Genetic variation in phenology of wild *Arabidopsis thaliana* plants

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Author Contribution

JRL conceived of the project with TEJ, DLDM assisted in experiments, VLD conducted experiments, and analyzed data, VLD and JRL led writing, all authors contributed to the interpretation and writing of the manuscript.

Data Accessibility Statement

Original data will be included as supplemental tables. Prior studies from which phenology data was sourced will be listed in a supplemental table.

Abstract

Phenology and the timing of development are often under selection, but at the same time influence selection on other traits by controlling how traits are expressed across seasons. Plants often exhibit high natural genetic variation in phenology when grown in controlled environments, and many genetic and molecular mechanisms underlying phenology have been dissected. There remains considerable diversity of germination and flowering time within populations in the wild and the contribution of genetics to phenological variation of wild plants is largely unknown. We obtained collection dates of naturally inbred *Arabidopsis thaliana* accessions from nature and compared them to experimental data on the descendant inbred lines that we synthesized from two new and 155 published controlled experiments. We tested whether the genetic variation in flowering and germination timing from experiments predicted the phenology of the same inbred lines in nature. We found that genetic variation in phenology from controlled experiments significantly, but weakly, predicts day of collection from the wild, even when measuring collection date with accumulated photothermal units. We found that experimental flowering time breeding values were correlated to wild flowering time at location of origin estimated from herbarium collections. However, local variation in collection dates within a region was not explained by genetic variation in experiments, suggesting high plasticity across small-scale environmental gradients. This apparent low heritability in natural populations may suggest strong selection or many generations are required for phenological adaptation and the emergence of genetic clines in phenology.

Introduction

Phenology, or the timing of the developmental transitions between an organism's life stages, directly and indirectly influences plant fitness and selection by determining the traits that are expressed at any point throughout the year (Donohue 2005). In seasonally variable environments, traits such as flowering time, growth rate, and dormancy can ameliorate harsh abiotic conditions by timing dormant periods to coincide with unfavorable seasons (e.g. drought escape (Ludlow 1989; Lawrence-Paul and Lasky 2024). Phenology may be under distinct selective pressures to maximize growth and resource acquisition during favorable conditions and reduce the risk of experiencing unfavorable conditions during sensitive growth stages, potentially leading to fitness tradeoffs between fast growing, resource acquisitive organisms and slow growing, resource conservative organisms (Stearns 1989; Franco and Silvertown 1996; Reich 2014; Salguero-Gómez et al. 2016). Phenology is determined by both endogenous and external cues, i.e. genetic and plastic variation (Amasino 2004; Andrés and Coupland 2012; Auge et al. 2018) but their relative importance in nature is unclear even in model plants.

A large portion of knowledge about the genetic basis for plant phenology comes from study of the model *Arabidopsis thaliana* (hereafter Arabidopsis). Common garden trials in both lab and field settings of inbred lines have uncovered genetic loci and genotype by environment interactions contributing to much of the observed variation in dormancy and flowering time (Juenger et al., 2005; Brachi et al., 2010; Fournier-Level et al., 2013; Ågren et al., 2017). Flowering time and dormancy are determined by complex, overlapping gene networks (Simpson and Dean 2002; Wilczek et al. 2010). These traits are also plastic (Juenger et al. 2005; Zhou et al. 2005; Wilczek et al. 2009): germination responds to temperature, photoperiod, moisture, and nutrient availability (Huang et al. 2010, 2018; Penfield and Springthorpe 2012; Footitt et al. 2013; Kenney et al. 2014), while flowering responds to multiple cues such as temperature and daylength (Thomas and Vince-Prue 1997; Lempe et al. 2005; Balasubramanian et al. 2006). The weight of environmental cues in phenological timing contributes to cascading effects on later phenological stages (Donohue 2005), where, for example, the timing of flowering within the year determines seed maturation environment and thus dormancy (Chiang et al. 2013; Springthorpe and Penfield 2015). In turn, germination timing can influence the expression of flowering time

by determining the seasonal environment during growth (Zhou et al. 2005; Li et al. 2010; Chiang et al. 2013).

The broad phenological variation described in experiments is observed in wild populations as well (Ratcliffe 1976; Simpson and Dean 2002; DeLeo et al. 2020). Arabidopsis can exhibit the life history of a winter annual, germinating in the fall, spending the winter as a rosette, and flowering in the spring. However, Arabidopsis can also germinate and flower in a single season. These shorter-lived plants can flower in spring, summer, or fall, and in some regions this fast life cycle enables multiple generations to complete within a year. This variation in life histories occurs in many annual plants (Baskin and Baskin 1988) and is made possible in part by a range of flowering times and germination traits that can vary independently from each other (Marcer et al. 2018; Martínez-Berdeja et al. 2020). Broad geographic clines in breeding values for components of phenology and allele frequencies of phenology QTL as well as evidence of selection on phenology QTL (Caicedo et al. 2004; Stinchcombe et al. 2004; Samis et al. 2012; Debieu et al. 2013; Fournier-Level et al. 2013; Ågren et al. 2017; Exposito-Alonso et al. 2018; Gamba et al. 2023; Lasky et al. 2024) support the conclusion that phenology is adapted to local environmental conditions in the wild. Yet, even within geographic proximity one can find genotypes with substantial genetic variation in flowering time (Alonso-Blanco et al. 2016; Méndez-Vigo et al. 2022).

It is not known to what degree genetic variation explains the phenology of wild Arabidopsis individuals, and there are several reasons to expect the phenology of a given genotype in experiments versus nature to be different (Wilczek et al. 2009). While genotype likely influences the phenology of an individual plant in the wild, interactions between stages as well as plasticity may limit the translation of genetic values of single stage phenology to natural phenology. First, there are interactions between germination and flowering time, both because of a shared genetic basis and because the timing of early life transitions influences environmental exposures in later life stages (Chiang et al. 2009; Huang et al. 2010; Springthorpe and Penfield 2015; Huo et al. 2016). Secondly, there is stochasticity in individual germination and flowering time (Jimenez-Gomez et al. 2011; Abley et al. 2020). Thirdly, spatial environmental variation is extensive in nature and genotype-environment interactions have major impacts on phenology. Maternal effects are an important source of plasticity especially for seed dormancy (Boyd et al. 2007; Chiang et al. 2013; Burghardt et al. 2016; Huang et al. 2018) and there is often variation in

phenology even under tightly controlled growing conditions. For these reasons, flowering time loci identified in common garden lab or field experiments might fail to predict flowering time variation among natural individuals (Chiang et al. 2013), possibly due to additional variation in dormancy (Huang et al. 2010; Chiang et al. 2013) and genotype-environment interactions across environmental gradients (Wilczek et al. 2009; Brachi et al. 2010a; Burghardt et al. 2015; Springthorpe and Penfield 2015). Genotypes differing in phenology in one set of conditions can have to the same expressed phenology in another set of conditions (Burghardt et al. 2016), and there is great diversity in germination and flowering date within populations and within genetic backgrounds (Brachi et al. 2013; Méndez-Vigo et al. 2013; Johnston and Bassel 2018; Abley et al. 2020).

The correspondence between genetic breeding values for phenological traits in controlled experiments and the phenology in nature of the same genotypes has been little studied owing to a lack of data. Here, we exploit measurements of wild phenology of individual plants from natural history collections (herbaria and seed stock centers) (Miller-Rushing et al. 2006; MacGillivray et al. 2010; Davis et al. 2015). Because Arabidopsis are naturally inbred, we can compare collection dates of wild individuals with phenology of nearly genetically identical descendants in controlled experiments. This comparison may provide a window into how genetic variation shapes phenology in natural populations against the forces of plasticity and GxE.

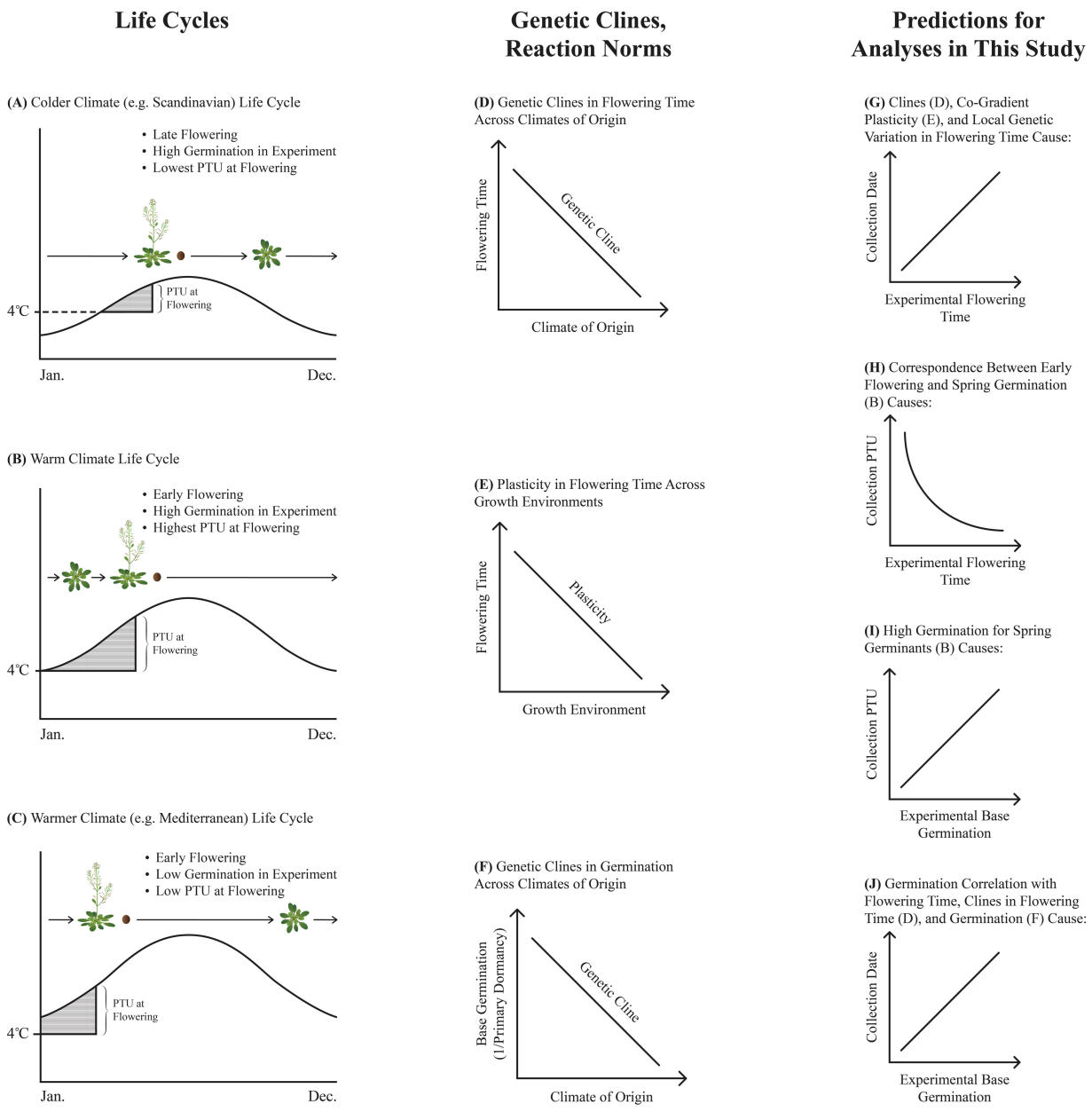


Figure 1*: Genotype and environment likely influence phenology of individual Arabidopsis plants in the wild. Genetics and environmental cues may determine how long a plant remains in vegetative growth (green) or how long a plant remains dormant as a seed (brown circles), with three representative life cycles shown from Scandinavia to the Mediterranean (A-C), with shaded areas showing accumulation of photothermal units (PTUs) in the growing season (e.g. temperature >4ºC). Existing knowledge of clines in flowering time and germination combined with plastic acceleration of flowering in warmer temperatures (D-F) lead to our predictions of*

143 *how flowering time and base germination rates in controlled experiments will correspond to*
144 *phenology in natural wild plants (G-J).*
145
146 We developed several hypotheses for how the phenology of individual plants in nature
147 would be related to genetic variation in phenological traits, elaborated in Figure 1. To
148 summarize, we expected that genetic clines in flowering time (early flowering in warmer
149 climates Figure 1D) and germination rates (Figure 1F), combined with plastic acceleration of
150 flowering in warmer conditions (Figure 1E) would lead to range-wide positive relationships
151 between breeding values for flowering time, germination rates, and collection dates (Figure 1G-
152 J). Locally, within populations, where much of the plasticity seen across the species range is
153 reduced, we expected genetic effects on flowering time would be positively related to collection
154 dates and that this relationship would be the most accurate signal of genetic effects on phenology
155 (Figure S1).
156
157 **Materials and Methods**

158 *Natural genotypes*

159 To complement published data (see below), we tested a set of 101 naturally inbred genotypes
160 ("ecotypes") with known collection date (either from the herbarium specimen label or recorded
161 in the Arabidopsis Biological Resource Center https://abrc.osu.edu/ database). 47 of these
162 ecotypes had not been studied in previous flowering time experiments and 75 had not been
163 included in previous germination experiments. Seed was ordered from ABRC (94 accessions) or
164 was germinated from herbarium sheets (7 accessions).
165 To improve germination, seeds from herbarium specimens were cold-stratified in tap
166 water at pH 7 and placed at 4°C for 7 d. Seeds were then directly sown into damp Fafard
167 germination mix and grown in Conviron growth chambers under 10/14hr, 18/22°C days/nights.
168 Seven herbarium accessions germinated using this protocol. In the second round of germination,
169 we surface sterilized seeds using standard protocols and then cold-stratified, as above. We plated
170 seeds onto MS+Gamborg's vitamins + agar plates containing 1% sucrose and 10uM GA4 and
171 then placed them in growth chambers, as above. Two more herbarium accessions flowered using

172 this protocol; these were transplanted to Fafard Germination mix. To induce flowering, plants
173 were exposed to 30d of 4°C with 10/18hr day/night cycles.
174
175 *Flowering and germination experiments*
176 Ecotypes were grown in common conditions prior to the flowering time experiment, and
177 flowering time replicates were descended from a single mother plant. For each ecotype, three
178 replicates were grown in separate pots. Seeds were stratified at 4°C for 5 days before sowing in
179 pots. Each pot was thinned to a single individual after the emergence of the second set of true
180 leaves. Plants were grown at 22°C under 16h days of fluorescent light in a walk-in Conviron
181 growth chamber (model MTPS). Day of bolting and day petals appeared were both recorded as
182 measures of flowering time.
183 Seeds from each replicate maternal plant in the flowering time experiment were collected
184 and stored separately in dry conditions until the germination trial. For each treatment, forty seeds
185 from each parent plant (or as many seeds as were available for replicates with low fecundity)
186 evenly divided across 2 plates were sown on filter paper in petri dishes and germinated at
187 23/18°C during day/night with constant 16h daylength in a Conviron growth chamber. In total,
188 1,752 plates and >50k seeds were assayed.
189 Seeds were subjected to cold stratification at 4°C in the dark for 3 treatment lengths: 2
190 weeks, 3 days, and 0 days. Cold stratification can break primary dormancy, however 2 weeks of
191 chilling can prompt secondary dormancy in the seedbank (Penfield and Springthorpe 2012). The
192 difference in germination rate between 3 days and 0 days of stratification can therefore indicate
193 primary dormancy while the difference in germination rate between 3 days and 2 weeks of
194 stratification may indicate secondary dormancy. As a caveat, lower germination after 2 weeks of
195 stratification could be due to other, unmeasured negative effects on germination, such as
196 bacterial infection. We staggered the planting so that all the plates came out of stratification on
197 the same day. The number of seeds that had germinated in each plate was recorded on days 1, 3,
198 5, 10, 14, 21, and 28. Seeds were considered germinated if the radicle was visible. Because
199 maternal plants flowered at different times, germination rates for this experiment may be
200 influenced by the length of the time between flowering and planting and any after-ripening that
201 may have occurred. We also tested for these maternal effects among individuals of the same
202 inbred line (see below).

203

204 *Phenology from published experiments*

205 We searched the literature for experiments on Arabidopsis that measured flowering time or
206 germination traits across different natural inbred lines (commonly referred to as Arabidopsis
207 "ecotypes"). The minimum number of ecotypes in any single experiment was 17. Our final set
208 used data from 38 previous studies (31 included some measure of flowering time, 15 included
209 germination) that, combined with our new experiments described above, included over 3,000
210 ecotypes for 86 flowering time experimental conditions and 66 germination experimental
211 conditions, although all ecotypes were used in only a subset of the trials and only 291 ecotypes
212 had a reliable date of original collection from the wild.

213 We used this dataset of phenology measurements from the literature to create an estimate
214 of genetic variation in flowering time and dormancy among ecotypes. We sought to gain
215 statistical power by combining data from different experiments. To make phenotypes
216 comparable, we standardized across treatments and experiments, transforming each flowering
217 time experiment such that the earliest flowering accession had a value of 0 and the latest
218 flowering accession had a value of 1.

219 We averaged standardized experimental flowering times across experiments, keeping
220 vernalized, non-vernalized, and field experiments separate. Here, we use 'vernalization' to
221 describe extended cold treatments applied to rosettes. Non-vernalized growing conditions
222 uncover genetic variation in flowering time due to vernalization requirements that is masked
223 under vernalized or fall-sown field conditions and may be important for determining phenology
224 and life history in the wild (Wilczek et al. 2009). However, Arabidopsis plants growing in many
225 natural settings are expected to experience changes in temperature and photoperiod that would be
226 more similar to vernalized and field experiments (Li et al. 2010). By keeping the three treatments
227 separate, we could test whether non-vernalized flowering time or vernalized flowering time was
228 more predictive of wild phenology. Field flowering time was aggregated across all seasons,
229 which may lead to less coherent estimates of field genetic flowering time if seasonal differences
230 lead to meaningful differences in flowering time, and also broken down further into spring (4
231 treatments over 2 experiments), summer (4 treatments, 1 experiment), and fall plantings (23
232 treatments, 9 experiments). To control for regional bias, this aggregation was performed both for

all experiments and without experiments that tested only ecotypes collected from a single country.

Data collection approaches for estimating germination rate and dormancy were more heterogeneous than for flowering time. Depending on the experiment, dormancy was reported as the number of days after planting until a set percentage of germination was reached, the percentage of seed germinated a given number of days after planting, the number of days of storage until a set percentage of germination, or the germination rate after a given number of days of storage. Because of the difference in metrics across experiments, dormancy values were standardized by rank within each experiment with zero indicating low to no dormancy and one indicating high dormancy. Measures that reported a percentage germinated were ranked in the opposite direction from measures that reported number of days until germination or the number of days of storage before 50% germination. It is reasonable to expect some relationship between different measures of dormancy (Ranal and De Santana 2006) and, indeed, we found that standardized rank-based metrics that increase with dormancy (such as days to 50% germination) were correlated with rank based on metrics that decrease with dormancy (such as percentage of seeds germinated after a set number of days, Pearson's $r = 0.485$).

These experiments captured variation in primary dormancy, or recalcitrance to germinate immediately after harvest. Secondary dormancy, or dormancy induced when a seed experiences conditions unfavorable to germination, has been studied in experiments that measured an increase in dormancy during storage. Primary and secondary dormancy could lead to different phenological and ecological outcomes (Martinez-Berdeja et al. 2020), so we averaged across experiments that measured primary or secondary dormancy separately to estimate each of these two traits. Secondary dormancy was not included in the Generalized Additive Models described below.

Wild phenology

The date of collection (for ecotypes maintained by ABRC) or collection date (for ecotypes grown from a known herbarium record) was used as an estimate of reproductive phenology of individuals in the wild (Primack et al. 2004; Davis et al. 2015). We excluded records from regions where Arabidopsis has been recently introduced, like North America and Japan. Accessions from outside the native range and or collected after the 320th day of the year (which

264 we deemed likely errors based on location, sometimes due to intentional plantings, and were
265 greater than 2 standard deviations from the mean) were removed. Our geographic limits also
266 excluded island accessions to the south of the Mediterranean, e.g. Cape Verde Island.
267 We hypothesized that genetic differences in wild phenology may be more apparent if we
268 account for spatiotemporal environmental fluctuations causing plasticity. Therefore, we
269 calculated photothermal units (PTUs) for each accession from the day of collection using
270 monthly climate time series data from CRU (Harris et al. 2014), beginning with January 1 of
271 each year, following the methods of (DeLeo et al. 2020) and (Burghardt et al. 2015). PTUs
272 integrate the temperature and light experienced by a plant at a given location through the
273 growing season and therefore may capture environmental cues relevant to phenology and better
274 describe genetic variation in development (Wilczek et al. 2009; Brachi et al. 2010). The models
275 described below use the square root of PTU because the resulting distribution was closer to
276 normal than the log transformation.
277
278 *Statistical comparison of collection date in the wild versus phenology in experiments*
279 We tested if genetic variation in normalized phenology measured on naturally inbred lines
280 explained variation in the wild phenology of the parent of the line (Figure 1 "Predictions"), using
281 linear regression between the normalized phenology breeding values (flowering time and
282 dormancy rank) and collection day. Vernalized, non-vernalized, and field flowering times were
283 modelled separately, because the genetic variation in phenology uncovered by each treatment
284 could relate to wild phenology in different ways. Under the hypothesis that vernalization and
285 field conditions better recreate environments a plant would expect at their geographic origin,
286 these genetic flowering time measures should be more positively related to wild flowering time.
287 Likewise, non-vernalized flowering times may better recreate the original temporal niches of
288 summer annuals and thus be positively related to flowering time in these ecotypes. However, it is
289 also possible that long non-vernalized flowering times indicate obligate winter annuals. In these
290 plants, non-vernalized flowering times would be negatively related to wild phenology since later
291 flowering times in non-vernalized experiments would indicate plants that overwinter and are
292 collected early the next year (Figure 1). To account for plasticity in phenology due to differences
293 among locations in the timing and progression of growing seasons, we also tested the

294 relationship between phenology breeding values and the estimated PTUs at the time of collection
295 from the wild, using linear regression.

296 We also implemented a set of models that allowed for geographic variation in the slope
297 of the relationship between wild and genetic variation in phenology. The hypotheses we
298 described above (Figure 1) may be true to different degrees in different populations, due to
299 geographic variation in GxE that, combined with microsite environmental variation, could
300 obscure genetic effects on phenology. Generalized Additive Models (GAMs) allow for
301 regression parameters to vary smoothly across space and thus can capture spatially varying
302 relationships (Yee and Mackenzie 2002; Yee and Mitchell 2006). We fit GAMs using the 'gam'
303 function in the 'mgcv' package in R (Wood 2006) using restricted maximum likelihood (REML),
304 although Generalized Cross Validation returned similar estimates. Model fitting allowed for
305 penalization of smooth terms to 0 so that uninformative covariates could be removed from the
306 equation. Residuals were plotted using the 'gam.check' function in 'mgcv' (Wood 2006), and
307 one ecotype (Nok-10) was removed from our analyses that was an extreme outlier based on its
308 residuals.

309 We also investigated how range-wide differences in wild phenology related to regional
310 differences in breeding values using a broader collection of ecotypes than just those with
311 documented collection dates. This comparison included stock center ecotypes with experimental
312 phenology data and known location-of-origin, but no recorded date of wild collection, and
313 herbarium specimens for which we had not germinated seed and grown plants to measure traits
314 in experiments. To do so, we first estimated wild flowering time for each stock center ecotype in
315 experiments using herbarium records near the ecotype collection location. These wild flowering
316 times were estimated from a previously published GAM with spatially-varying intercepts of
317 herbaria collection dates from 2,655 Eurasian Arabidopsis records used in (DeLeo et al. 2020),
318 which included year of collection as a nuisance variable, as collection date has changed over
319 time across the range of Arabidopsis (DeLeo et al. 2020). Values of the smooth intercept surface
320 were extracted at the coordinates of stock center ecotypes having an experimentally measured
321 flowering time. These estimated wild flowering times were regressed against breeding values for
322 flowering times under vernalized, non-vernalized, and field experiments and rank primary
323 dormancy. In addition, for these same data we performed Spearman rank correlation between
324 estimated wild flowering times and phenology breeding values.

Next, we tested for the effects of genetic variation in flowering time and dormancy on phenological variation *within* populations in the wild. By first accounting for geographic variation in mean flowering time, we aimed to isolate within-population variation (Figure S1). This analysis was in essence asking whether genetic variation explains natural phenological variation within populations. We built a GAM for the dependent variable of collection date which included covariates of experimental flowering time, dormancy, and a spatially varying intercept:

Equation 1.

$$Y_{ij} = \mu_j + \beta_1 \text{Flowering Time}_{ij} + \beta_2 \text{Primary Dormancy}_{ij} + \beta_3 \text{Elevation}_j + \varepsilon_{ij}$$

The spatially varying intercept term μ_j smoothed across latitude and longitude estimates spatial variation in mean collection date (due to e.g. environmental gradients). The upper limit on degrees of freedom for the spatially varying intercept was increased to 45 following the recommendations of Wood (2006). In models that allowed for higher degrees of freedom, the effective degrees of freedom did not meaningfully increase. Elevation was included because of its known importance to flowering times and spring onset (Vidigal et al. 2016; Gamba et al. 2023) and the high resolution of elevation data compared to smooth variation in GAM parameter surfaces. Spatial variation in coefficients for flowering time and dormancy covariates (Eq 1) were not significant, so a simpler model using a constant coefficient was used in our analyses. Field, vernalized, and non-vernalized flowering times were tested in separate versions of the model and tested different hypotheses with regards to wild phenology. To compare among the three measures of flowering time, a version of Equation 1 was fit on a subset of ecotypes that had all three flowering time measures and Akaike's Information Criterion (Akaike 1974) was compared.

In the wild, both flowering time and dormancy contribute to phenology. Thus, we also tested whether including interactions between flowering time and primary dormancy improved the model:

Equation 2.

$$Y_{ij} = \mu_j + \beta_1 \text{Flowering Time}_{ij} * \beta_2 \text{Primary Dormancy}_{ij} + \beta_3 \text{Flowering Time}_{ij} * \text{Primary Dormancy}_{ij} + \beta_4 \text{Elevation}_j + \varepsilon_{ij}$$

Finally, given the importance of plasticity in response to temperature in the timing of germination and flowering time, PTU might better capture genetic variation in phenology in the wild (Figure 1 "Predictions"). Therefore, we also tested the models above using PTU in place of collection day to correct for climate.

Plasticity due to maternal effects

Maternal conditions may be an important source of phenological plasticity; thus we used our dormancy experiment to examine how variation in flowering time among maternal plants influences germination. For each ecotype and for each maternal replicate, we used R packages 'drc' (Ritz et al. 2015) and 'drcSeedGerm' (Onofri et al. 2018) to fit a logistic function to germination counts to estimate three parameters: maximum germination proportion, time to 50% germination, and slope of the germination curve. A Generalized Linear Mixed Model was fit using the lmer package in R (Bates et al. 2015) to estimate the influence of relative flowering time of maternal replicates of each ecotype on germination traits. Because plasticity or responsiveness to environmental cues may confer greater fitness in some environments and not others (Alpert and Simms 2002; Baythavong 2011), germination traits and variation were regressed against maternal flowering time and location of origin. We performed hierarchical clustering of a distance matrix of phenotypic variation among ecotypes to group ecotypes that reacted similarly to different stratification treatments.

Results

Genetic correlation among phenology traits in controlled experiments

Across all compiled experiments (both previously published and our new experiments) mean flowering times of ecotypes measured in vernalized and non-vernalized experiments were strongly positively correlated (Pearson's $r = 0.90$, $p < 0.001$). Although fall-sown field trials might be expected to expose plants to cold seasonal temperatures that give vernalization cues, standardized flowering time breeding values in both non-vernalized and vernalized experiments

were similarly correlated to published field experiment values ($r_{\text{non-vernalized}} = 0.77$, $r_{\text{vernalized}} = 0.72$, $p < 0.001$ for both). Primary and secondary dormancy were negatively correlated, but not significantly so ($r = -0.15$, $p = 0.11$). Despite known interactions between flowering time and dormancy due to seed maturation environment and pleiotropy of causal loci, only non-vernalized flowering time was modestly correlated with primary dormancy ($r = -0.17$, $p = 0.02$, Figure 2). For a heatplot of all phenotypes, see Figure S2.

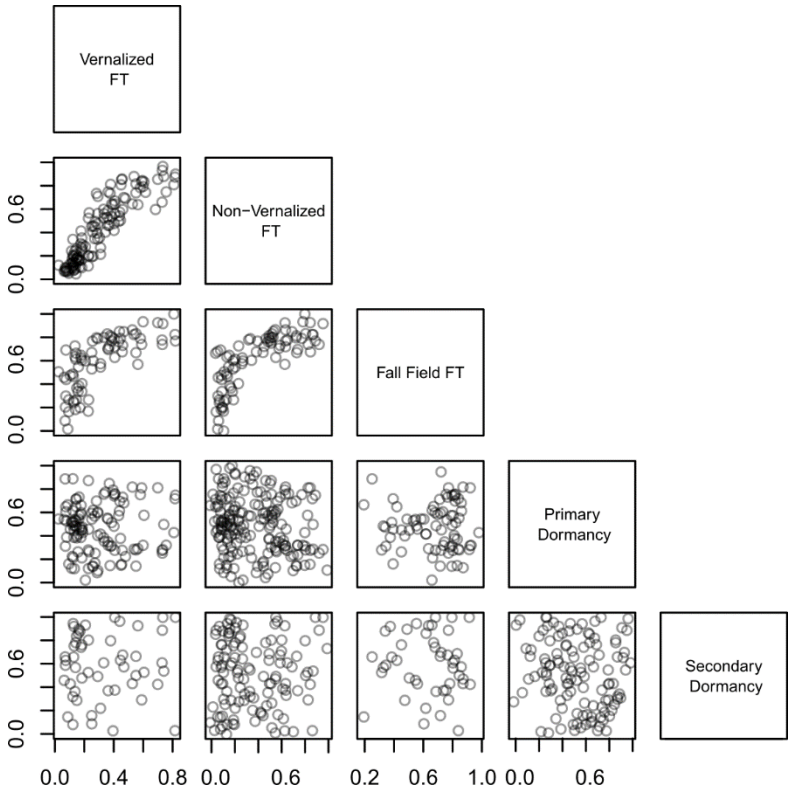


Figure 2: Standardized phenology traits combined across multiple published controlled experiments combined with our new experiments. Standardized flowering times were correlated across treatments (vernalized, non-vernalized, field), but they were mostly unrelated to dormancy.

Comparing wild phenology with breeding values in experiments

Experimental breeding values for phenology traits were modest predictors of collection day in simple linear models, with the only significant predictors being non-vernalized flowering time (estimated slope = 27.7 days/standardized flowering time, $r^2 = 0.09$, $p < 0.001$) and primary

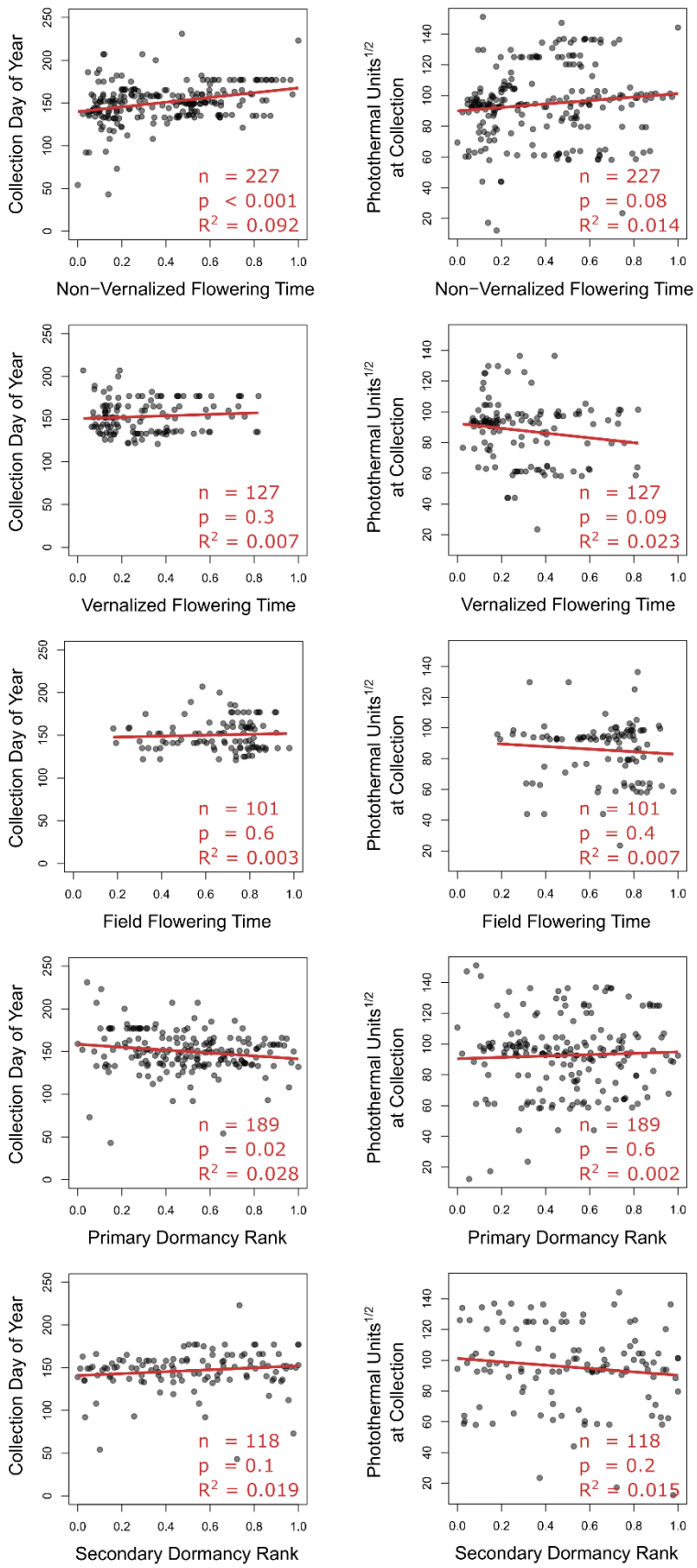
401 dormancy (-17.1 days/standardized rank dormancy, $r^2 = 0.02$, $p = 0.02$). Flowering time breeding
402 values under vernalized or field conditions did not significantly predict day of collection with a
403 linear model ($p > 0.05$) (Figure 3). Flowering time and germination measured within individual
404 experiments did not predict wild collection day better than our averaged breeding values,
405 suggesting some power was gained by combining individual experiments into standardized
406 values (Figure S3).
407 Because environmental differences among locations can influence phenology (Fournier-
408 Level et al. 2013), we also calculated PTU at collection and compared to traits from experiments.
409 However, PTU at collection was not significantly predicted by any phenology traits (Figure 3).
410 Thus, while ecotypes with later flowering time breeding values tend to be collected later in the
411 year, these later flowering ecotypes are not collected at higher PTU, i.e. later in local growing
412 seasons. The fact that phenology breeding values are correlated with date, but not PTU at
413 collection, is consistent with a hypothesis that geographic climate variation maintains clines in
414 breeding values due to local adaptation and clines in collection date due to plasticity (co-gradient
415 variation), generating a spurious breeding value-collection date correlation. That PTUs, which
416 account for variation in seasonality among site, show no relationship with breeding values
417 suggests a weak role for genetic variation in phenology in nature.

418

419 *Figure 3: Date of collection (left*
420 *panels) and PTUs (photothermal*
421 *units) at collection (right panels)*
422 *of wild plants compared to*
423 *standardized breeding values for*
424 *phenological traits from*
425 *experiments. Collection day of*
426 *wild plants was positively related*
427 *to non-vernalized flowering time*
428 *in experiments. Collection day*
429 *was negatively related to*
430 *primary dormancy. The values*
431 *shown for field flowering time*
432 *experiments were calculated*
433 *from fall-sown experiments only.*

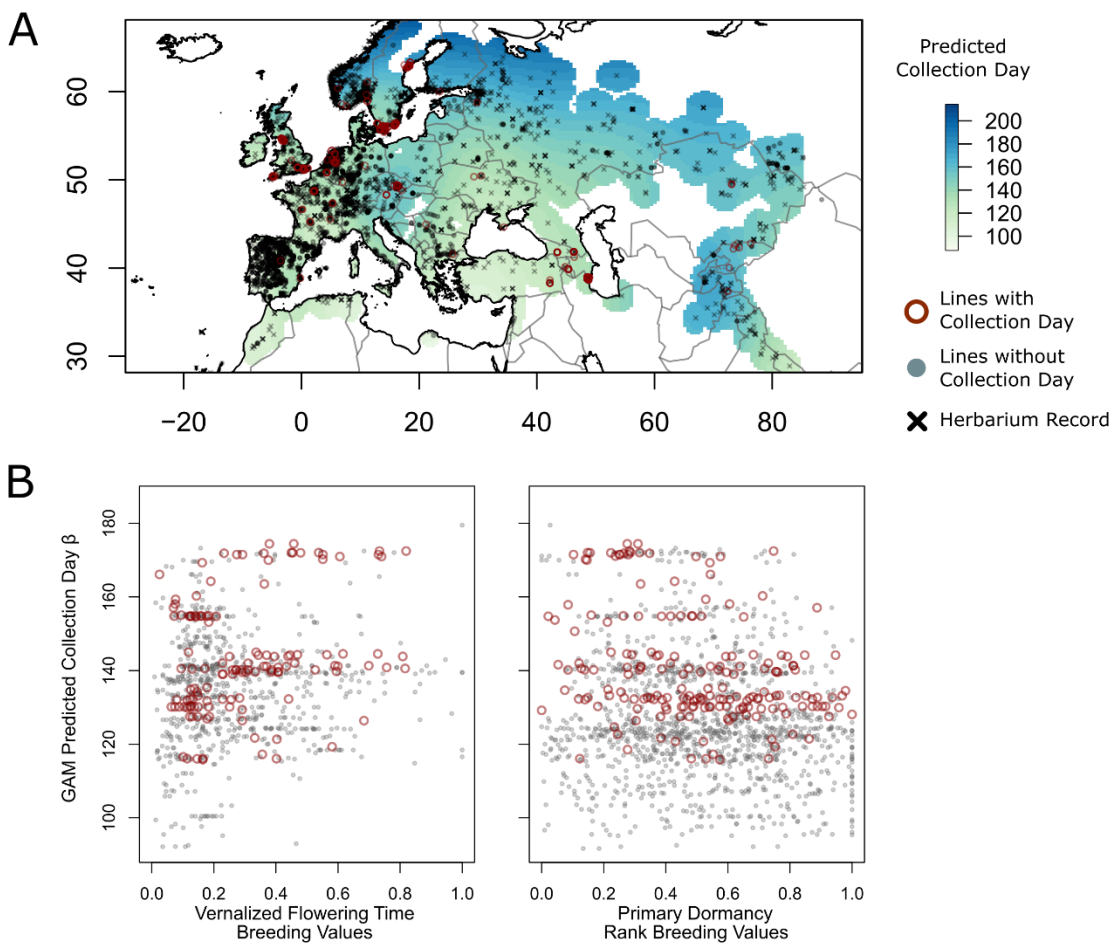
434

435



Flowering time and dormancy breeding values predict regional variation in collection date

Our model of collection date above was limited to the 227 ecotypes with an available collection date and experimental phenology data. To expand our predictions across more of Arabidopsis' range, we tested whether genetic variation in flowering time in experiments predicts local mean collection dates of herbarium specimens across the landscape. First, we found that local mean collection dates across a landscape from >2500 herbarium collections were positively correlated with actual collection days of individual stock center ecotypes (which were not used in fitting the model, $\rho = 0.51$, $p < 0.001$ Figure S4), validating these local predictions of phenology in the wild based on herbarium collections.



448 *Figure 4: Genetic variation in flowering time experiments predicts collection dates of nearby*
449 *herbarium specimens. A) There is geographic variation in collection dates as shown by*
450 *estimated mean date from GAMs with spatially varying means, with earlier collections (light*
451 *green) around the Mediterranean* (DeLeo et al. 2020). *B) Local mean collection dates were*
452 *positively correlated with genetic variation in vernalized flowering time experiments (*$\rho = 0.29$*, p*
453 *< 0.01), and all ecotypes from locations with earlier mean collection dates in the wild (largely*
454 *Mediterranean) had rapid flowering breeding values. Primary dormancy breeding values were*
455 *negatively correlated with local mean collection dates (*$\rho = -0.30$*, p < 0.001).*

456

457 As with the collection dates of the original maternal sources of stock center lines,
458 breeding values for non-vernalized flowering times weakly but significantly predicted local
459 mean herbarium collection dates ($r^2 = 0.10$, $p < 0.001$). For comparison, latitude was a better
460 predictor of mean herbarium collection date ($r^2 = 0.37$, $p < 0.001$). These patterns were similar
461 for vernalized, non-vernalized, and field measurements, although not significant for field trials
462 when fall plantings were grouped with spring and summer plantings ($r^2 = 0.07$, $p = 0.002$, $r^2 =$
463 0.10, $p < 0.001$, and $r^2 = 0.02$, $p = 0.13$, respectively). These results show that range-wide genetic
464 variation in phenology measured in controlled environments corresponds to actual phenology of
465 plants in nature. However, because plants in nature from different regions experience different
466 environments these patterns cannot determine the degree to which genetic variation causes the
467 variation in natural phenology as opposed to genetic variation being confounded with
468 environmental variation that causes phenological plasticity in nature (Figure 1).

469

470 *Variation in phenology breeding values does not predict local, within-region variation in wild*
471 *collection dates*

472 To reduce the influence of genotype-environment confounding among regions in our analyses,
473 we next examined whether genetic values can explain variation in natural phenology *within*
474 regions (Figure S1). We used GAMs that included spatially varying intercepts and an elevation
475 covariate to account for the among population variation in environmental effects on collection
476 dates, leaving local variation to be potentially explained by genetic variation. However, we found

that normalized flowering time (slope of non-vernalized flowering time = -8.2, p = 0.098) and dormancy (slope of dormancy = 2.2, p = 0.51) were not significantly related to day of collection in this GAM (Equation 1), although elevation effects were significant (estimated slope of elevation = 0.012, p = 0.049, Table S1). In comparing models using vernalized, non-vernalized, and field experiments, we found that standardized non-vernalized flowering times led to lower AIC compared to other flowering time measures but higher AIC than a model without flowering time at all, although the difference was slight (difference < 2 for an AIC of 530). We used non-vernalized flowering times in the final models, in part because there were more ecotypes with non-vernalized flowering times (184 for non-vernalized vs 113 for vernalized or 78 for field).

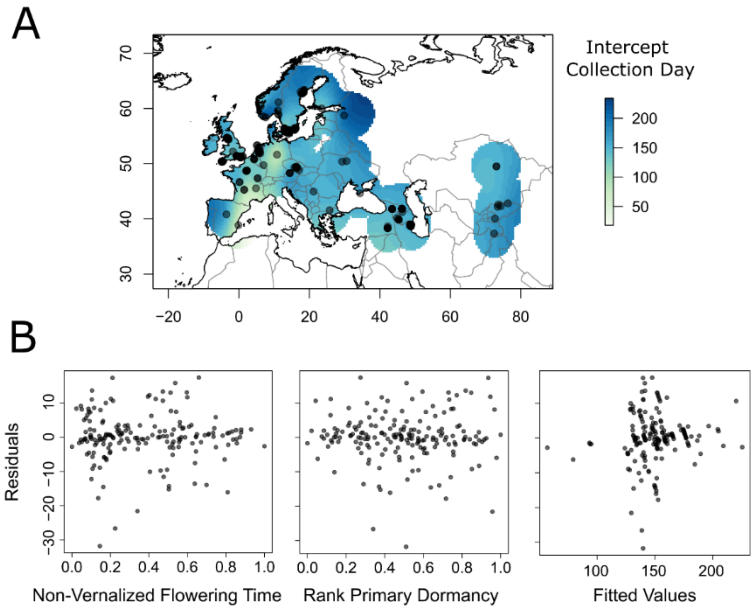


Figure 5: Top: Spatial variation in day of collection for 227 ecotypes with known collection dates. A) Color represents the intercept value for collection day at a location after fitting a linear (i.e. non-spatially varying) relationship with elevation, experimental primary dormancy and non-vernalized flowering time. Much of the variation in collection day was explained by the spatially varying intercept rather than being explained by experimental flowering time. B) Residuals of GAM predicting collection day were not related to non-vernalized flowering time or dormancy, suggesting that genetic phenology values in experiments do not explain the local variation in phenology in nature.

496 Although we found that flowering time, dormancy, and an interaction between the two
497 were not significantly related to collection day, including phenology breeding values did lower
498 the AIC and slightly increase the deviance explained by the model. This suggests that dormancy
499 and flowering time could be related to variation in phenology within regions. However, most of
500 the variation was described by the spatially varying intercept –a model with no more than a
501 spatially varying intercept had a deviance explained of 80.1% – which could account for a large
502 plastic response to geographic climate gradients (Figure S4).

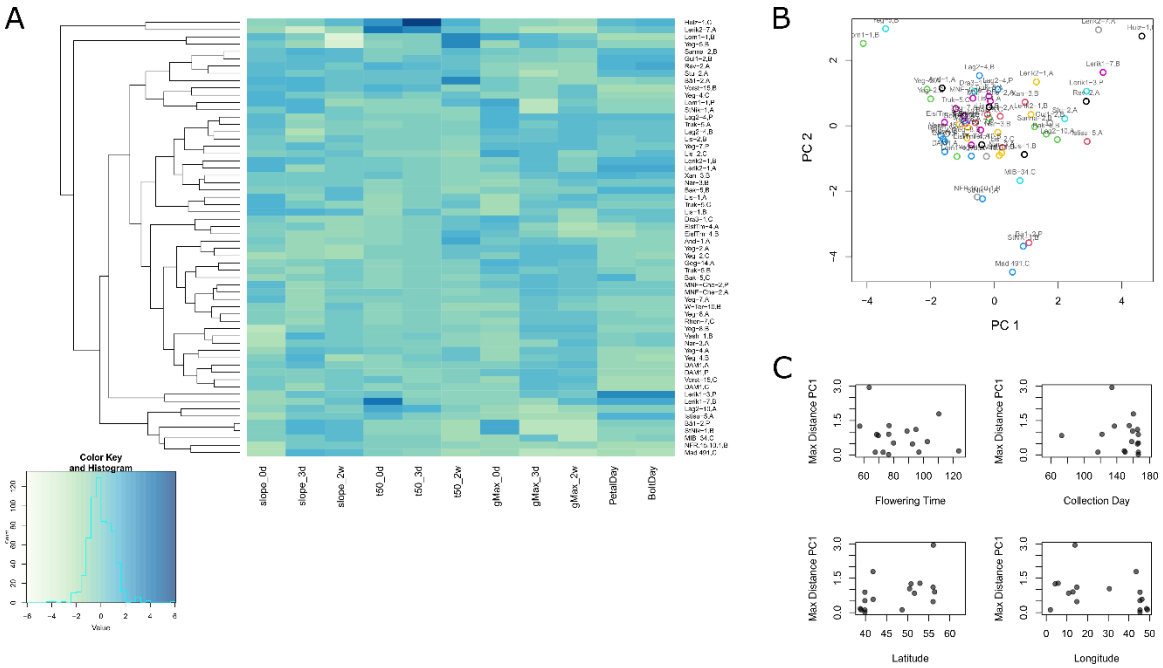
503 As with the linear models, we also tested whether genetic variation in phenology in
504 experiments could explain local variation PTU of collection date in nature. Again, we found that
505 flowering time and dormancy were not significantly related to wild PTU at collection in GAMs
506 with spatially varying intercepts. Elevation was negatively related to PTU (slope: -0.018
507 $\sqrt{PTU}/m$, $p < 0.01$), although it was positively related to day of collection (slope: 0.012 days/m,
508 $p < 0.05$).

509

510 *The role of maternal effects*

511 Finally, when we looked at phenological variation within genetic backgrounds due to different
512 maternal replicate plants, we found that some ecotypes had more consistent phenology across
513 maternal lines. For example, DAM1 maternal replicates clustered together in germination across
514 cold treatments, whereas Yeg-7 replicates did not (Figure 6A). Cold stratification altered
515 expression of variance among maternal sources. In Yeg-7, for example, maternal lines P vs A
516 had similar total germination following 3 days or 2 weeks of stratification but different
517 germination for 0 days stratification. Thus, stratification (or lack of stratification) could
518 contribute to diverse phenology for similar genetic backgrounds under natural conditions. The
519 first and second principal components in an analysis of flowering and germination traits
520 explained 24.55% and 17.85% of the variation respectively, but again maternal replicates of
521 ecotypes did not always cluster in PC space (Figure 6B). The variance in phenology for an
522 ecotype was not related to collection date or geographic origin (latitude and longitude, Figure
523 6C), suggesting that clinal variation in maternal plasticity effects do not confound our earlier
524 large-scale analyses comparing breeding values with phenology in nature. Nevertheless, these
525 effects may further obscure genetic contributions to phenology in nature.

Figure 6: Variation in experimental phenology among maternal lines. A) Early flowering lines tended to have lower germination under 0 days stratification relative to 3 days or 2 weeks stratification, and later-flowering lines tended to have longer times to 50% germination. However, there was variation across traits. Replicate maternal lines of the same ecotype did not always cluster. B) Maternal lines of the same ecotype did not always cluster together in PCA of phenology traits. Lines belonging to the same ecotype are represented with the same color, although colors are not unique across ecotypes. PC1 was positively associated with time to 50% germination at 0 days and 3 days stratification and flowering time and negatively associated with time to 50% germination at 2 weeks stratification and max germination under all treatments. C. Flowering time, collection day, or geographic origin did not explain variance among maternal lines.



Discussion

Plant phenology comprises multiple traits that contribute to fitness. In Arabidopsis, flowering time and germination can vary independently to create a landscape of possible life histories across environments (Debieu et al. 2013; Marcer et al. 2018; Martínez-Berdeja et al. 2020). This variation in phenology is likely partly maintained by selection, given that the traits vary geographically in association with climate (Fournier-Level et al. 2011; Vidigal et al. 2016; Exposito-Alonso 2020) and biotic pressures (Lyons et al. 2015; Davila Olivas et al. 2017), QTL show evidence of local adaptation (Gamba et al. 2023; Lasky et al. 2024), and phenology has been correlated with fitness (Korves et al. 2007; Stock et al. 2015). Given the evidence that genetic variation in life history may be adaptive, we investigated to what degree experimentally measured genetic variation in Arabidopsis phenology predicts phenology of plants in the wild, based on collection dates of natural history records.

The influence of genotype and environment on flowering time and dormancy have been well studied experimentally in Arabidopsis. Yet, common garden and controlled environment experiments must be designed thoughtfully to highlight genetic differences between ecotypes that are relevant to selection in natural environments (Karrenberg and Widmer 2008). While experimental design has increasingly recognized the importance of field conditions to acquire a measure of phenology that is more representative of natural environments (Wilczek et al. 2009; Brachi et al. 2013; Poorter et al. 2016), there has been little comparison of controlled experiments, field or otherwise, to phenology in wild, naturally cycling Arabidopsis individuals. We found that flowering time and dormancy breeding values are weakly, but statistically significantly, related to date of collection and capture variation in phenology but primarily among populations in the wild, explaining little within populations. However, much of this large-scale relationship is likely due to spurious genetic correlations with environment, which also drives plasticity in the wild.

Genetic variation in flowering time and dormancy weakly predict variation in wild phenology among populations

569 Across the species range, both primary dormancy and non-vernalized flowering time
570 significantly, but weakly, predicted collection dates. The predictive power of phenology breeding
571 values was not improved by substituting collection day for PTU at collection, suggesting that
572 temperature and photoperiod variation among locations (captured by PTU) explains a large
573 portion of the geographic variation in collection date, which would explain why genetically later
574 flowering ecotypes were not necessarily collected at higher PTU. Spring onset differs among
575 locations, and so one calendar day at a higher latitude or elevation may be earlier in the season
576 than at a lower latitude or elevation. By recording temperature and daylight above a threshold,
577 PTUs represent how much of a growing season has passed by a given date. Thus, plants collected
578 at lower PTUs may be early flowering (developmentally) despite a later collection date. Higher
579 PTU may indicate plants growing as summer or fall annuals that germinated later in the year. In
580 that case, ecotypes with higher PTUs would likely be fast cycling, earlier flowering plants. While
581 we did find a negative correlation between flowering time breeding values in field and vernalized
582 experiments versus PTU, we see the opposite in non-vernalized experiments. This last
583 observation is somewhat surprising, since we expected plants that flower later in the absence of
584 vernalization to grow as winter annuals in the wild and flower early in the spring (Figure 1).
585 Higher PTUs in these presumptive winter annuals may suggest that plants in these locations
586 regulate their life histories to flower later in the year than expected by temperature and daylight
587 alone. There is some evidence that later flowering ecotypes could in fact be more flexible in their
588 flowering time relative to germination than early flowering ecotypes (Miryeganeh et al. 2018),
589 which could hide the signal of early spring flowering we expected in late flowering Arabidopsis.

590 Both dormancy and flowering time were weakly, but significantly, related to date of
591 collection across the species range, but phenological transitions are not independent of each
592 other. As seen in individual experiments (Martínez-Berdeja et al. 2020), we found that the
593 average breeding values for primary dormancy and non-vernalized flowering time were
594 negatively correlated (Figure 2). Furthermore, dormancy is strongly affected by environmental
595 conditions during seed maturation (Penfield and Springthorpe 2012; Huang et al. 2015;
596 Burghardt et al. 2016). Thus, interaction between flowering time and dormancy in the wild could
597 stem from both genetic pleiotropy and environmentally induced interactions. Despite our
598 expectation for interactions between germination and flowering traits, models of collection day
599 that included an interaction between flowering time and dormancy had a higher AIC and did not

600 explain more of the deviance than a model with both traits separately. Including interactions
601 between flowering time and dormancy in our models did not help to explain collection date in
602 the wild.

603 When we expanded our original set of observations of wild phenology by predicting wild
604 flowering times from a smoothed surface of collection dates fit to herbarium records, we found
605 that predicted wild collection date was positively related to flowering time breeding values. In
606 our linear models, average flowering time breeding values was more closely related to this
607 estimated day of collection than was the actual date of collection of ecotypes in experiments.
608 However, a large portion of phenological variation within populations remained unexplained
609 even with phenological breeding values. We attempted to avoid records that were unusually
610 young or old by only using specimens that had both flowers and fruits, and both herbarium and
611 seed collections spanned nearly the entire year (days 5-350 for herbarium records, 43-346 for
612 seed collections). Still, herbarium records are known to skew slightly towards earlier in seasons
613 (Daru et al. 2018), while seed collections must be collected long enough after the initiation of
614 flowering to allow for the development of some mature seeds. A single observation during
615 reproduction, common for natural history collection vouchers, cannot resolve the uncertainty
616 around when plants begin their vegetative growth in the wild, making it difficult to describe
617 phenology of the full life cycle. Nevertheless, maintained collections could perhaps provide a
618 useful counterpoint observation to fine tune models of landscape phenology.

619

620 *Within populations, breeding values do not predict phenology in the wild*

621 Because Arabidopsis exhibits substantial local within-population variation in phenology (Brachi
622 et al. 2013; Alonso-Blanco et al. 2016; DeLeo et al. 2020), we asked how genetic variation in
623 phenology was related to phenological variation within populations. This within-population
624 phenological variation has fitness consequences. Variation contributes to population persistence
625 in variable environments, as when different germination behavior provides bet hedging in the
626 seedbank (Cohen 1967; Gremer and Venable 2014). Individuals at the tails of the distribution
627 could also play an important role in overall population fitness by maintaining potentially
628 adaptive variation (Jump et al. 2009).

We found that neither dormancy nor flowering time breeding values from experiments were significantly related to collection date when we accounted for geographic variation in local mean collections dates in a GAM. However, flowering time and dormancy did improve GAMs for collection date over a model that included only geographic location and elevation, suggesting that genetic variation in flowering time does explain a minor fraction of the phenological variation within regions.

In our models, there was very little difference between non-vernalized, vernalized, and field-measured flowering times in predicting collection dates, despite the expectation that field experiments recreate conditions similar to nature. Data from field experimental trials were available for a smaller set of ecotypes than non-vernalized and vernalized indoor trials, and our model lacked Iberian ecotypes with field experimental data and date of collection, limiting the usefulness of this measure across the range. Our model provided weak evidence that the breeding values for flowering time and dormancy explained within-population variation in wild collection day. However, our set of ecotypes with known collection day was 258, potentially still too few to detect strong statistical significance given the noise arising from plasticity in responses to local environmental gradients.

Plasticity and maternal effects

Finally, we examined the role of plasticity and within-ecotype diversity in the expression of phenological traits to account for the range of phenological variation we observe in the wild. By comparing the stratification response of seeds from maternal replicates within ecotypes, we aimed to describe the variance in expression of germination traits within a genetic background. Hierarchical clustering of germination traits across 100 ecotypes under three stratification treatments showed that for some genetic backgrounds, seeds from different maternal sources had little variance in germination traits (speed, total germination, ds50) and clustered together. Other ecotypes had more diversity in phenology expression among maternal lines.

Variation in phenology may not be adaptive in regions where there is harsh seasonality or environmental conditions are less variable year to year (Cohen 1967). Similarly, observations of phenology in the wild could be temporally variable because of year-to-year environmental

658 differences (Walker et al. 1995; Hu et al. 2017; Postma and Ågren 2018) or genetically diverse
659 individuals germinating from the seedbank (Ratcliffe 1976). Among replicate individuals in the
660 same controlled experiment, variation in phenology is likely due to responses to very subtle
661 environmental differences and some developmental stochasticity. We expected to see more such
662 variation among ecotypes from regions with favorable growing conditions year-round. However,
663 we did not find a geographic association between variance in germination traits and flowering
664 time among replicate individuals of an ecotype. Instead, there may be geographically consistent
665 selection on the canalization of phenology.

666

667 *Conclusion*

668 We demonstrated that experimental measures of genetic phenology in Arabidopsis explain little
669 phenological variation in wild populations, suggesting low heritability or extensive rank
670 changing genotype-environment interactions across microsites. Differences in Arabidopsis
671 phenology across the species range are often cited as evidence of the adaptive importance of
672 phenology (Stinchcombe et al. 2004; Fournier-Level et al. 2011; Samis et al. 2012; Brachi et al.
673 2013; Exposito-Alonso 2020). Incorporating information on between-population and within-
674 population phenology diversity helps to clarify how selection may be acting on phenology across
675 the landscape and how influential plasticity is in the phenology of this annual herb. We found
676 that flowering time and dormancy weakly predict wild flowering time, and the two together do
677 an incomplete job of predicting wild flowering time even when photoperiod and temperature are
678 accounted for. Yet, genetic flowering times among populations across the species range are
679 associated with phenological variation in natural history collections, apparently due to
680 confounding between genotype and environment (Jones et al. 2024). While phenological
681 plasticity is often of large magnitude, it may not be enough to maintain fitness under
682 environmental change (Zettlemoyer et al. 2024). Ultimately, controlled experiments on many
683 plants have suggested phenology is under selection in nature, but understanding more subtle
684 environmental differences and stochasticity may help to clarify the evolution of phenology and
685 translate genetic values into reliable predictions with and between populations.

686

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921 **Supplemental Information**

922 Figure S1. An illustration of the importance of accounting for local mean flowering dates when
923 testing for correlations between breeding value and observed flowering dates in the presence of
924 co-gradient variation (correlated plasticity and genetic clines). In both A and B there is co-
925 gradient variation, but in A the breeding values predict variation in actual natural phenology
926 (flowering date) while in B the breeding values do not predict local phenology independent of
927 plasticity and environmental differences among sites.

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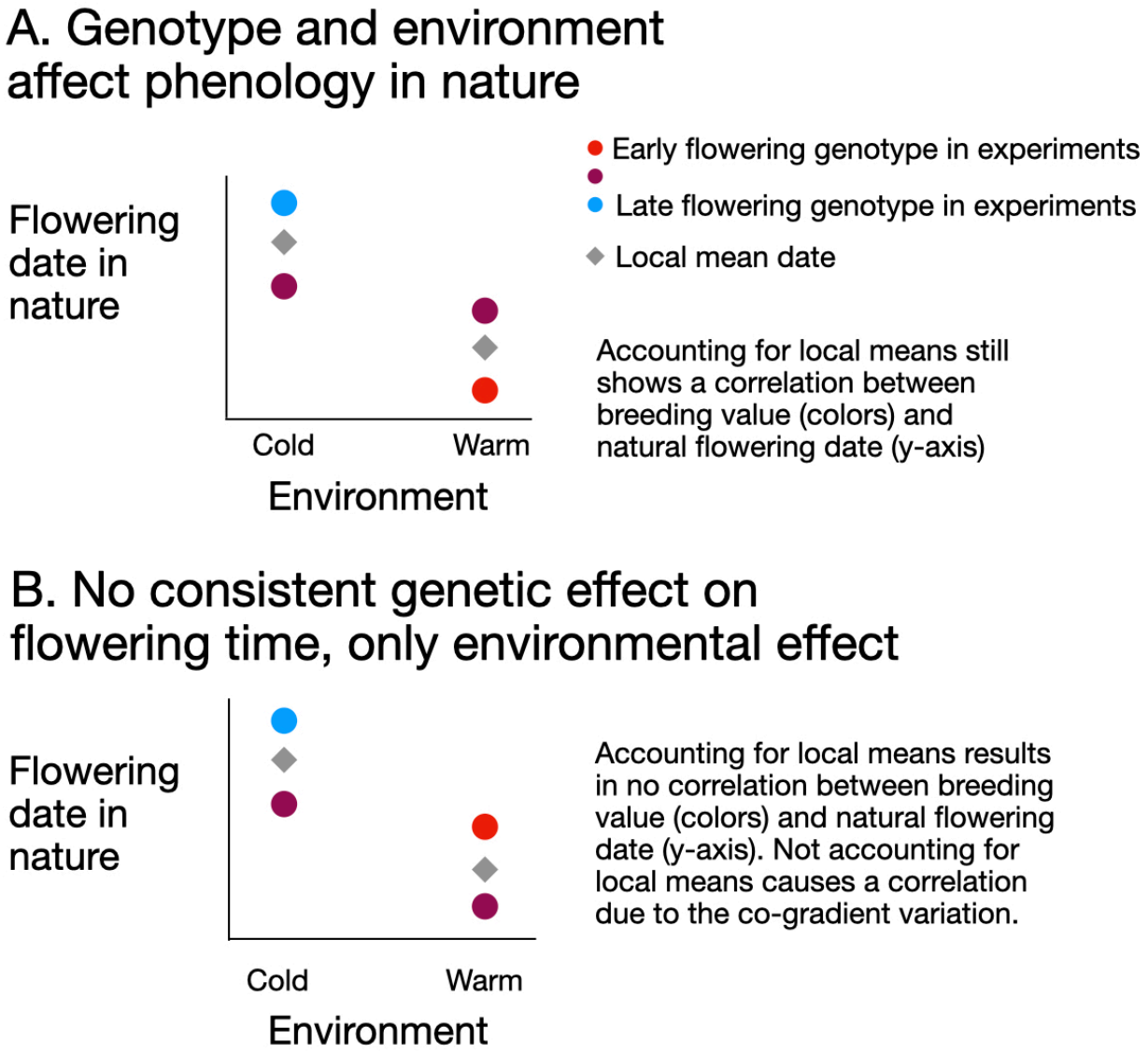

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Figure S2. 42 ecotypes have measures of vernalized, non-vernalized, and field flowering times as well as primary and secondary dormancy traits (standardized trait units are shown). The latest flowering accessions tend to be late flowering across all treatments, but primary and secondary dormancy vary independently from flowering time.

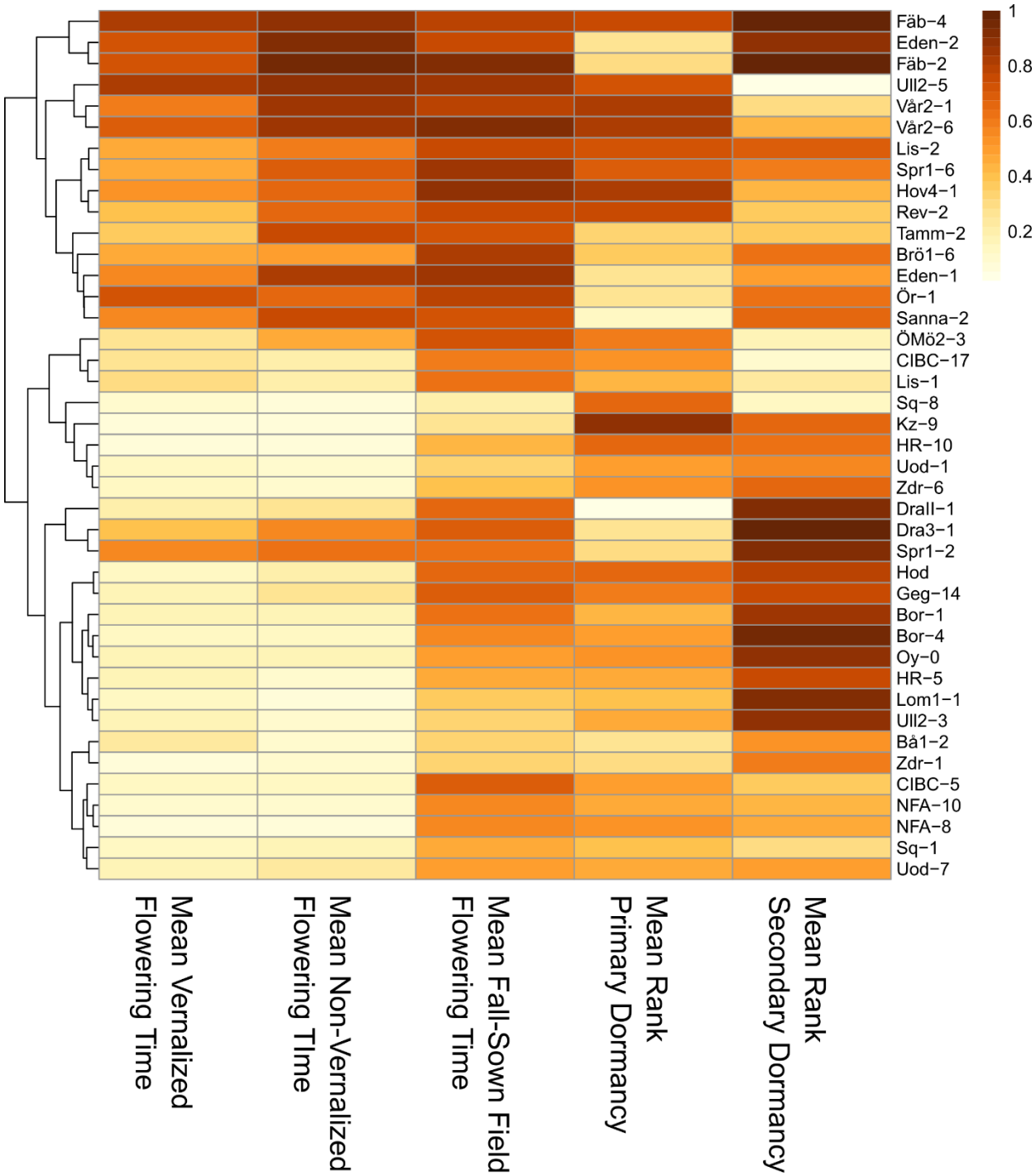

Figure S3. Flowering times and germination rates within experiments do not predict wild phenology better than average values across multiple experiments. Left, non-vernalized flowering times at 16°C (using the same criteria to remove collection day and geographical outliers as in the full set of accessions) (Alonso-Blanco et al. 2016)(Alonso-Blanco et al. 2016)(Alonso-Blanco et al. 2016) did not significantly predict collection day ($p > 0.2$, $n = 173$). Right, germination rates after 4 days of stratification at 4°C (Martínez-Berdeja et al. 2020)(Martínez-Berdeja et al. 2020)(Martínez-Berdeja et al. 2020) did not significantly predict collection day ($p > 0.1$, $n = 84$).

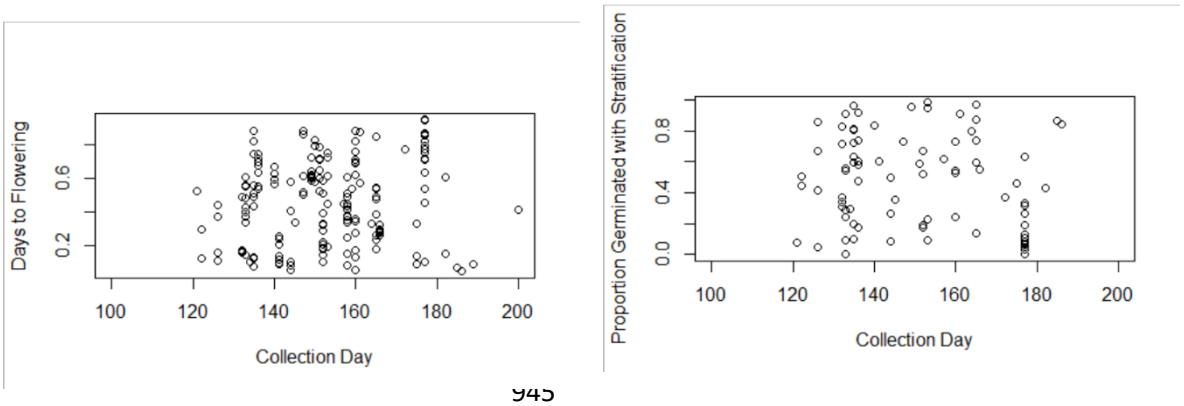

Figure S4: Predicted collection day vs. actual collection day. A) Difference between collection day and predicted collection day in ecotype accessions is designated by the size of the black circles. Accessions from Turkey were collected earlier than expected in our model, while expectations for Western Europe were closer to observed values. Herbarium records on which the model was built are shown as gray circles. B) Density plots of predicted collection days (black) and actual collection days (gray). Collections tended to be later than model predictions. C) Relationship between non-vernalized flowering time and collection day. Both predicted (black) and observed (gray) collection days are positively related to non-vernalized flowering times, but the observed collection days have greater range and a lower slope. Model-predicted flowering times underestimate the variation in collection days, which might be due to actual collection days picking up plants on the tails of the flowering distribution.

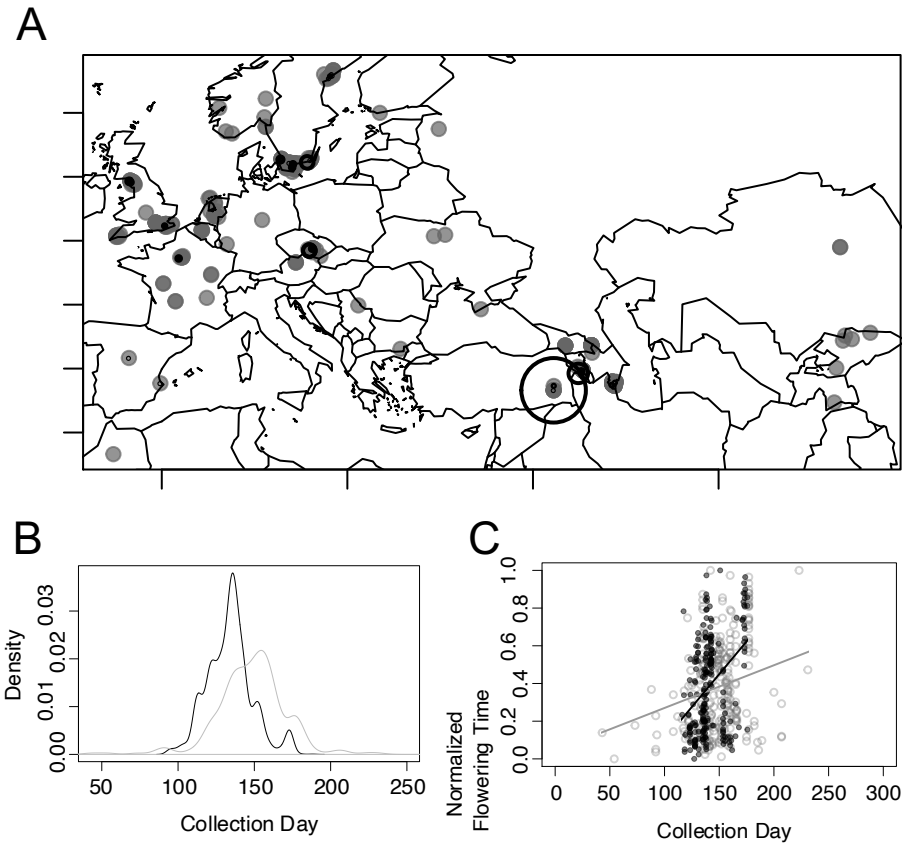

Figure S5. Spatially varying intercept of models of within-population phenological variation. The spatially varying intercept absorbed most of the phenological variation in collection date and photothermal units. Red, dashed lines represent areas where collection day was earlier while green, dotted lines represent areas where collection day was later. Collections are represented as circles. The model attributes a great deal of variation to geographic location, despite including phenological breeding values. Elevation was more important in predicting photothermal units than collection date.

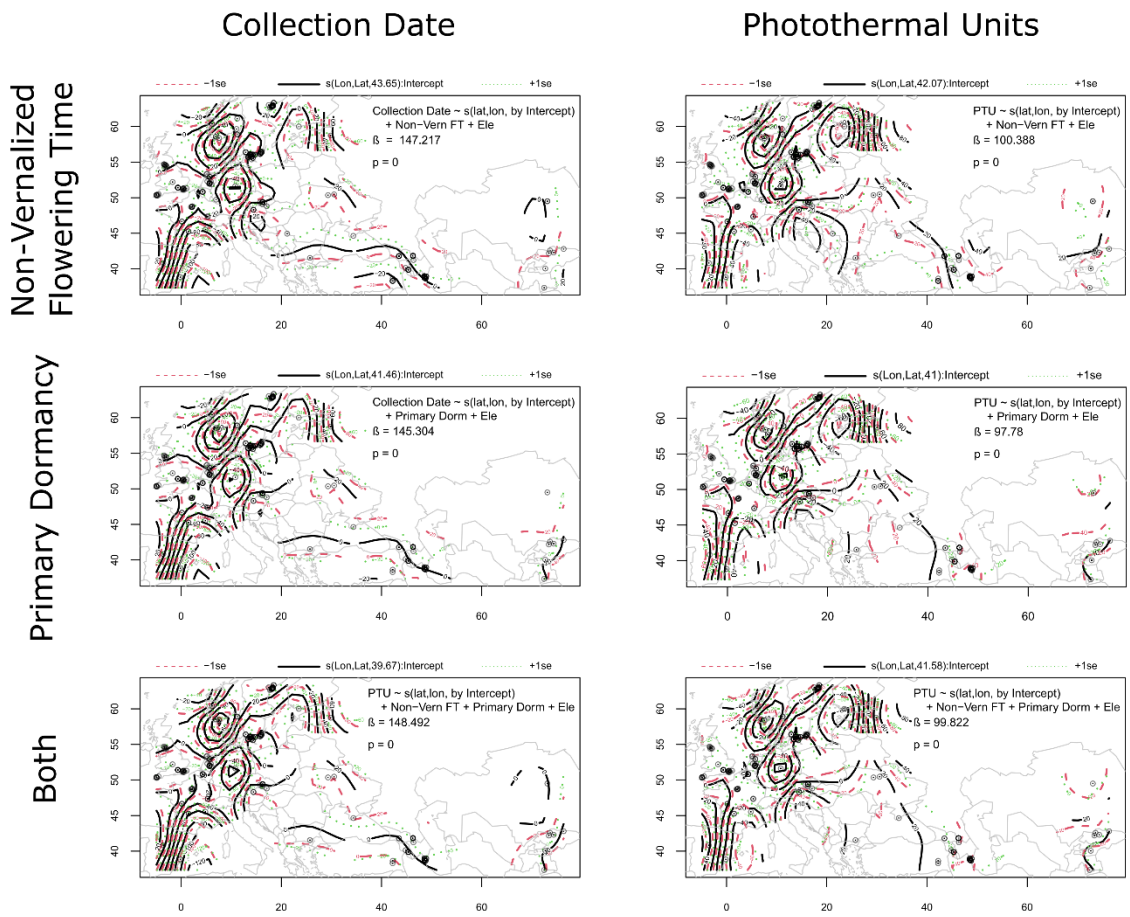

Table S1: Model statistics for other variables in models of within-population phenological variation. P-values for the estimates are given in parentheses. Phenology breeding values did not significantly predict within-population variation in phenology.

Model	Non-Vernalized Flowering Time	Primary Dormancy	FT*Dormancy	Elevation	Adj R^2
Collection Date ~ μ + FT + Elevation	-3.25 (0.35)	NA	NA	**0.0148 (0.006)**	0.883
$PTU^{1/2}$ ~ μ + FT + Elevation	-1.21 (0.75)	NA	NA	**-0.0147 (0.006)**	0.876
Collection Date ~ μ + Dormancy + Elevation	NA	1.98 (0.56)	NA	**0.0126 (0.04)**	0.876
$PTU^{1/2}$ ~ μ + Dormancy + Elevation	NA	2.56 (0.48)	NA	**-0.0181 (0.009)**	0.858
Collection Date ~ μ + FT + Dormancy + Elevation	-8.19 (0.10)	2.19 (0.51)	NA	**0.0120 (0.05)**	0.881
$PTU^{1/2}$ ~ μ + FT*Dormancy + Elevation	-5.41 (0.56)	5.39 (0.43)	-6.86 (0.66)	**-0.0183 (0.009)**	0.864