were returned to the long-day greenhouse. We recorded the number of days to the onset of flowering (hereafter known as days to flowering), scored as the number of days from germination to the opening of the first flower. For the plants that received vernalization, we subtracted the 28 days they spent in the cold. At flowering, we harvested the plants and measured the following traits: node of the first flower, longest leaf, and number of elongated branches (including both stolons derived from the rosette and branches originating from the primary inflorescence). We then cut plants at soil level, dried them for a minimum of 3 days at 95°C, and weighed them to estimate aboveground biomass. We terminated the experiment after 4 months, at which point we scored any plants that had not flowered as nonflowering, and harvested and measured them for the same set of traits as above.

Statistical Analyses

We used linear mixed models to determine the effects of ecotype, population, family, and stratification treatment on germination timing (Proc Mixed; SAS, 2013) and germination proportion (Proc Glimmix for binomial distribution with logit link function). We included population (nested within ecotype) and family (nested within population and ecotype) as random effects, and the other factors as fixed effects. Analysis of the effect of stratification used a planned comparison contrasting noncold treated seeds (0 days stratification) to the mean of the cold treated seeds (5, 10, and 15 days stratification). The effect of life history was examined with contrast statements that compared annuals to the mean of the two perennial groups.

Although cold treatment of seeds can sometimes be referred to as vernalization when investigating the effect on flowering (Michaels and Amasino, 2000), for consistency we refer to cold treatment of seeds as stratification, and cold treatment of rosettes as vernalization. For all traits we used linear mixed models (except for flowering proportion, where we used a generalized linear mixed model) to estimate variance components for ecotype, population, family, stratification treatment, vernalization treatment, and the relevant two-way and three-way interactions. As above, population (nested within ecotype) and family (nested within population and ecotype) were treated as random effects, and the other factors as fixed effects. If interactions did not explain a significant amount of variation ($\alpha = 0.05$), we removed them by stepwise backward elimination. For these analyses, we included days to germination as a covariate in the model to evaluate whether variation in germination timing affected traits expressed later in life.

For the models above, we estimated best linear unbiased predictors (BLUPs) for families for each trait within each treatment. We use these BLUPs to investigate covariation among traits across all families (rather than within populations), because our replication of families within populations is low.

To investigate covariation between days to germination and all other traits, we calculated Pearson correlations between germination timing and each of the other traits using family BLUPs (standardized to a mean of 0 and standard deviation of 1 within each stratification environment). We tested for differences in the

strength of the pairwise correlations among treatments using a test of heterogeneous slopes. Slopes did not differ across treatments for any of the pairwise traits; therefore, we present a single correlation using the pooled data from all treatments.

Lastly, to investigate whether families respond similarly to stratification and vernalization, we examined plasticity in flowering proportion. For this study, we define plasticity as the difference in the mean trait value across treatments for each full-sib family. We calculated family-level directional plasticities for flowering proportion across stratification or vernalization treatments by subtracting the proportion of plants flowering without cold (0 days stratification or vernalization) from the proportion of plants flowering with cold (15 days stratification or 28 days vernalization) for each family. We then calculated the Pearson correlation coefficient between the plasticity in flowering proportion across stratification treatments and the plasticity in flowering proportion across vernalization treatments.

To test for effects of local climatic factors on phenology, we examined the relationship between a population's germination and flowering phenology and environmental conditions at the population's original location. We obtained 19 bioclimatic variables from the WorldClim data set derived from long-term observations of temperature and precipitation (1950–1990) mapped to a resolution of 2 arc-min (Hijmans et al., 2005). We extracted climate variables for each population's latitude and longitude coordinates using the 'rgdal' package in R (Bivand et al., 2014). Because climate variables frequently covary, we conducted principal component analysis using z-scores, and retained the first two principal components (PC1 and PC2, Proc Princomp, SAS 9.4 2013). We then used general linear models (Proc Mixed) to test for the effects of PC1, PC2, ecotype, stratification, vernalization, and their interactions, on the proportion and timing of germination and flowering. Population and family (nested as above) were included as random factors. We used contrast statements to estimate and compare partial regression coefficients across stratification and vernalization treatments.

RESULTS

Stratification environment and life history affect germination

Both perennial groups had consistently higher germination than annuals across all stratification treatments (Figure 1A and Table 1). However, for the timing of germination, there was a significant interaction between ecotype and stratification treatment. In annuals, the addition of cold accelerated germination by 2.39 ± 0.40 days (0 vs. 5, 10, 15: $t_{66.8} = 6.04$, $P < 0.0001$), but perennials were unaffected (coastal: $t_{37.7} = 1.61$, $P = 0.12$; perennial: $t_{39.9} = -0.45$, $P = 0.65$; Figure 1B). As a result, with no cold, annuals germinated later than both perennial groups (delayed by 4.07 ± 0.85 days; A vs. CP, P: $t_{29.5} = 4.80$, $P < 0.0001$); and with 15 days of stratification there was no difference in germination timing between annuals and perennials ($t_{30.1} = 1.83$, $P = 0.08$). Populations and families differed in both germination traits (Table 1). In all four stratification treatments, families with earlier germination also had higher germination proportions ($r = -0.43$, $P < 0.0001$), and the strength of this association was not significantly different across treatments (data not shown).

Stratification and vernalization affects flowering traits

Populations and families were variable for all phenological (flowering proportion and flowering time), developmental (node of first flower), and morphological (longest leaf, branch number, and aboveground biomass) traits measured (Table 2). The timing of germination significantly covaried with all downstream traits, except for the proportion of plants that flowered (Table 2).

TABLE 1. Summary of linear models of the influences on germination traits for *Mimulus guttatus*. The F-values are reported for fixed effects, Wald Z-statistics are reported for random effects. Nonsignificant terms removed from the model are indicated with "—", terms that remain in the model to reflect experimental design, but are not significant are indicated with "NS". *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Sources of Variation	Germination Proportion	Days to Germination
Stratification	$F_{3,2497} = 2.62^*$	$F_{3,138} = 10.17^{***}$
Life History	$F_{2,20} = 6.34^{**}$	$F_{2,19.8} = 5.42^*$
LH × Strat.	—	$F_{6,135} = 7.19^{***}$
Population (LH)	$Z = 2.55^{**}$	$Z = 2.27^*$
Family (Pop., LH)	$Z = 1.54$	$Z = 3.47^{***}$
Family (Pop., LH) × Strat.	—	$Z = 3.05^{**}$
Block	NS	NS

TABLE 2. Summary of linear models of the influences on phenological, developmental, and morphological traits for *Mimulus guttatus*. The F-values are reported for fixed effects, Wald Z-statistics are reported for random effects. Nonsignificant terms removed from the model are indicated with "—", terms that remain in the model to reflect experimental design, but are not significant are indicated with "NS". *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Sources of Variation	Flowering Proportion	Days to Flowering	Node of First Flower	Leaf Size	Branches	Biomass
Stratification	$F_{3,382} = 3.60^*$	$F_{3,163} = 29.66^{***}$	NS	$F_{3,180} = 4.60^{**}$	$F_{3,184} = 7.75^{***}$	$F_{3,193} = 17.07^{***}$
Vernalization	$F_{1,65} = 12.87^{***}$	$F_{1,30.9} = 20.46^{***}$	NS	$F_{1,11.3} = 14.89^{**}$	$F_{1,1756} = 24.50^{***}$	$F_{1,51.7} = 4.35^*$
Strat. × Vern.	$F_{3,356} = 5.90^{***}$	$F_{3,164} = 29.72^{***}$	NS	$F_{3,198} = 5.44^{**}$	$F_{3,1757} = 6.51^{***}$	$F_{3,209} = 10.78^{***}$
Life History	$F_{2,19.9} = 1.21$	$F_{2,19.4} = 8.95^{**}$	$F_{2,20.5} = 7.95^{**}$	NS	NS	$F_{2,19.8} = 1.94$
Population (LH)	$Z = 2.80^{**}$	$Z = 2.96^{**}$	$Z = 2.67^{**}$	$Z = 2.57^{**}$	$Z = 3.00^{**}$	$Z = 2.92^{**}$
Family (Pop., LH)	$Z = 3.21^{***}$	NS	NS	$Z = 2.47^{**}$	$Z = 2.51^{**}$	NS
LH × Strat.	$F_{6,378} = 3.01^{**}$	$F_{6,163} = 2.66^*$	$F_{6,198} = 3.44^{**}$	—	$F_{6,182} = 5.19^{***}$	—
LH × Vern.	$F_{2,64.3} = 7.78^{***}$	NS	$F_{2,106} = 4.81^*$	—	NS	$F_{2,63.9} = 4.29^*$
LH × Strat. × Vern.	NS	—	—	—	—	—
Family (Pop., LH) × Strat.	NS	NS	NS	$Z = 5.43^{***}$	$Z = 2.97^{**}$	$Z = 4.17^{***}$
Family (Pop., LH) × Vern.	NS	$Z = 2.49^{**}$	$Z = 3.03^{**}$	$Z = 2.67^{**}$	—	$Z = 3.97^{***}$
Family (Pop., LH) × Strat. × Vern.	$Z = 4.93^{***}$	$Z = 4.58^{***}$	$Z = 2.49^{**}$	$Z = 2.05^*$	—	$Z = 3.71^{***}$
Block (Vern.)	NS	NS	NS	$Z = 1.76^*$	NS	NS
Days to Germination	NS	$F_{1,1570} = 135.69^{***}$	$F_{1,1832} = 24.75^{***}$	$F_{1,1889} = 56.06^{***}$	$F_{1,1851} = 190.79^{***}$	$F_{1,1874} = 122.31^{***}$

The addition of cold at either seed (stratification) or rosette (vernalization) stages significantly affected all traits (Table 2). Flowering time was accelerated in plants that received cold at either life stage (Figure 2). Specifically, stratification accelerated days to flower by 4.9 ± 0.5 (0 vs. 5, 10, 15: $t_{166} = 9.25$, $P < 0.0001$) and

vernalization accelerated days to flower by 2.8 ± 0.6 days (0 vs. 28: $t_{30.9} = 4.52$, $P < 0.0001$). The addition of the maximum combination of cold—15 days of stratification and 28 days of vernalization—decreased flowering time by 9.8 ± 1.0 days (0/0 vs. 15/28: $t_{157} = 9.95$, $P < 0.0001$).
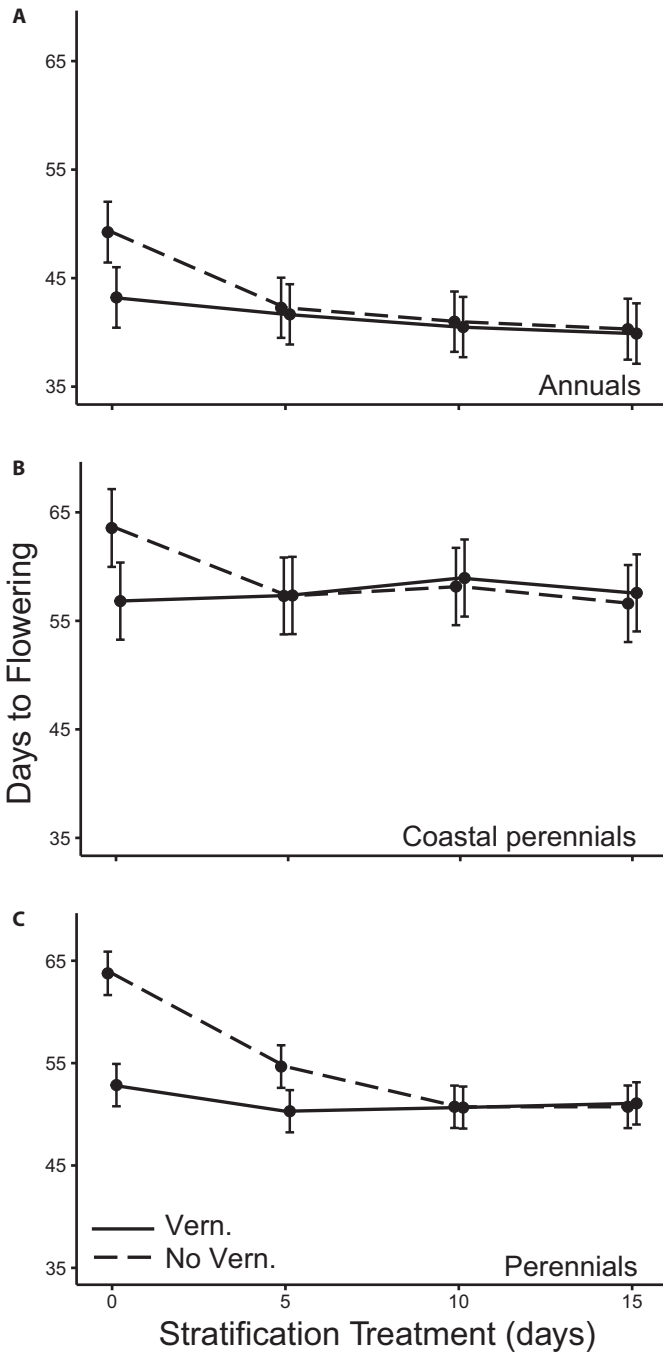

FIGURE 2. Relationship between the days to flowering and stratification and vernalization treatments for *Mimulus guttatus* (A) annuals, (B) coastal perennials, and (C) perennials. The four stratification treatments are shown on the *x*-axis, solid lines indicate vernalization, dashed lines indicate no vernalization. Plotted values are adjusted to account for other variables in the model (see Table 2 for the complete list of all model terms).
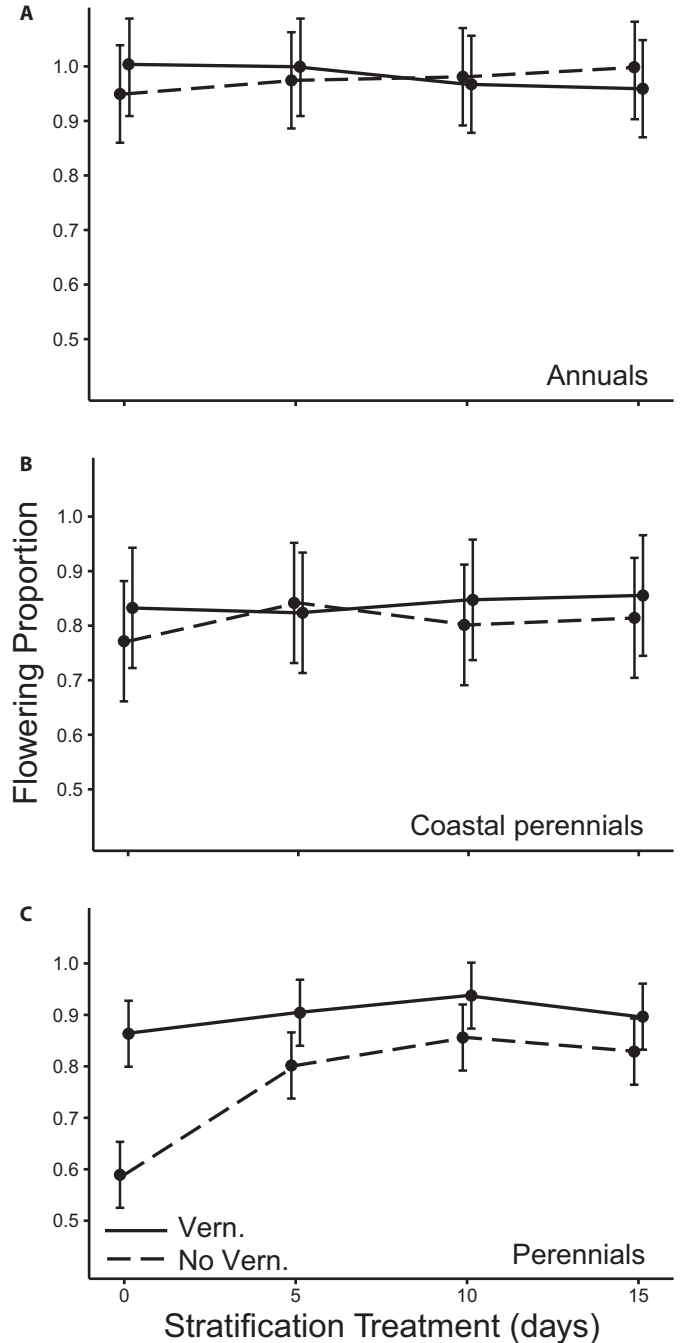
FIGURE 3. Relationship between the proportions of plants flowering and stratification and vernalization treatments for *Mimulus guttatus* (A) annuals, (B) coastal perennials, and (C) perennials. The four stratification treatments are shown on the *x*-axis, solid lines indicate vernalization, dashed lines indicate no vernalization. Plotted values are adjusted to account for other variables in the model (see Table 2 for the complete list of all model terms).

Annual plants flowered at close to 100% regardless of stratification or vernalization treatments (Figure 3A). Likewise, coastal perennials flowered in equal proportions regardless of stratification or vernalization treatment (Figure 3B). Lastly, almost all perennial plants flowered following vernalization regardless of stratification treatment (Figure 3C). It is important to note that for perennials that did not receive vernalization, stratification increased the proportion of plants that flowered. Thus, compared to plants that received no cold, the addition of cold at either stage increased the number of plants that flowered and advanced their time to flowering. The increase in proportion flowering is evident in family-level plasticity to the different environments. Families differed in both the direction and degree of their plasticity responses to stratification or vernalization, where exposure to either cold treatment altered flowering proportion by up to 90%. Populations responded to stratification and vernalization in consistent ways, that is, plasticity to one treatment accurately predicted the response to the other treatment (Figure 4).

For all traits, except node of first flower, the main effects of stratification, vernalization, and their interaction were significant (Table 2). Annual plants were the smallest at flowering, as estimated by aboveground biomass, followed by perennials, and lastly coastal perennials were the largest. Average plant size decreased with stratification (-0.044 ± 0.008 grams; 0 vs. 5, 10, 15: $t_{192} = 5.66$, $P < 0.0001$), vernalization (0 vs. 28: -0.023 ± 0.010 grams; $t_{51.7} = 2.09$, $P < 0.05$) and the addition of maximum cold at both life stages (-0.089 ± 0.015 grams; 0/0 vs. 15/28: $t_{171} = 5.85$, $P < 0.0001$).

Associations with climate and correlations among traits

The 19 WorldClim bioclimatic variables were summarized by the first two principal components, which collectively explained 74% of the variance. The first principal component (PC1) explained 42% of the variance, with large positive loadings representing precipitation variables and negative loadings representing temperature
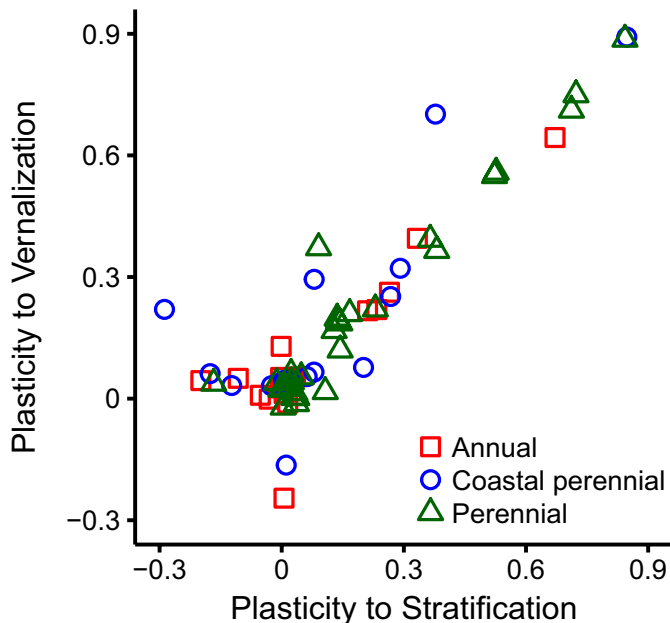

FIGURE 4. Correlation between the 65 families' plasticity to stratification and their plasticity to vernalization for flowering proportion ($r = 0.89$, $P < 0.0001$).

seasonality (Appendix S3). The second principal component (PC2) explained 32% of the variance, with positive loadings for temperature variables and variation in precipitation stability. Latitude, longitude, and elevation were strongly correlated with PC1 (latitude: $r = 0.76$, $P < 0.0001$; longitude: $r = -0.82$, $P < 0.0001$; elevation: $r = -0.55$, $P < 0.01$). Latitude and elevation, but not longitude, were correlated with PC2 (latitude: $r = -0.57$, $P < 0.01$; longitude: $r = -0.11$, $P = 0.61$; elevation: $r = -0.66$, $P < 0.001$).

The bioclimatic PC scores affected germination and flowering differently across stratification and vernalization treatments (Appendix S4; see significant Strat. or Vern. × PC interaction terms). For all four traits analyzed (germination proportion, germination timing, flowering proportion, flowering timing) none of the partial regression coefficients for the effect of PC scores differed significantly from zero, however they differed significantly in pairwise comparisons between treatments (Appendix S4). In particular, germination timing varied negatively with PC2 in the presence of cold, but in the absence of cold there was a very weak positive response, so that plants from environments with higher PC2 scores accelerated their germination in response to cold (Figure 5A). In contrast, days to flowering varied positively with PC1 in the presence of cold stratification (Figure 5B) and vernalization (Figure 5C), but showed weaker relationships in the absence of cold, so that plants from environments with low PC1 scores accelerated their flowering in response to cold (Figure 5B, C). Finally, flowering proportion varied negatively with PC2 in the presence of cold stratification and vernalization (Figure 5D, E), and positively in the absence of cold, so that plants from environments with low PC2 scores flowered at a greater proportion with cold at either stage.

Germination timing showed strong correlations with other traits, and these associations were not significantly different across treatments (data not shown). In addition to families with earlier germination having higher germination proportions ($r = -0.43$, $P < 0.0001$), they also had higher flowering proportions ($r = 0.18$, $P < 0.0001$). Early germination was also associated with delayed flowering time ($r = -0.22$, $P < 0.0001$), the production of more nodes prior to flowering ($r = -0.14$, $P < 0.001$), and larger leaves and aboveground biomass ($r = -0.27$, $P < 0.0001$, $r = -0.14$, $P < 0.01$). The only trait that was not associated with germination timing was the number of branches produced ($r = -0.03$, $P = 0.51$).

DISCUSSION

Surviving the first winter of life is a crucial hurdle for plants, and especially challenging if the risks of overwintering as a seed versus as a rosette vary unpredictably because of spatial or temporal heterogeneity. Given such variability, selection is unlikely to act independently on stratification and vernalization pathways. Although many studies describe how plant phenology is attuned to patterns of seasonality, few have experimentally manipulated the timing and duration of cold at different life stages. Furthermore, selection on phenology is expected to vary for plants with ephemeral life cycles versus those with perennial life strategies. Here we show that germination is flexible in *Mimulus guttatus*, potentially as a buffer against environmental uncertainty. We further show that for perennial plants, responses to cold at either seed or rosette stages are coordinated, and either mechanism can synchronize flowering. Below we discuss these findings in the context of the environments
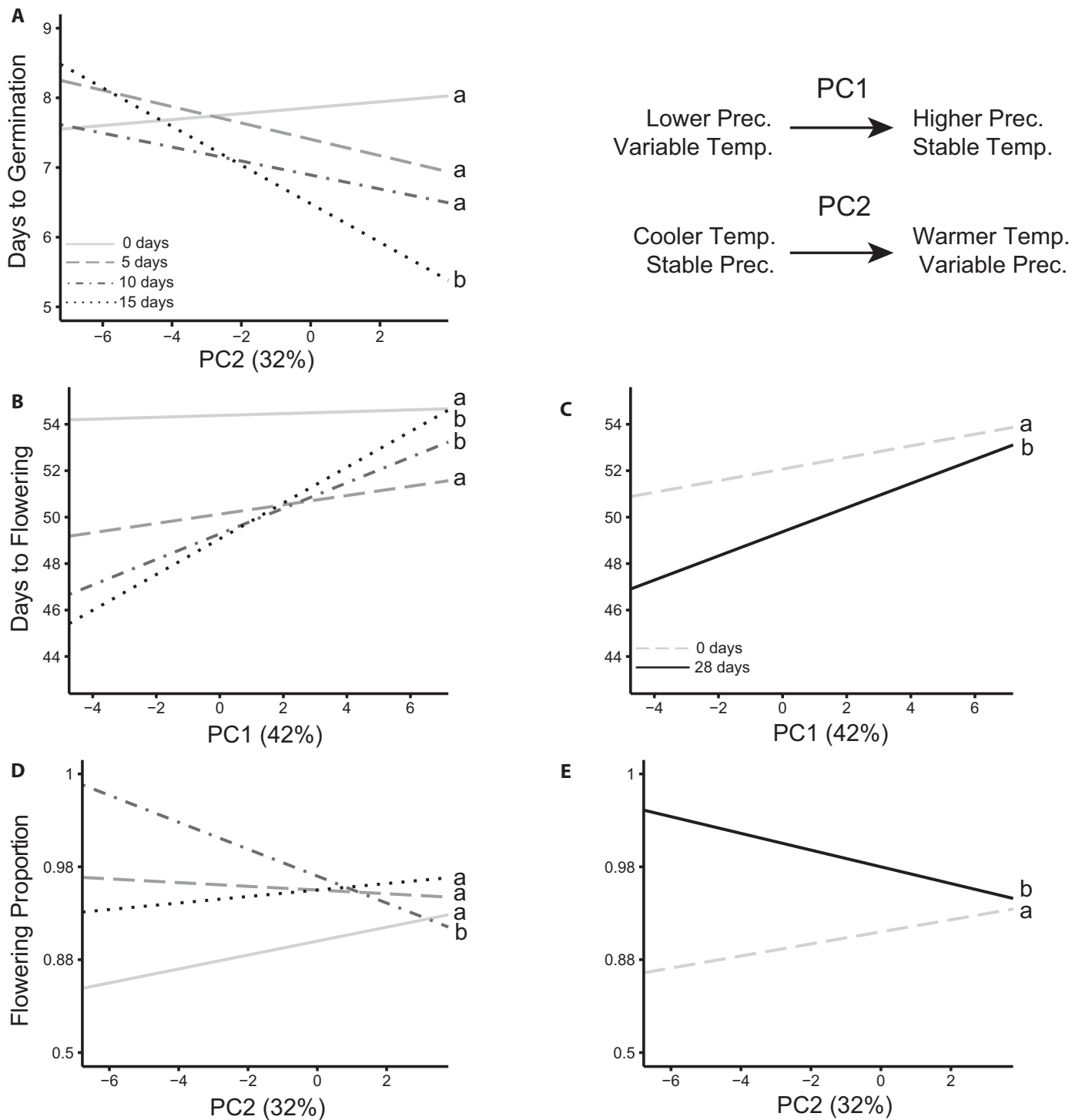
FIGURE 5. Relationship between (A) PC2 score and days to germination for each stratification treatment, (B) PC1 score and days to flowering for each stratification treatment, (C) PC1 score and days to flowering for each vernalization treatment, (D) PC2 score and flowering proportion for each stratification treatment, and (E) PC2 score and flowering proportion for each vernalization treatment. Different lowercase letters denote significant differences (adjusted using Bonferroni correction for multiple tests) in the slopes for pairwise contrasts between 0 days stratification and 5, 10, or 15 stratification treatments; or 0 days and 28 days vernalization. In the upper right corner we indicate the predominant loading characteristics of PC1 and PC2.

experienced by these plants in nature, and the evolutionary implications of using seasonal cues to time phenological events.

Our results show that annual *M. guttatus* may behave as both winter and short-lived summer annuals with seeds germinating in autumn and in spring, and both cohorts of plants completing flowering in late spring/early summer. In the annual populations of *M. guttatus*, flowering occurs rapidly following snowmelt and there is strong selection to flower early to avoid summer drought.

Thus, autumn germination allows plants to take the full advantage of the spring growing season. However, if seed do overwinter prior to germination, then stratification accelerates germination and time to flower, overcoming the handicap from a delayed start. Overall, the germination proportions for *M. guttatus* in this experiment were comparable to those observed in a recent study that included germination proportion in the spring in field conditions (Peterson et al., 2016). We expected perennial *M. guttatus* to have the most flexible germination because it lives in environments that are wet year-round, and indeed, we found this in both its overall proportion of seeds germinating and their lack of response to stratification.

We found a highly significant effect of germination timing on all later morphological and flowering traits and biomass (Table 2), and a strong relationship between a family's germination timing and the proportion of seeds that germinated. Moreover, we also found that early germination was associated with later flowering time and larger plant size (as estimated by node number, leaf size, and aboveground biomass). A similar result was found by Zhou et al. (2005), when they staggered planting, and thus germination, in a field experiment and found delaying germination led to relatively earlier reproduction and smaller sizes. This reiterates the central idea that selection is unlikely to act on single phenological events, but rather on overall life strategies. Several studies have found that the timing of germination influences fecundity, in both natural conditions and controlled environments (Baskin and Baskin, 1972; Kalisz, 1986; Galen and Stanton, 1991; Simons and Johnston, 2000; Donohue et al., 2005c). Simons and Johnston (2000) found that in *Lobelia inflata* (which like *Mimulus*, also has tiny seeds) the effect of germination timing on fecundity and biomass is mediated through both direct effects and through correlations with seed size. We did not measure seed size in our experiment, and it is certainly possible that it is correlated with germination timing. Nonetheless, our results suggest that the rate of germination might be under strong selection, as has been found in other studies (Donohue et al., 2005b; Huang et al., 2010; Postma and Ågren, 2016; Yuan et al., 2016).

Although field experiments would be necessary to identify the benefits of rapid germination, several processes might be involved. Germination timing may covary with dormancy level, so that selection for reduced dormancy also leads to faster germination (Baskin and Baskin, 1998). Second, because we found that seed germination strongly affects later size and flowering traits, germination may be genetically or physiologically constrained to covary with plant growth and development. Rapid seed germination may reflect a feedback loop where selection is operating on other later life stages (for example, flowering time or seed dispersal time), and altering its germination schedule is a means to favorable niche construction (Donohue et al., 2005a). Also, in *M. guttatus*, the primary mechanism of seed dispersal is water, with perhaps very limited dispersal by wind or animals (Lindsay, 1964; Vickery et al., 1986). In artificial flow experiments with *M. guttatus*, Truscott et al. (2006) found that ungerminated seed sink rapidly in water. Thus it might be beneficial for seed to germinate quickly to avoid sinking and to safeguard an establishment site. Further experiments would be required to assess these possibilities.

For some species, delayed germination or seed dormancy can be part of an overall bet-hedging strategy (Cohen, 1966; Simons and Johnston, 2006; Gremer and Venable, 2014; Gremer et al., 2016) where environmental unpredictability selects for variable germination strategies that maximize fitness in the long-term. Given the more restrictive environments of the annual populations, this may explain their lower germination proportion. There is limited evidence that seed banks exist for *M. guttatus*, although transient 2- to 3-year seed banks might occur (Vickery, 1983; Truscott et al., 2006), and seed are resilient and remain viable following submersion in rivers and passage through bird and deer digestive systems (Lindsay, 1964; Vickery et al., 1986). However, in our experiment we cannot rule out the possibility that the lower germination in annuals is because we miss a necessary cue for germination; for example, other cues used by plants to stimulate germination include heat, darkness, pH, fire, or smoke (Keeley et al., 1985; Pierce et al., 1999; Clarke et al., 2000; Staden et al., 2000). Ultimately, the adaptive value of delayed germination relies on the relative risk of death in the seed bank versus survival as a rosette and reproductive success (Masuda and Washitani, 1992). It is likely that seeds and rosettes have different over-winter mortality depending on spatial or temporal heterogeneity in environmental conditions. This heterogeneity may have direct fitness consequences for the seed, but may also affect other aspects of the plant's life history (Marks and Prince, 1981; Donohue, 2002). For example, a 3-year field experiment by Mojica et al. (2012) showed strong spatial (at the scale of meters) and temporal variability of survival and fecundity in plants that varied for flower size.

To help explain the environmental cues that may be driving differences in stratification and vernalization, we investigated the role of a suite of bioclimatic variables (based on the geographic coordinates of the source populations). We found that locations with warmer temperatures and more variable precipitation (PC2) were more responsive to stratification for time to germination. These types of environments may select, directly or indirectly, for more constrained germination windows. These warmer environments with more variable precipitation might promote seed dormancy, ensuring late germination when the summer drought is over and autumn rains begin. In contrast, we found that plants from environments with lower precipitation and more variable temperatures (PC1) were more responsive to cold at both seed (stratification) and seedling (vernalization) stages to accelerate the time to flowering. Precipitation is known to be a major driver of flowering time differences in *M. guttatus* as a drought-avoidance strategy, and our results suggest that especially in drier environments, cold contributes to accelerating the time to flowering.

Because our results show limited evidence that seed germination is season specific, plants may experience cold at different life stages (i.e., at seed, or rosette, or both for perennials that do not flower their first year). This is similar to *Lobelia gattingeri* (Baskin and Baskin, 1979) and *Campanula americana* (Baskin and Baskin, 1984), where seeds are capable of germinating in either autumn or spring. However, in contrast to *C. americana*, which has an absolute requirement for vernalization regardless of prior stratification, we find significant interactions between stratification and vernalization treatments on traits expressed during later life stages, which may mean that selection is acting across combinations of cold cues. This is most apparent in the perennial ecotype, which cycle repeatedly through the seasons and where either stratification or vernalization promote faster flowering and synchronize flowering time. The similar flowering response induced by stratification and vernalization suggests that the over-wintering success of seeds versus rosettes fluctuate sufficiently to maintain alternative over-wintering strategies. Furthermore, at the family level we find a strong correlation in plasticity to stratification and plasticity to vernalization, suggesting that there might be shared genetic pathways for stratification and vernalization.

The results reported here provide evidence that responses to stratification and vernalization may be synergistic traits. Similar results were recently reported in *Capsella bursa-pastoris* linking seed secondary dormancy to flowering time (Toorop et al., 2011), and in *A. thaliana* showing coevolution of seed dormancy and flowering time on the Iberian peninsula (Marcer et al., 2017). Although in *A. thaliana* extensive genetic work identified largely independent molecular mechanisms for stratification (e.g., *DELAY OF GERMINATION 1* (*DOG1*); Bentsink et al., 2006; Huang et al., 2010) and vernalization (e.g., *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*); Simpson and Dean, 2002; Caicedo et al., 2004), more recent studies are suggesting that overlapping molecular pathways may indeed coordinate seed dormancy and vernalization-induced flowering (Chiang et al., 2009; Huijser and Schmid, 2011; Huo et al., 2016; Auge et al., 2017; Blair et al. 2017). Further work looking at candidate gene expression and/or using whole transcriptome sequencing might elucidate whether shared genetic pathways are involved in the response to cold at the two life stages in *M. guttatus*.

If the optimal germination season varies unpredictably among years, then germination variance may be an adaptive diversification strategy. Furthermore, if the risks of overwintering as a seed versus as a rosette vary unpredictably with small spatial heterogeneity, then phenotypic variance will be advantageous. Given such population variability, selection unlikely acts independently on stratification and vernalization pathways. The interactions between stratification and vernalization in our experiments, and their correlated plasticity, suggest that either stage of cold might be equivalent in terms of synchronizing flowering and growth. Our research here paves the way for several avenues of future work including studying the fate and fitness of autumn versus spring germinants in field conditions, and whether the genetic architecture of stratification and vernalization include shared molecular pathways.

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AUTHOR CONTRIBUTIONS

MJR and JF participated in design and execution of the research including data collection, analysis, and interpretation, and preparation of the manuscript.

DATA ACCESSIBILITY

Data used to conduct statistical analysis are available in the online supplement (Appendix S5).

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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