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Research

Environmental heterogeneity generates intrapopulation variation in life-history traits in an annual plant

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Summary

- Environmental variation affects a plant's life cycle by influencing the timing of germination and flowering, and the duration of the growing season. Yet we know little information about how environmental heterogeneity generates variation in germination schedules and the consequences for growth and fecundity through genetic and plastic responses.
- We use an annual population of *Mimulus guttatus* in which, in nature, seeds germinate in both fall and spring. We investigate whether there is a genetic basis to the timing of germination, the effect of germination timing on fecundity, and if growth and flowering respond plastically to compensate for different season lengths.
- Using sibling families grown in simulated seasonal conditions, we find that families do not differ in their propensity to germinate between seasons. However, the germination season affects subsequent growth and flowering time, with significant genotype-by-environment interactions (G × E). Most G × E is due to unequal variance between seasons, because the spring cohort harbours little genetic variance. Despite their different season lengths, the cohorts do not differ in flower number (fecundity).
- Heterogeneous environments with unpredictable risks may maintain promiscuous germination, which then affects flowering time. Therefore, if selection at particular life stages changes with climate change, there may be consequences for the entire life cycle.

Introduction

Environmental variation is ubiquitous in nature. Because plants are sessile, one way they can exert some control over the effects of their local environment is by shifting the timing of different life events. The timing of key life stage transitions, like germination and flowering, are important adaptations to seasonality, and the maintenance of variation within populations suggests variable selection in space and time. Environmental variation can maintain polymorphism in life-history strategies within populations through balancing selection, where fitness effects of alleles are context dependent, or spatial and temporal variation shifts selection and determines beneficial alleles (Christiansen, 1974; Gillespie & Turelli, 1989; Delph & Kelly, 2014). Other models have identified conditions under which natural selection favours the maintenance of phenotypic variance, with an essential component in these models being that the environment is heterogenous and phenotypic variation can buffer against unpredictable selection (Cohen, 1966; Levins, 1968; Gillespie, 1973; Slatkin & Lande, 1976; Real, 1980).

When the environment fluctuates over small temporal or spatial scales from the perspective of an organism, selection acts to maximise fitness across the array of environments encountered (Bull, 1987; Frank & Slatkin, 1990). Several mechanisms may be involved, such as adaptive phenotypic plasticity, selection against developmental homeostasis or bet hedging. A core requirement for adaptive phenotypic plasticity is the existence of reliable cues, so that organisms can use environmental information to accurately predict future selection on the plastic trait (Moran, 1992; Scheiner, 2013). Alternatively, bet hedging traits are expected to evolve when the association between phenotype and fitness cannot be accurately predicted at an earlier developmental stage (Cohen, 1966), and selection maximises long-term geometric mean fitness by reducing the variance in fitness among generations (Simons & Johnston, 2006; Starrfelt & Kokko, 2012). Cohen's (1966, 1967) classic papers on bet hedging use a model of annual plants that maximise their reproduction in randomly varying environments by having a proportion of seeds germinate and experience the current environment, and a proportion remain dormant as a hedge against unsuitable conditions. Seed dormancy can involve physiological, morphological or anatomical mechanisms (Baskin & Baskin, 1988; Willis *et al.*, 2014) and in many temperate species cold exposure breaks dormancy and ensures spring germination (Vleeshouwers *et al.*, 1995; Finch-Savage & Leubner-Metzger, 2006).

Germination time has served as a useful theoretical and empirical trait to examine the joint effects of environmental and genetic factors on phenotypic variance. It has long been recognised that in seasonal environments early germination is inherently risky due to increased risk of mortality, but early germinators who

survive generally have higher fecundity than late germinators, owing to their competitive advantage (Arthur *et al.*, 1973; Harper, 1977; Silvertown, 1985). In species that germinate in a single season, both viability and fecundity selection generally favour earlier germination, however, as demonstrated by both Kalisz (1986) and Biere (1991), there is significant temporal and spatial heterogeneity in the direction and intensity of selection. Recent theory demonstrated that genetic trade-offs (antagonistic pleiotropy) between viability and fecundity fitness components can maintain genetic variation in a population (Brown & Kelly, 2018). Their model also indicated that spatial heterogeneity in resources can maintain variation and obscure the negative genetic correlations between fitness components (Brown & Kelly, 2018). This occurs because microclimate variation changes the potential growth rate (and therefore the vigour) of plants and obscures the correlation between traits (Van Noordwijk & De Jong, 1986).

The research here is motivated by the observation that, within an annual population of *Mimulus guttatus* (synonym: *Erythranthe guttata*), seedlings germinate in both the fall and spring (Fig. 1a). Therefore, two life cycles coexist in the population: plants that germinate with fall rains and overwinter as a vegetative rosette (winter annual) and plants that overwinter as a seed and germinate in the spring following snowmelt and warming (spring annual). For both life cycles, flowering occurs in spring followed by seed ripening and death with the onset of summer drought. Similar variation in life cycle has been demonstrated in *Arabidopsis thaliana*, mostly segregating between populations, but there is also some evidence for polymorphic strategies within populations (Kronholm *et al.*, 2012; Montesinos-Navarro *et al.*, 2012; Burghardt *et al.*, 2016). However, unlike *A. thaliana*, there is no clear geographic pattern associated with dormancy in *M. guttatus* (Rubin & Friedman, 2018), suggesting that small-scale environmental heterogeneity might be important in maintaining strategies.

When considering selection on the timing of events, it is crucial to recognise that phenological events are part of an integrated life cycle (Ehrlén, 2015). For annual plants that complete their entire life cycle within a single year and have only one chance at reproduction, there is strong selection on timing flowering to coincide with optimal environmental conditions (Rathcke & Lacey, 1985; Forrest & Miller-Rushing, 2010). Even if selection is acting on the timing of eventual flowering, this timing may be driven by decisions earlier in an organism's life cycle, and feedbacks may exist between life stages (Donohue *et al.*, 2010; Ehrlén, 2015). For example, the timing of seed germination can affect flowering time (Donohue, 2002) and fecundity (Kalisz, 1986; Galen & Stanton, 1991; Purrington & Schmitt, 1998), which subsequently affects the maternal environment of seed maturation (Galloway & Burgess, 2009; de Casas *et al.*, 2012), which then feeds back into the degree of seed dormancy (Roach & Wulff, 1987; Kendall & Penfield, 2012; Postma & Ågren, 2016), with strong maternal effects and transgenerational plasticity (Galloway & Etterson, 2007; Wadgymar *et al.*, 2018a). Therefore, examining covariation between traits, including those at different developmental stages, can be important for understanding selection. However, in part because of the challenges of studying germination in nature, few studies have considered the heritability of variable germination schedules under environmental variation, and the consequences on plant growth and fecundity through genetic covariation and/or plastic responses.

Here, we test the hypothesis that flexible germination timing enables plants to cope with environmental heterogeneity, and imposes variable flowering strategies. Any response to selection on germination timing or correlated traits will depend on the occurrence of genotype × environment interactions and the presence of genetic correlations with other traits that are under simultaneous selection. Therefore our hypothesis (above) generates several predictions. First, germination timing will be driven by environmental variation, that is families contain seeds that germinate equally well in both seasons, or sets of seed that are capable of germinating in fall or spring (i.e. evidence of bet hedging within families). An alternative to this scenario is that families differ in their propensity to germinate in the fall or spring (i.e. there is genetic variation in dormancy). Second, plants compensate for different growing season lengths (a consequence of different emergence times) through other correlated traits, including growth and flowering time, so that adaptive phenotypic plasticity results in similar reproductive success across seasonal cohorts. An alternative to this is that fall-germinating plants, which experience a longer growing season, have higher fecundity. Studying the role of environmental heterogeneity in maintaining polymorphic life-history strategies, and the relations between key life-

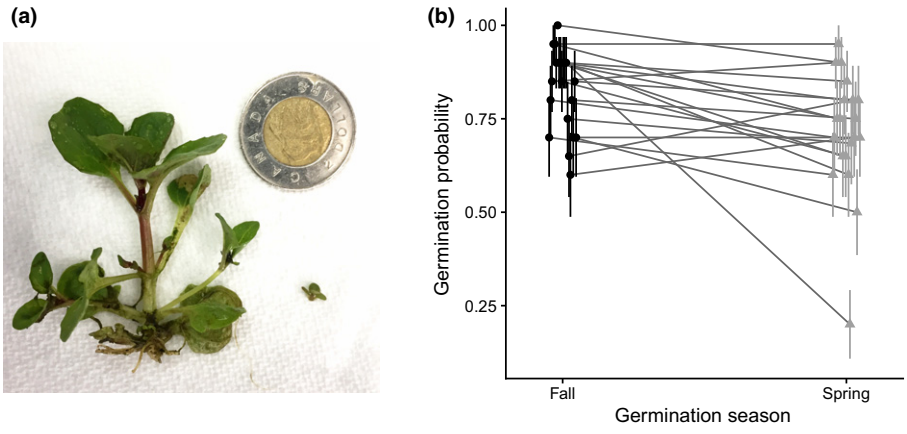

Fig. 1 (a) Photograph from the field (*Mimulus guttatus* population SKZ, British Columbia, Canada) showing a plant that germinated in the fall (left) and a seedling that germinated in the spring (right). Canadian two-dollar coin shown for scale. Photograph taken on 4 April 2015. (b) Germination probability by season for plants grown in a growth chamber in simulated natural conditions. Points represent family means with SE.

stage transitions, helps us better assess the potential for plants to modify their life-cycle phenology under climate change.

Materials and Methods

We used the seep monkey flower *Mimulus guttatus* (DC.; synonym: *Erythranthe guttata* (Fisch. ex DC.) G.L.Nesom), which is an hermaphroditic herbaceous plant that is widely distributed across western North America. Annual populations reproduce exclusively via seeds, by which plants make showy bee-pollinated flowers that can each contain hundreds of ovules that develop into seeds. Previous research on 23 populations of *M. guttatus* demonstrated that germination occurs with or without stratification and suggests that both annuals and perennials can germinate in either the fall or spring (Rubin & Friedman, 2018). For this study, we used a single annual population (SKZ) located near Skutz Falls in Cowichan River Provincial Park, Vancouver Island, British Columbia, Canada (48°46′955″N, 123°57′173″W). In this population, we have observed that seedlings germinate in both the fall and spring, timed with the onset of the fall rainy season or spring snowmelt/warmth (M. J. Rubin, pers. obs.; Fig. 1a). In June 2015, we collected open-pollinated seed from 20 plants to use in this study.

We simulated the natural environment for this population in a walk-in growth chamber (Environmental Growth Chambers, Chagrin Falls, OH, USA) using ControlNet software, Environmental Growth Chambers, Chagrin Falls, OH, USA, which allows for dynamic daily programmes that vary across days. We downloaded the hourly local temperature and humidity data for 3 yr (2014, 2015, and 2016) from http://climate.weather.gc.ca for the weather station closest to the SKZ population (North Cowichan station, Climate ID: 1015630, WMO ID: 71927) located at 48°49′27.010″N, 123°43′08.009″W. We generated hourly averages for temperature and humidity across the 3 yr and used the average value to create a unique program for each day that corresponded to 1 October to 7 July. We obtained the day length for each day based on the latitude of the population and timing of sunset and sunrise. For each daily cycle, we programed 10 steps, six of the steps occurred at regular 4-h intervals and the remaining four steps occurred at sunrise, 30-min post-sunrise, 30-min pre-sunset and sunset. We selected the ramping option between all steps which allowed temperature and humidity to gradually increase or decrease (rather than rapid changes at the onset of each step). During the first and last 30 min of each day, we set only 50% of the lights on to simulate twilight. To coarsely simulate variation in light intensity across the year, we used all growth lights for days that corresponded to 1 October through to 31 October and 6 March through to 12 July. For days that corresponded to 1 November to 15 March, we used 50% of the lights, except for the weeks corresponding to 1 January to 28 February, we used 0% lights to imitate snow cover at the site. For practical reasons, we accelerated the timeframe between 1 November to 28 February by having one experiment day correspond to the average of four calendar days. The simulated experiment ran from calendar days 1 October to 7 July (279 d) but, because of our accelerated winter, our experiment ran for 195 d.

We planted two germination cohorts that corresponded to the two timeframes in which germination occurs in this population – in early October (fall) and early March (spring). For each germination cohort, we planted single seeds from 20 siblings from each of 20 families ($n_{season} = 400$) in separate 6-cm pots filled with moist Fafard 4P growing mix (Sun Gro Horticulture, Agawam, MA, USA). Pots were placed randomly into 13 flats (treated as a blocking factor in analyses). We sieved the top layer of soil (7-mm sieve) to remove large particulates to standardise germination environment. To simulate overwintering as a seed, we cold stratified the spring cohort seed at 4.0°C in the dark for 14 d, which corresponded with the time when the chamber was at 0% light. For both germination cohorts, we misted flats twice daily, and we bottom-watered flats for 1 h d^{-1} for the duration of the experiment until the onset of summer drought. At this point, all watering ceased. Plants were not fertilised for the duration of the experiment. To ensure adequate sample sizes for assessing germination, we used double the number of individuals for this stage, and following germination of the spring cohort we randomly culled pots to 10 replicates per family from each cohort.

We observed plants daily, and recorded the date of germination (the day when green from cotyledons was first visible), date of flowering (the day the first flower was fully open) and senescence (the day the last flower withered). We use flowering to refer to the calendar day of first flowering and flowering duration is calculated as the number of days between first flowering and senescence. On the day of first flower, we counted the number of leaves and measured the length of one leaf on the second developmental node. At senescence, we counted the total number of flowers as an estimate of fecundity.

To assess whether plants within and between cohorts differed in the viability of seed – an additional measure of fitness – we conducted a set of crosses when plants from the fall and spring cohorts were flowering. In addition, because parent environments were controlled and their life cycle known, the offspring generation afforded us an opportunity to better assess genetic and residual variance for germination, which could represent genetic variation and/or diversifying strategies. We performed 40 independent crosses from each of the following categories: fall ♀ × fall ♂, spring ♀ × spring ♂, fall ♀ × spring ♂, spring ♀ × fall ♂ ($n = 160$). To avoid inadvertent pollination, we used only newly opened flowers, we emasculated these and we separated plants after pollination. When seed were almost dehiscing, we collected the seed pods and kept them in coin envelopes in a controlled incubator set to 21°C. We randomly selected 15 full-sib families from each of the four categories (60 families total) and aliquoted six pools of 15 seed from each. We used three of the replicate pools of seed as is (fall germination), and for the other three replicates we cold-treated seed for 15-d at 4°C in the dark to simulate overwintering (spring germination). We then planted the replicate pools of seed ($n = 5400$) on Petri plates containing 0.5% agar and we placed them in a growth chamber set to 11.5 h of light day-1 with a daytime temperature of 15.9°C and nighttime temperature of 5.9°C. These conditions represented the average temperature and day length of the time of year when the parent seed germinated. Petri plates were divided into fourths,

and contained seed from four families. At the same time each day, we recorded the number of seed that germinated, and calculated the probability of germinating for each batch.

Data analysis

We determined the least-squares means and examined the effect of seasonal germination cohort on each measured trait separately using a mixed model analysis (SAS PROC GLIMMIX for germination probability with a binomial distribution and logic link function, and SAS PROC MIXED for the remaining traits: SAS Institute Inc., Cary, NC, USA). Before analysis, we square-root transformed the data for variables that are counts (leaf number, branch number, flower number) and, for ease of interpretation, we present back-transformed estimates. The models included germination season as a fixed effect and family, family × season, and block as random effects. For each trait, we estimated genetic variance among maternal families (V_G) and residual variance (V_R) using restricted maximum likelihood. To test the statistical significance of random effects, we compared the −2 Res Log-Likelihood of models with or without the random effect of interest. A significant family × season interaction could have three different sources. It could arise due to imperfect correlation of family means across environments (i.e. crossing reaction norms), or because variances among families are unequal in the two environments, or, finally, because residual variances are unequal between the two environments (Fry, 2004). We fitted models allowing both among- and within-family variances to differ between the two environments and compared the unconstrained model with constrained models to identify terms. For each model, we assessed significance using likelihood ratio tests. In addition, we estimate the proportion of the family × season (G × E) variance that is explained by crossing reaction norms vs changes in genetic variance between seasons using Cockerhams's equation (Cockerham, 1963; Johnson, 2007):

$$\sigma^2_{GEI} = \frac{\sum_{i=1}^{h}\sum_{j=1}^{h}[2\sigma_i\sigma_j(1-r_{ij}) + (\sigma_i - \sigma_j)^2]}{h(h-1)},$$

where σ_i and σ_j are the square-root of genetic variance in treatments i and j, r_{ij} is the genetic correlation between treatments and h the number of treatments. The first half of the equation is variance due to imperfect genetic correlation between treatments and the latter half is the magnitude of the difference in genetic variance between treatments.

We estimated the genetic variance by multiplying the family variance component by the inverse of the expected relatedness of sibling offspring (Lynch & Walsh, 1998). Because we used open-pollinated seed, we estimated the relatedness of offspring using previous studies of mating in *M. guttatus*. Therefore we assumed that 40% of seed were selfed and 60% outcrossed, and that one-third of the outcrossed seed had shared paternity (Ritland & Ritland, 1989; Dudash & Ritland, 1991; Willis, 1993; Ivey & Carr, 2005). This resulted in a calculated estimate of genetic variance as 2.5 times the family variance component (i.e. 40% selfed, 40% half-sibs, 20% full-sibs, $r = 0.4$; Rubin *et al.* 2019). We then calculated broad-sense heritability (H^2) as the estimated genetic variance divided by the total phenotypic variance (Lynch & Walsh, 1998).

The estimates of genetic variance are based on seed from field-collected maternal families and therefore maternal environmental effects could inflate the estimates. One mechanism to account for this is to test for an association between seed size – which can reflect maternal provisioning – and traits expressed later in life. Therefore we measured the area of a random sample of field-collected seed for each of the 20 maternal families included in the experiment before germination. For each maternal family, we imaged 46–130 seed (mean: 73) by placing the seed onto a sheet of white paper and taking an overhead image. We used IMAGEJ software to estimate the mean seed size (area) for each sample from the images. The mean seed size across families ranged from 0.09 to 0.19 mm^2. For each seasonal cohort, we calculated correlation coefficients using family means between seed size and germination day, probability, leaf length, leaf number, branch number, flowering time, flowering duration and total flower number. Despite the large variation in seed size, in both cohorts there were no significant correlations between seed size and any of the traits we measured. This suggests that maternal provisioning is unlikely to strongly influence measures of growth, phenology and fecundity across seasonal cohorts.

We were interested in genetic correlations among traits, as well as more complex models of trait interactions. Therefore, to assess whether patterns of variation among traits differed across the seasons we used two analytical approaches. First we estimated pairwise phenotypic and genetic Pearson correlation coefficients between traits, in each season independently (PROC CORR in SAS). The genetic correlations used family means based on best linear unbiased predictors (BLUP), which were calculated using generalised linear mixed models for each trait and season separately. Next, to examine more complicated interactions, we used structural equation modelling (SEM) using AMOS 17.0, IBM SPSS, Chicago, IL, USA.. We designed a saturated model that contained all biologically meaningful connections among traits. We controlled for a potential effect of number of experimentally pollinated flowers by including this as a covariate in the saturated path model. We permuted all possible models to identify the best-fit model based on the Bayesian Information Criterion (BIC) for each season separately using standardised phenotypic data. After identifying the best-fit model for each seasonal cohort, we tested the model fit with both the data from the same season and the data from the alternative season. The program AMOS uses a chi-squared test to determine if the data fitted the best-fit path model. We then tested the model fit for the spring or fall cohort best-fit model using the alternative cohort's data.

Results

Life cycle, growth and fecundity

The probability of germination was high in both fall and spring cohorts, with an 83% probability of germination in the fall, and 72% in the spring (Fig. 1b). The difference between the seasons

is largely driven by a single family that had *c.* 90% germination in the fall but only *c.* 20% germination in the spring (Fig. 1b; discussed below). For both germination cohorts the probability of flowering was 100% (data not shown). However, the timing of first flowering (i.e. the onset of flowering) differed significantly between the cohorts (Table 1). The fall cohort started flowering 17 d earlier than the spring cohort (Fig. 2). All plants overlapped in flowering, that is the earliest flowering plant in the fall cohort was still flowering when the last flower from the spring cohort began flowering. Plants continued flowering until their highly synchronous death, which was largely governed by the reduction in water availability (i.e. the simulated onset of drought; Fig. 2). Because of the difference in the start of flowering, but not the end of flowering, the fall plants had a significantly longer duration of flowering than the spring plants (fall mean ± SE: 60.73 ± 1.06; spring mean ± SE: 46.77 ± 0.57; Table 1).

In addition to the differences in phenology, the two cohorts differed in growth strategies (Table 1). Plants that germinated in the fall made significantly more leaves than spring plants (fall mean ± SE: 7.26 ± 0.34; spring mean ± SE: 4.18 ± 0.11) and slightly more branches (fall mean ± SE: 3.55 ± 0.21; spring mean ± SE: 3.10 ± 0.11), presumably because they had a longer growing season. By contrast, spring plants made significantly larger leaves than fall plants (fall mean ± SE: 18.10 ± 0.52; spring mean ± SE: 24.32 ± 0.35), suggesting adaptive phenotypic plasticity to compensate for the reduced growing season they experienced. Therefore, despite the large differences in flowering time, duration of flowering, and plant size, the fall and spring cohorts had the same average fecundity, measured as the total number of flowers (fall mean ± SE: 34.30 ± 1.36; spring mean ± SE: 31.89 ± 1.03; Table 1). In general, total flower number exhibited substantial phenotypic variation in both cohorts.

Genetic (co)variation and heritability

We detected significant genetic variation (family effect) for all traits (Table 1). We also identified a significant family × season interaction for all traits, except flower number. However, the cause of the interaction was not consistent across traits (Table 1; Supporting Information Table S1; Fig. 3). In most cases, unequal among family variances explained most of the G × E variance (Table 1) and crossing reaction norms (i.e. imperfect genetic correlations) were important for only some traits. Comparing V_G and V_R directly (Table S1) illustrates the unequal genetic variances across seasons, where fall-germinating plants have a much higher genetic variance and lower residual variance, and therefore higher heritability than spring-germinating plants (Table 2; Fig. 3).

Genetic correlations between traits change in the fall and spring cohorts, perhaps because of the lack of variation in the spring. In the fall cohort, there is a significant positive genetic correlation between leaf number and flowering time ($r_G = 0.84$***; Table S2), so that the latest flowering plants had the most leaves. In the fall, there was also a significant positive genetic correlation between leaf number and leaf length ($r_G = 0.60$**; Table S2), so that families that made the most leaves also made the largest leaves. By contrast, for spring plants there was no pattern for these pairs of traits, and overall there were very few significant genetic correlations.

Path analysis

To understand if the structure of trait covariation differed between the germination cohorts, we used SEM to identify a best-fit model for each cohort. We started with a saturated model (Fig. 4a) and produced a reduced best-fit model (Fig. 4b, c). For both seasonal cohorts, the data fit the reduced model (i.e. no significant difference between the data and the model: fall: $\chi^2_{15} = 11.5$, $P > 0.05$; spring $\chi^2_{14} = 11.5$, $P > 0.05$). In both the best-fit models, flower number was directly and positively affected by leaf length, branch number, and flowering duration (Table S3). There were several other trait associations shared between the two models: earlier germination resulted in greater leaf length, greater leaf length was associated with earlier flowering time, earlier flowering time was associated with longer flowering duration, and more leaves were associated with more branches. However, the models also differed in several ways, in particular the relations among traits related to growth and

Table 1 Results of mixed models evaluating the influence of season, block, genetic effects and their interactions on germination, growth and flowering traits in *Mimulus guttatus*.

	Season	Block	Family	Family × Season	Proportion of G × E due to crossing reaction norms	Proportion of G × E due to unequal among family variance
Germination proportion	**$F_{1,19} = 9.46$***	1.04	2.41	**6.35****	0.97	0.03
Germination day	**$F_{1,18.6} = 188.36$*****	0.3	**28*****	**29.8*****	0.14	0.86
Leaf length	**$F_{1,19.5} = 129.87$*****	2.2	**4***	**7*****	0.2	0.8
Leaf number	**$F_{1,18.9} = 106.41$*****	0.5	**46.4*****	**67.5*****	0.04	0.96
Branch number	**$F_{1,18.8} = 9.01$****	0.3	**30.4*****	**31.4*****	0.59	0.41
Flowering time	**$F_{1,20.3} = 362.31$*****	**10.4****	**28.4*****	**34*****	0.18	0.82
Flowering duration	**$F_{1,18.6} = 268.31$*****	**6.2****	**19.5*****	**21.3*****	0.36	0.64
Total flower number	$F_{1,18.7} = 4.12$	1.1	**27*****	1.3	0.85	0.15
Death date	$F_{1,11.6} = 0.21$	0.1	**8.7****	**8.7*****	0.99	0.01

F-values are shown for fixed effects (season), and chi-squared values are shown for random effects (block, family, family × season interaction). Significant values are indicated in bold with *P*-values represented as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

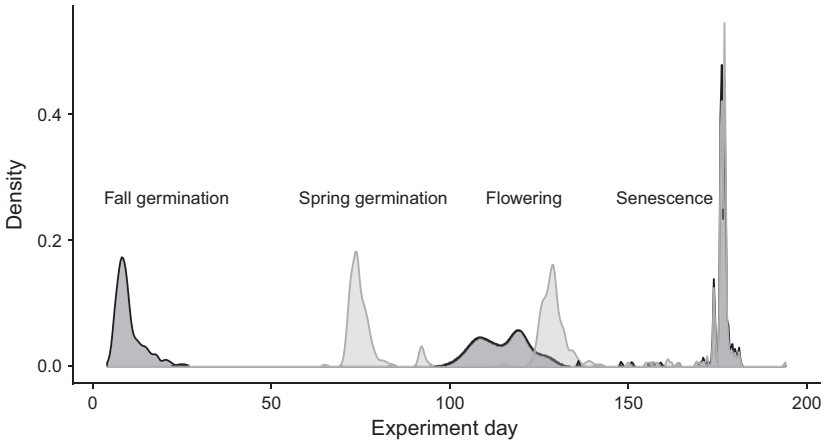

Fig. 2 Density plots showing the duration of the experiment with the distributions of germination, onset of flowering, and senescence, by seasonal cohort for *Mimulus guttatus*. The fall cohort is shown in dark grey and the spring cohort in light grey. The break between fall and spring germination represents winter.

development strategies (leaf number and leaf length, germination day and flowering time; Fig. 4, Table S3). When we permuted the data and best-fit models across germination cohorts, the data from one cohort did not fit the model from the alternative cohort (i.e. data were significantly different in the model: fall data with spring model: $\chi^2_{15} = 63.7$, $P < 0.001$; spring data with fall model: $\chi^2_{14} = 41.8$, $P < 0.001$), indicating that the structure of trait covariation differs across the fall and spring cohorts.

Offspring generation

When we examined germination in the next generation, we found no difference in germination probability among the different cross types, with or without cold exposure (Cross: $F_{3,52.9} = 1.41$, $P = 0.25$; Treatment × Cross: $F_{3,285} = 1.20$, $P = 0.31$), except for one family that had cold-induced dormancy (see next paragraph). All cross types also had similar germination timing (Cross: $F_{3,53} = 1.078$, $P = 0.36$), although exposure to cold accelerated germination (Treatment: $F_{1,287} = 133.61$, < 0.001, no Treatment × Cross interaction). Therefore, offspring from the two seasonal cohorts do not differ in their germination characteristics. However, we did find significant variation among families in their germination timing (No Cold: $\chi^2_1 = 12.1$, $P < 0.001$; Cold $\chi^2_1 = 21.9$, $P < 0.0001$), but not in their germination probability (No Cold: $\chi^2_1 = 0.2$, $P = 0.32$; Cold $\chi^2_1 = 1.5$, $P < 0.11$). When we examined variance components, it was evident that for germination probability there was both low genetic variance and high residual variance, leading to an absence of heritability (Table 2). For germination timing, genetic variance was higher, but there was still substantial residual variance (Table S1), especially considering the highly homogenous environments (Petri dishes with agar).

We discovered one family that had substantially reduced germination after cold exposure (i.e. cold-induced dormancy). Recall in the parent generation, one family had *c.* 20% germination in the spring but average germination in the fall (see Fig. 1b). From this family, two fall-germinating plants and two spring-germinating plants served as either maternal or paternal parents for the offspring generation. In three of these four offspring families, their seed germinated at 95% probability after both 0- and 15-d stratification. However, in the remaining one offspring family, only 4% seed germinated after 15-d stratification, but 100% seed germinated after 0-d stratification. These segregation ratios suggest that this family harboured a dominant allele for cold-induced dormancy. As expected, the offspring seed that displayed dormancy originated from a fall-germinating plant, because it is unlikely the two spring-germinating parents would carry the dormancy allele (or else they would not have germinated).

Discussion

In herbaceous plants, the timing of germination and flowering are critical phenological decisions, and yet few studies of natural populations have simultaneously investigated the maintenance of variation in both traits. Here we studied the mechanisms maintaining phenotypic and genetic variation in the joint timing of these life-history stages, and how they influence the entire life cycle, in a single annual population of *M. guttatus*. Although distinct cohorts of germination in the fall and spring exist in the population, families do not differ in their propensity to germinate in one season or the other. Instead, all families contained seed that could germinate in both seasons. Furthermore, we discovered significant plasticity in growth strategies that may help mitigate the consequences of different growing season lengths. We found that fall-germinating plants flowered significantly earlier; but the two cohorts did not differ in overall flower number (fecundity). Finally, there are considerable evolutionary implications of two cohorts coexisting in the population, since the spring cohort harbours very little genetic variance and low heritability. In the following section we discuss how these findings relate to the maintenance of alternative growth strategies and the efficiency of selection in heterogeneous environments.

Efficacy of selection

For all growth and reproductive traits, we found substantially less genetic variance and lower broad-sense heritability in the spring cohort compared with the fall cohort. Because of our experimental setup, the genetic composition of both cohorts is identical, so

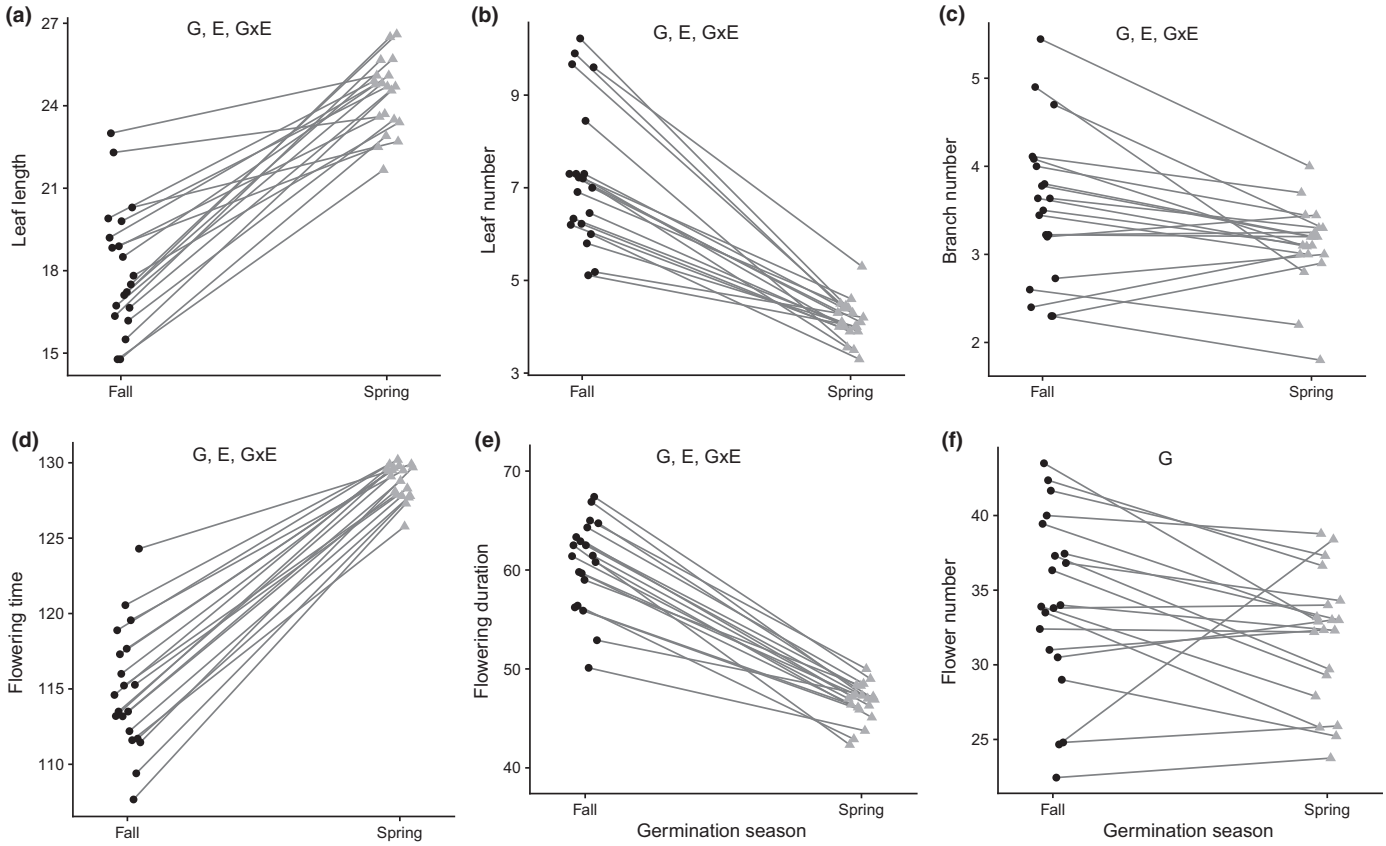

Fig. 3 Reaction norms illustrating growth (a–c) and reproductive (d–f) traits by germination season for *Mimulus guttatus*. Points and lines represent family means. The letters above each plot (G, E or G × E) indicate whether there are significant genotype (family), environment (germination season) or genotype-by-environment interaction effects influencing each trait, as indicated in Table 1.

that the reduction in genetic variance and heritability is wholly driven by the different environments experienced by the plants. It is well known that heritability estimates are specific to a particular environment (Falconer & Mackay, 1996). What was unique about our findings was that the environment experienced by a plant was driven by an earlier life-history trait, the probability of germination across seasons. However, apart from one family that had cold-induced dormancy, there was no significant plasticity among families in their propensity to germinate in the fall or the spring, and the variation within families was substantially greater than between families. Taken together, our results suggest that selection on the spring cohort will be ineffective in driving evolutionary change. The consequences of this for the population's ability to respond to selection will depend on the proportion of flowering plants that germinate in each season. Nonetheless, the lack of genetic variance and low heritability in the spring cohort may help explain the maintenance of phenotypic variation within the population.

Some of the best empirical research on the mechanisms maintaining genetic variation within a population comes from studies on the Iron Mountain (IM) population of *M. guttatus*. This work has demonstrated that environmental fluctuations in water availability determine growing season length, and impose variable selection on flowering time (Mojica *et al.*, 2012; Lee *et al.*, 2016; Troth *et al.*, 2018). In years with abundant moisture, large, late-flowering individuals are favoured through fecundity selection but, in more typical years with drought, smaller, earlier flowering plants are favoured. The result is that the population maintains allelic variation for flowering time and flower size. Interestingly, our results suggest an alternate (but not mutually exclusive) route to maintaining variation in flowering time. Within the fall cohort we found a positive genetic correlation between larger plants and late flowering (Table S2), but if we had been unaware of the seasonal difference in germination we would have detected an overall negative relation between plant size (leaf number) and flowering onset (Fig. 5). This is akin to studies that find that individuals that flower early are often larger than late-flowering individuals (Forrest, 2014), with one explanation being that unequal access to resources means that these individuals are in overall better condition (Ehrlén & Münzbergová, 2009). Indeed, the difference in germination destines plants to experience different growing season lengths, and allows plants to grow larger. Therefore, at least some of the covariance between flowering time and size is environmental and weakens any response to selection on flowering time (Rausher, 1992; Austen *et al.*, 2017).

Our calculations of heritability used an estimate of relatedness that reflected a mix of selfed and outcrossed seed based on data from other *M. guttatus* populations (Ritland & Ritland, 1989;

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Table 2 Least-squared means (± SE) and broad-sense estimates of heritability (H^2), for measured traits in two seasonal cohorts of *Mimulus guttatus*.

	Fall (LSMean ± SE)	Spring (LSMean ± SE)	H^2 Fall	H^2 Spring
Germination proportion	0.82 ± 0.05	0.74 ± 0.05	0.09	0.05
Germination day	10.05 ± 0.39	15.72 ± 0.41	0.4	0.16
Leaf length	18.1 ± 0.52	24.32 ± 0.35	0.35	0
Leaf number	7.26 ± 0.34	4.18 ± 0.11	0.9	0.11
Branch number	3.55 ± 0.2	3.10 ± 0.11	0.47	0.29
Flowering time	114.80 ± 0.94	128.81 ± 0.38	0.6	0.13
Flowering duration	60.73 ± 1.06	46.77 ± 0.57	0.5	0.08
Total flower number	34.30 ± 1.36	31.89 ± 1.03	0.48	0.29
Death date	175.57 ± 0.40	175.45 ± 0.43	0.16	0.21
Offspring				
Germination probability			0.01	0.2
Germination day			0.59	0.78

For the offspring generation, fall and spring were simulated as the presence of stratification (cold) or no stratification.

Dudash & Ritland, 1991; Willis, 1993; Ivey & Carr, 2005). There may be high variance in relatedness within and among populations, and here we may have either overestimated or underestimated the degree of selfing, which would change our estimates of heritability. An additional caveat is that we used flower number as a measure of fitness, which is not necessarily equivalent to fecundity, as flower number may not uniformly reflect final seed set. For example, even if flowers were visited equally by pollinators and received sufficient pollen for full seed set, plants may vary in their ability to provision seed. It is possible that fall plants could mature more seed because of their larger size. In addition, it is well established in annual populations of *M. guttatus* that later flowering plants often die from summer drought before they adequately provision their seed (Hall & Willis, 2006; Mojica *et al.*, 2012). Therefore, if drought sets in early, the spring-germinating plants will experience stronger negative selection because of their later flowering time. Another consideration is that in our experiment, total flower number for both cohorts was likely inflated compared with natural plants, because greater access to resources, a lack of competition, and the absence of pollination may have encouraged plants to continue making flowers at the expense of setting seed.

Plastic growth strategies

Among our most important findings was that total flower number was largely unaffected by germination season (Table 1). In addition, there was no significant G × E for this trait (Table 1) suggesting that all families harbour considerable developmental flexibility in growth strategies to maintain a set number of flowers, or reproductive homeostasis. A similar phenomenon was observed in an experiment with imposed drought, in which despite smaller plant size under drought conditions, plants had equivalent reproductive fitness (Heschel *et al.*, 2004). They showed that drought-stressed plants achieved equivalent reproductive success either through plasticity in water-use efficiency or in root biomass allocation. Additionally, Hughes & Simons (2014) found adaptive phenotypic plasticity for reproductive behaviour under constrained growing season length. In our study, we found that spring-germinating plants had substantially larger leaves than fall-germinating plants, despite their shorter growing season (Table 2; Fig. 3). Rapid leaf expansion may be one mechanism by which spring-germinating plants increase photosynthetic capacity to then invest resources in reproduction.

Plants can increase their photosynthetic capacity by expanding existing leaves and/or by making new leaves. Because of their shorter growing season, spring-germinating plants make a minimal number of leaves, but they are larger than in fall-germinating plants. This situation suggests that they are avoiding the construction costs of new leaves, and gaining photosynthetic capacity by expanding leaves. If season length is an index of resource availability, then we essentially find alternate investment strategies between leaf size and leaf number (Scott & Aarssen, 2012). Analogously, research on flower longevity shows that, under low resources, plants make fewer flowers and maintain these for longer, while under high resources, plants invest in more flowers (Spigler & Woodard, 2019). We found very high heritability for leaf number in the fall cohort, and strong genetic correlations between leaf number and other traits (Tables 2, S2); suggesting the potential to respond to selection with coordinated growth strategies. One of the consequences for differences in leaf number is the concomitant difference in node number and meristem availability (Scott & Aarssen, 2012; Baker *et al.*, 2014). Because they have more leaves, fall-germinating plants have more meristems that could become reproductive, but they remain dormant, and branch number is only slightly higher (Table 1). By contrast, spring-germinating plants on average develop reproductive stems from all possible nodes. Therefore, more leaves in the fall-germinating plants are not facilitating more reproductive branches or greater fecundity. Therefore it appears that the value of a leaf varies, perhaps because leaves made before winter are older and have lower photosynthetic activity, or they are at greater risk of damage and herbivory (Harper, 1989).

Our experiment simulated natural abiotic conditions, but we were missing selective pressures caused by biotic agents. Exposure to herbivores exerted significant selection via survival in a field experiment, particularly on annual plants (Popovic & Lowry, 2019). In the population we studied, fall-

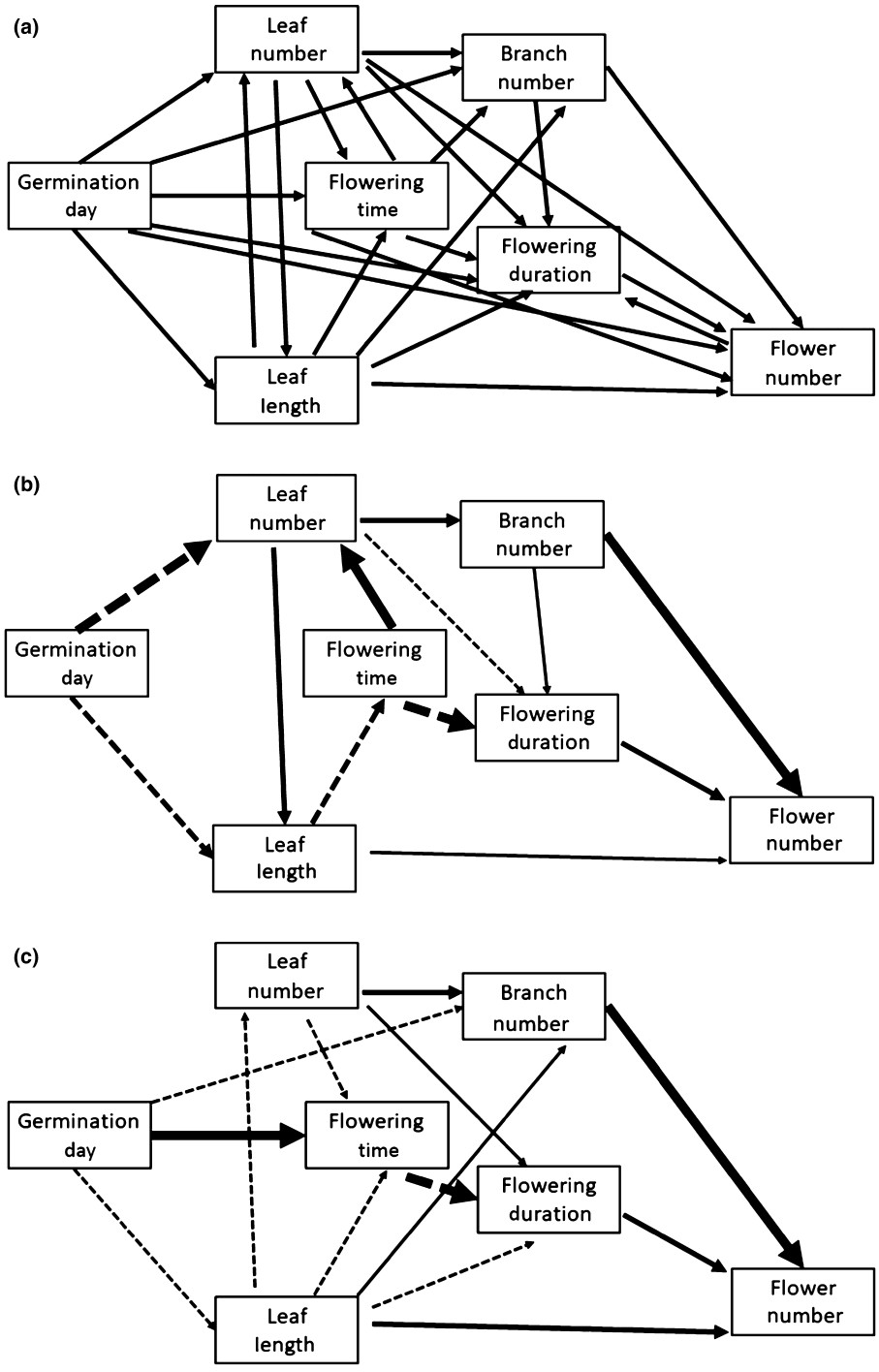

Fig. 4 Saturated path model showing all possible trait interactions (a) and reduced, best-fit path model for the fall (b) and spring (c) germination cohorts in *Mimulus guttatus*. Single-sided arrows indicate hypothesised directionality of the effect. Solid lines indicate a positive coefficient between the two traits and dashed lines indicate a negative coefficient. The width of the line represents the magnitude of the coefficient grouped into three classes: 0.00–0.20 and 0.00 to −0.20 are shown in the thinnest line, 0.21–0.44 and −0.21 to −0.44 in the medium line and >0.45 and < −0.45 in the thickest line.

germinating plants may have greater exposure to herbivores and pathogens. In a common garden field experiment located *c.* 100 km from this site, plants experienced substantial herbivory from slugs in early spring (M. J. Rubin, pers. obs.). Promiscuous germination might mitigate the costs associated with exposure to pathogens and herbivores over winter and in early spring. Additionally, in populations with short growing seasons, decreased allocation to herbivore resistance is part of a rapid growth strategy (Kooyers *et al.*, 2017). Because of their longer experienced growing season, fall-germinating plants may increase allocation to defence, which then comes at the cost of rapid growth, as indicated by their smaller leaf size compared with spring plants. In *Arabidopsis*, a variety of stress response genes, including defence-related genes, were upregulated during the processes of acclimation and de-acclimation to cold (Kuwabara & Imai, 2009; Miki *et al.*, 2019). Future research that compares allocation to defence in fall and spring-germinating plants, and the implications of this for growth and reproduction, will clarify the role of biotic selection pressures.

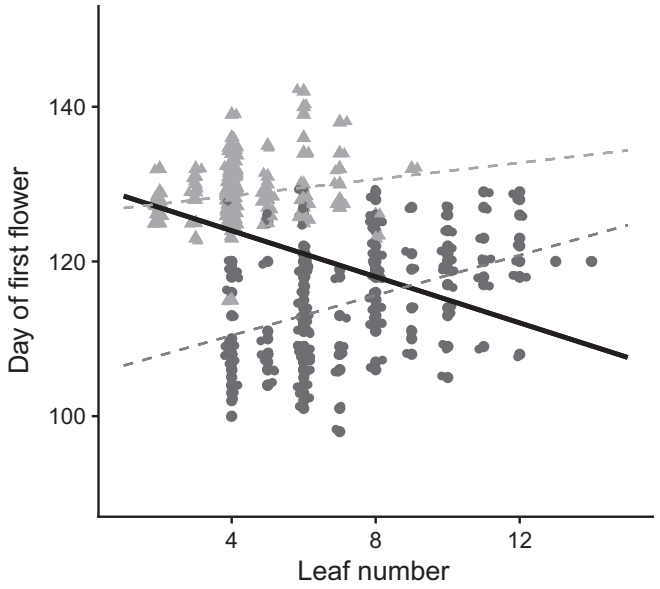

Fig. 5 Scatterplot of the relation between leaf number and day of first flower for individual plants of *Mimulus guttatus*. The black circles are plants that germinated in the fall and the grey triangles are plants that germinated in the spring. The dotted lines indicate the relation between the traits within each cohort (fall: $y = 1.29x + 105.25$, $t_{344} = 8.11$, $P < 0.001$; spring: $y = 0.53x + 126.37$, $t_{372} = 1.77$, $P > 0.05$). The thick black line shows the relation that would be observed if germination season were unknown ($y = -1.49x + 129.94$, $t_{375} = 8.19$, $P < 0.001$).

Annual plants in heterogeneous environments

Our results indicated that plants in this population were capable of producing seed that could germinate in both the fall and spring. Explaining this strategy implies that germination flexibility is advantageous compared with producing seeds that germinate predominantly in one season or the other. The benefit of a promiscuous strategy lies in the relative risks of germinating in the fall and dying during a severe winter, balanced with the rewards for germinating in the fall, and establishing and growing before spring germination. Similar results were found for annual poppies (*Paper dubium*: Arthur *et al.*, 1973), in which fall germinators have a fecundity advantage over spring germinators in some years, but in other years spring germinators have a survival advantage. Therefore there is no long-term advantage of germinating in one season or the other, and plants produce seed that can germinate in either season.

Our experiment was unable to clarify the mechanism underlying seasonal germination probabilities. The uniformly high germination across treatments in the offspring generation (except for one family which expresses cold-induced dormancy) argues against seed pods containing a mix of seed that germinate in either the fall or the spring. Following a period of after-ripening, all seed can germinate immediately with exposure to fall conditions and available water, unlike many species with classic bet hedging strategies (Clauss & Venable, 2000; Venable, 2007). The question, then, is why we observe a cohort of spring-germinating seed in the field? It is possible that we erased the signature of diversifying germination in the offspring generation by creating an environment that was too permissive (agar in Petri dishes). In the parent generation in which seed germinated on soil, the probability of germination ranged from 60% to 95%. Diversified germination strategies could be achieved by extreme sensitivity of seeds to their environment (Childs *et al.*, 2010), and microgradients within apparently homogeneous growth chambers caused over 30% of the variation in the timing of germination (Simons & Johnston, 2006). The microtopography of soil (Oomes & Elberse, 1976) and seed orientation (Sheldon, 1974) can substantially influence germination. If seeds differ in their sensitivity then the heterogeneity of soil might be an important component to maintaining variation in germination characteristics. Further work is necessary to distinguish this from truly random environmental variation, in which spring-germinating plants are an arbitrary set of seed that lacked exposure to water in the fall.

Germination and flowering time in annual plants are two life-history strategies that have received substantial attention because of their intimate association with fitness. In general, germinating (i.e. nondormancy) and flowering later are both riskier, but more rewarding, strategies. In models of the coevolution of seed dormancy (germination probability) and flowering time in an annual plant, the optima for the two characteristics are interdependent (Ritland, 1983). In particular, a higher value in one characteristic favours a compensating lower optimal value in the other, due to a compromise between risks and expectations over the entire life cycle. Although there was not strict dormancy in our population, fall germination was likely the riskier, but more rewarding strategy, compared with spring germination. In support of Ritland's model, plants that germinated in the fall flowered significantly earlier than spring-germinating plants, suggesting that the risks were balanced.

Our research showed that fall- and spring-germinating cohorts differed in subsequent growth and phenology, suggesting alternate life-cycle strategies. Intriguing new evidence in support of this comes from recent work in the IM population of *M. guttatus*, which also contains both fall- and spring-germinating cohorts (Mojica & Kelly, 2010). Nelson *et al.* (2018) sequenced the DNA of pools of fall and spring seedlings, and showed that the groups differed in copy number variation of a tRNA ligase gene, with the spring cohort carrying more copies (*high*+ category). Therefore, unlike our study, they found evidence for cohort differences suggestive of genetic differences underlying germination strategies. It is possible that diverse mechanisms underlie observed variation in seasonal germination among populations, as is the case for *A. thaliana* (Montesinos-Navarro *et al.*, 2012). However, in accordance with our findings, Nelson *et al.* (2018) showed with a different set of experimental plants, that genotypes of the *high*+ category were selected against in dry years that favoured early flowering, but favoured in years with a longer growing season. These results are similar to our finding that spring-germinating plants flowered significantly later than fall-germinating plants, and suggest strong coordination between life stages. In addition to temporal fluctuations in the end date of the growing season, there may also be significant fluctuations in the

start date of the season, both of which may act to maintain variation in life-history traits.

Both germination and flowering phenology are an integrated part of an organism's life history, and, by changing the timing of different events within the life cycle, plants can exert some control over the effects of their local environment. Phenology has received substantial attention in recent years due to the role that climate change has on phenological responses and the risks imposed by phenological mismatch (e.g. Miller-Rushing *et al.* 2010; Johansson *et al.*, 2013; Wadgymar *et al.*, 2018b). In our study, the significant effect of seasonal cohort (i.e. germination time) on flowering time and growth strategies, but not fecundity, suggest that this population has at least some potential to buffer against changes in the start of the growing season caused by environmental variation. The fall and spring cohort are subject to opposing risks – frost damage and/or overwinter survival for the fall cohort vs early onset of drought for the later flowering spring cohort – and so the long-term response will be determined by the frequency of these events. Selection for earlier flowering under climate change has been well documented (Parmesan & Yohe, 2003; Giménez-Benavides *et al.*, 2011; Anderson *et al.*, 2012), and it is likely that other aspects of life history are under correlated selection, including the timing of germination. We suggest that future research includes examining selection acting on the start of the growing season, coordination between life stages, and the effect on the life cycle as a whole.

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Author contributions

JF and MJR conceived of and designed the study. TEM and MJR performed the research. JF and MJR conducted statistical analyses. JF, TEM and MJR interpreted the data, and JF and MJR wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Table S1 Genetic variance (V_g), residual variance (V_r), and chi-squared values for log-likelihood tests for measured traits in two seasonal cohorts of *Mimulus guttatus*.

Table S2 Phenotypic correlations and family-level correlations for germination, growth, and flowering traits measured in *Mimulus guttatus* in two germination cohorts.

Table S3 Partial regression coefficients for each significant path for the fall and spring seasonal germination cohort in *Mimulus guttatus*.

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