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1 RESEARCH PAPER:

2 Fluctuating, warm temperatures decrease the effect of a key floral repressor on flowering
3 time in *Arabidopsis thaliana*

4

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32 SUMMARY:

33 • The genetic basis of growth and development are often studied in constant laboratory
34 environments; however, the environmental conditions that organisms experience in
35 nature are often much more dynamic.

36 • We examined how daily temperature fluctuations, average temperature, day length,
37 and vernalization influence flowering time of 59 genotypes of *A. thaliana* with allelic
38 perturbations known to affect flowering time. For a subset of genotypes we also
39 assessed treatment effects on morphology and growth.

40 • We identified seventeen genotypes, many of which have high levels of the floral
41 repressor *FLC*, that bolted dramatically earlier in fluctuating—as opposed to
42 constant—warm temperatures (mean=22°C). This acceleration was not due to
43 transient *VIN3*-mediated vernalization, differential growth rates, or exposure to high
44 temperatures, and was not apparent when the average temperature was cool
45 (mean=12°C). Further, in constant temperatures, contrary to physiological
46 expectations, these genotypes flowered faster in cool environments than warm ones.
47 Fluctuating temperatures often reversed these responses restoring faster bolting in
48 warm conditions. Independently of bolting time, warm fluctuating temperature
49 profiles also caused morphological changes associated with shade avoidance or "high
50 temperature" phenotypes.

51 • Our results suggest that previous studies have overestimated the effect of the floral
52 repressor *FLC* on flowering time by using constant-temperature laboratory
53 conditions.

54

55 Keywords (5-8): *Arabidopsis thaliana*, flowering time, *FLOWERING LOCUS C*,
56 fluctuating temperature, phenotypic plasticity, *FRIGIDA*, shade avoidance, life history

57

58

59 INTRODUCTION

60 In natural environments, temperatures fluctuate diurnally with the lowest
61 temperatures often at dawn and the warmest in the afternoon. The magnitude of the
62 difference between temperature minima and maxima varies by location and season.
63 Fluctuating day/night temperatures have been shown to influence growth rates and
64 phenology in many insect species (Hagstrum & Milliken, 1991; Brakefield & Mazzotta,
65 1995; Radmacher & Strohm, 2011; Malek *et al.*, 2015; Spanoudis *et al.*, 2015;
66 Vangansbeke *et al.*, 2015) and a few plants (Thingnaes *et al.*, 2003; Pyl *et al.*, 2012; Liu
67 *et al.*, 2013). Yet much of what we know about the pathways regulating growth or
68 phenology in response to temperature has come from experiments in which temperature
69 conditions were held constant. It remains an open question 1) how these genetic pathways
70 contribute to development in more complex environments, and 2) whether we have
71 missed important regulatory pathway behaviors by considering only the effects of
72 contrasting constant temperatures. Taking such a perspective is vital for the accurate
73 prediction of growth and development in natural contexts as climates change. Here we
74 assess the effect of fluctuating temperatures on flowering time in *Arabidopsis thaliana*.
75 We do so across a wide range of mutants in the "flowering-time pathway" to ascertain if
76 known genes characterized in constant conditions have similar effects in fluctuating
77 temperatures. Along the way, we also consider if changes in growth and morphology can
78 account for the flowering patterns we observe.

79 In *A. thaliana,* the genetic pathways and environmental factors that influence the
80 timing of reproduction (bolting—often referred to as flowering) have been particularly
81 well studied. This pathway combines information from internal and external cues
82 (reviewed in Jarillo & Pineiro, 2011; Srikanth & Schmid, 2011; Andrés & Coupland,
83 2012). Genetic signals indicating season (temperature, day length, cold exposure) and
84 biotic environment (light quality) converge on key integrator genes including
85 *SUPPRESSOR OF OVEREXPRESSOR OF CONSTANS 1* (*SOC1*), and *FLOWERING*
86 *LOCUS T* (*FT*). When the expression of these integrators is high the meristem
87 irreversibly switches from a vegetative state to the reproductive state.

88 Temperature influences reproduction in multiple ways. First, increasing ambient
89 temperature tends to accelerate flowering (Halliday *et al.*, 2003; Salome & McClung,

90 2004; Salome *et al.*, 2010; Capovilla *et al.*, 2015). Recent work suggests that this pattern
91 is partially driven by temperature-dependent nucleosome occupancy preventing
92 expression of floral promoting genes (Kumar & Wigge, 2010) and/or increasing floral
93 repression due to alternative splicing in cool temperatures (Lee *et al.*, 2013; Pose *et al.*,
94 2013). However most of this work has been conducted on mutants in standard genetic
95 backgrounds that are early bolting. Accessions vary in the extent to which they accelerate
96 flowering at moderate temperature increases (Lempe *et al.*, 2005) and this variation is
97 frequently linked to the genes *FLOWERING LOCUS C* (*FLC*—a potent bolting
98 repressor) and the closely related repressor *FLOWERING LOCUS M* (*FLM*)
99 (Balasubramanian *et al.*, 2006). Mutations in autonomous pathway genes have also been
100 shown to reduce the difference in bolting time between constant cool and warm
101 treatments (Blazquez *et al.*, 2003).

102 Second, even though low temperatures delay bolting in an immediate sense, long-
103 term exposure to cold temperatures can ultimately accelerate bolting once favorable
104 conditions such as warm temperatures and long days return. This process is called
105 vernalization or winter chilling and occurs via epigenetic down regulation of floral
106 repressors (Song *et al.*, 2013; Pyo *et al.*, 2014). Vernalization effects on flowering depend
107 on the expression levels of floral repressors during vegetative development. Accessions
108 with high expression of *FLC*, for example, bolt later when not exposed to an extended
109 period of cold, whereas low floral repression accessions bolt at similar times with and
110 without a prolonged cold cue. Low floral repression ecotypes can occur due to variation
111 at the *FLC* locus itself (Michaels *et al.*, 2004; Li *et al.*, 2014) or via loss of function of the
112 *FLC* activator *FRIGIDA* (*FRI*), which has occurred fairly recently and repeatedly
113 (Johanson *et al.*, 2000; Toomajian *et al.*, 2006). Accessions vary both in the length of
114 cold required to repress *FLC* (Shindo *et al.*, 2006) and the upper bound of temperatures
115 that can satisfy this requirement (Wollenberg & Amasino, 2012; Song *et al.*, 2013).

116 In addition to flowering time, temperature also has a strong effect on *A. thaliana*
117 growth. When grown at low temperatures, wild type *A. thaliana* rosettes are compact,
118 hypocotyls are short, and leaves are horizontal to the soil surface (Patel & Franklin,
119 2009). However, when plants are grown at warmer temperatures photosynthetic rates
120 increase (Bunce, 2008) as do leaf addition rates (Granier *et al.*, 2002). Further increasing

121 temperatures, causes a suite of morphological changes: hypocotyls are elongated (Gray *et*
122 *al.*, 1998), petioles are lengthened (van Zanten *et al.*, 2009) and leaves are elevated above
123 the soil surface (Patel & Franklin, 2009). Similar morphological changes also occur in
124 response to light quality changes indicating the presence of neighbors and thus often
125 called the "shade avoidance" response.
126 Given the importance of temperature for flowering and development, it is perhaps
127 to be expected that diurnal fluctuations in temperature will have important consequences
128 for these traits. Patterns of gene expression shift diurnally, and changes in temperature
129 and co-expression may shape developmental responses (Filichkin *et al.*, 2015).
130 Interestingly, the pathways that determine bolting time and circadian rhythms share a
131 genetic basis: genes originally implicated in the flowering time pathway, in particular
132 *FLC* and some autonomous pathway mutants, have been shown to influence circadian
133 rhythms (Edwards *et al.*, 2006; Salathia *et al.*, 2006) and temperature cycles can entrain
134 the circadian clock (Barak *et al.*, 2000). On the other hand, *A. thaliana* may have
135 sophisticated compensatory mechanisms that allow it to develop similarly regardless of
136 temperature fluctuations.
137 A few previous studies addressed how alternating constant day/night temperatures
138 influence plant growth, morphology, and bolting time. Two experiments showed that
139 metabolism, photosynthesis, and growth primarily depend on daytime temperatures (van
140 Zanten *et al.*, 2009; Pyl *et al.*, 2012). In contrast, other experiments showed the transition
141 to flowering is accelerated by warm nights (Thingnaes *et al.*, 2003; Chew *et al.*, 2012;
142 Thines *et al.*, 2014) and that the difference in temperature between night and day can
143 influence hypocotyl and petiole elongation (Thingnaes *et al.*, 2003).
144 In field experiments conducted across Europe, we observed that genotypes
145 defined as late flowering in the lab including those with a functional *FRIGIDA* allele and
146 those with mutations in the autonomous pathway were far less delayed than in
147 corresponding laboratory experiments (Wilczek *et al.*, 2009). We hypothesized that this
148 effect may be caused by fluctuations in temperature experienced across the day. Here we
149 test that hypothesis using a panel of *Arabidopsis thaliana* flowering time mutants in the
150 Landsberg *erecta*, Columbia, and Columbia $FRI_{\text{Sf-2}}$ backgrounds. We address the
151 following questions: 1) How do fluctuating temperatures influence flowering time? 2) Do

152 these effects depend on day length, average temperature, or exposure to winter chilling?
153 3) How does mutational perturbation in different genes known to effect flowering time
154 affect flowering responses to thermal fluctuations? 4) Are those responses associated
155 with growth or morphology differences?
156

157 MATERIALS AND METHODS

158 We performed three experiments to test phenotypic responses of *Arabidopsis*
159 *thaliana (L.) Heynh* to fluctuating temperatures (see Figure 1 for summary). Experiment
160 1 focused on a diverse collection of loss of function mutants in two genetic backgrounds
161 that were primarily early flowering. Experiment 2 tested whether high temperatures
162 explained the acceleration observed in Experiment 1. Experiment 3 focused on genotypes
163 known to have high floral repression. This experiment also included assessments of
164 biomass accumulation of two focal genotypes.
165

166 *Temperature treatments:* We tested temperature fluctuations in chambers where
167 temperature profiles were controlled to closely mimic recorded ground temperatures in
168 Norwich, UK (Wilczek *et al.*, 2009). We chose this location because *A. thaliana* cohorts
169 germinate and establish in multiple seasons (spring, summer, and fall) in this location
170 (Wilczek *et al.*, 2009; Wilczek *et al.*, 2010). Arabidopsis rosettes grow extremely close to
171 the soil surface until bolting, so ground-level temperatures represent the conditions they
172 experience more accurately than air temperatures. Examination of Norwich temperatures
173 revealed that daily temperature fluctuations in the summer can span 20°C in one day—
174 frequently ranging from 12-32°C—and temperature profiles in the spring and fall
175 commonly range 12°C in a day—frequently ranging from 6-18°C.
176 We created variable temperature profiles to mimic temperatures in summer (avg.
177 22°C) and spring/fall (avg. 12°C) by identifying criteria from the Norwich daily
178 temperature profiles that defined the profile shape such as absolute daily maxima and
179 minima and the timing of those maxima and minima in relation to the day length. Profiles
180 were optimized using Solver in Excel to match these criteria while maintaining the same
181 average profile temperature. The shape of the fluctuating profile in long-days and short

182 days differed so timing of the maxima and minima of the profiles would correspond with
183 natural conditions (Fig. 1a).
184 Control plants were grown in constant conditions reflecting the average
185 temperature of the variable profiles. This is a relevant comparison because plants in the
186 constant treatments accumulate the same number of degree hours per day (a common
187 time-unit for plant growth) as plants grown in the variable treatment. These four
188 temperature treatments were crossed with two day lengths: short days (8 hour day/16
189 hour night) and long days (16 hour day/8 hour night), and two vernalization pretreatments
190 (not vernalized and vernalized) for a total of sixteen environmental treatments (Fig 1b).
191
192 *Overall experimental setup (see Figure 1c and text below for experiment-specific*
193 *details):* Seeds for all experiments were bulked in common maternal conditions in a
194 walk-in chamber under both fluorescent and incandescent bulbs with a 14 hour 22°C light
195 period and a 10 hour dark 20°C night period. Plants were fertilized and watered as
196 needed.
197 Seeds were stratified for 96 hours at 4°C in the dark in 0.125% agar solution.
198 Subsequently, seeds were sown into randomized positions in fifty cell trays (four trays
199 per treatment replicate) into a 4:1 promix: perlite media and placed into short day 22°C
200 conditions for four days to synchronize germination. Randomized blank positions were
201 left empty for the vernalized seedlings (see below). After four days of common
202 germination conditions, experimental treatments commenced.
203 Plants in vernalization treatments were started four weeks earlier than non-
204 vernalized plants. These seeds were stratified and germinated as outlined above and then
205 were placed into a 4°C short-day chamber (see experiment specifics for lengths). The
206 pots with the vernalized seedlings were moved into the experimental conditions on the
207 day the experimental treatments were begun, and after three days of acclimatization, the
208 vernalized seedlings were pricked out into randomized cells in the experimental trays.
209 Plants grown in long days received twice the daily total photon flux of plants
210 grown in short days. Trays were rotated twice weekly, watered as needed, and fertilized
211 sparingly with ¼-strength 50 ppm Cal Mag. Temperature sensors (HOBO® data
212 loggers—Onset, Cape Cod) confirmed continuity of treatments.

213 We recorded days to bolting (DTB), days to first flower opening (DTF), rosette
214 leaf number at flowering (RLN), and cauline leaf number at flowering (CLN). All data
215 have been deposited on Dryad. Bolting date was determined by macroscopic inspection
216 of the meristem and flowering date was the point at which the first flower unfurled its
217 petals past parallel. RLN and CLN were determined by counting primary leaves and
218 cauline leaves at flowering respectively. RLN and CLN are combined to get total leaf
219 number (TLN). In warmer temperatures, late flowering plants produced many secondary
220 leaves, particularly in short days, preventing an accurate estimate of RLN. In these cases,
221 we did not report a RLN. Data collection was blind except in circumstances that required
222 identification.
223 Individuals were removed from the experimental trays after flowering to avoid
224 shading other plants. Most experimental treatments were terminated when the last
225 individual flowered. However in some treatments, after an extended period of time, a few
226 genotypes were left that showed no sign of bolting, were growing extremely slowly, and
227 were dying. At this time treatments were terminated.
228
229 *Experiment 1:* We included 36 loss-of-function flowering time mutants and near
230 isogenic lines (NILs) in Columbia (Col) and Landsberg *erecta* (L*er*) genetic
231 backgrounds. We used mutants implicated in the photoperiod, autonomous, vernalization,
232 temperature sensing, and light-quality sensing pathways. Both Col and L*er* have weak
233 floral repressor (*FLC*) expression (early flowering genotypes). Therefore, we also
234 included a high floral repression line (late flowering genotype) where a strong *FRIGIDA*
235 allele from the San Feliu-2 ecotype was introgressed into the Columbia background (Col
236 $FRI_{\text{Sf-2}}$). A complete list of genotypes can be found in Table S1.
237 All eight environmental treatments were used. In addition, a subset of genotypes
238 was exposed to a 28 day vernalization treatment. Treatments were replicated in at least
239 two E7/2 growth chambers (Conviron—Winnipeg). There were 4 replicates of each
240 genotype/vernalization treatment in each chamber for a total of 8 genotypic replicates per
241 treatment. Due to differences in chamber age, light intensities differed among pairs of
242 replicate chambers. In each pair, one chamber produced ~120-130 µmols of
243 photosynthetically active radiation (PAR) and the other produced ~190 µmols. These

244 differences lead to a slightly lower R/FR (1 vs. 1.3) in the dimmer chamber, but did not
245 cause rank-order reversals in bolting time.
246 In addition to the previously listed measurements, we also recorded leaf blade
247 length and total length of the longest leaf at bolting. On several genotypes (Col, L*er*, and
248 *phyB-1*) we measured hypocotyl length fourteen days after seed sowing. Experimental
249 treatments were terminated after 162 days.
250
251 *Experiment 2:* To test whether high temperatures could explain the earlier
252 flowering of late bolting lines, we grew Col and the Col $FRI_{\mathrm{Sf\text{-}2}}$ NIL in constant and
253 fluctuating 27°C long day (16 hour) treatments. The temperature varied from 22°C –
254 32°C in the fluctuating treatment. These treatments were compared to a 22°C constant
255 long day treatment. No plants were exposed to vernalization. Due to space constraints, 12
256 replicates of each genotype were grown in one chamber replicate. The experiment was
257 terminated after 116 days.
258
259 *Experiment 3:* We used 23 flowering time mutants and NILs exclusively in the
260 Columbia genetic background. Six of the genotypes overlapped with those in Experiment
261 1 and many were late flowering genotypes (see Table S1). Experiment 3 was identical to
262 Experiment 1 except: a) irradiance levels were nearly twice as high (280 PAR for one
263 replicate and 300 PAR for the second replicate); b) the cool temperature fluctuating
264 treatments were omitted because they did not differ from the constant cool treatments; c)
265 vernalization treatments lasted 40 days; and d) data were not collected on
266 leaf/blade/hypocotyl length.
267 Additionally, to assess the influence of the treatments on growth rates and size at
268 bolting, we collected aboveground biomass data on two genotypes (Col and Col $FRI_{\mathrm{Sf\text{-}2}}$).
269 We harvested subsets of plants at bolting and at multiple time points before bolting. To
270 span development, sampling intervals were longer for later flowering genotypes and
271 treatments. At each time point, we harvested 8 replicate individuals of each genotype in
272 each treatment by cutting the plant from the root at soil surface level. Plants were dried in
273 an oven at 70°C for 2 weeks and each individual was weighed. When plants were very
274 small, we pooled replicates for measurement and divided by the number of plants.

275

276 *Statistical Analysis*

277 Several different measures associated with the reproductive transition can be
278 used. We focus on days to bolting because 1) bolting is the first macroscopically visible
279 marker of the reproductive transition, 2) calendar time is the most relevant trait scale for
280 ecological processes, and 3) leaf number counts became unreliable for late-flowering
281 plants critical to this paper. However, for most genotypes, days to bolting, days to
282 flowering, and total leaf number at flowering were highly correlated (Fig S1).

283 We used sixteen treatment combinations of four binary factors (abbreviations
284 summarized Fig 1b). Hereafter the treatments are labeled with those abbreviations (*e.g.*
285 22VarLDV refers to 22°C average temperature, fluctuating temperatures, long day
286 photoperiod, and vernalization). Unless otherwise noted, all treatments subsumed within
287 a label are included. For instance, 12SD refers to all treatments that were at 12°C average
288 temperature and in short day photoperiods (12ConSDNV, 12VarSDNV, 12ConSDV,
289 12VarSDV). Experiment 1, 2, and 3 use identical notation except for Experiment 2 had
290 an additional average temperature of 27°C.

291 We also often employed genotype by fluctuation interaction terms (genotype x
292 fluctuation) in our analysis to test whether specific genotypes responded differently to
293 fluctuating temperatures than the wild type control. For all analyses, we corrected for
294 multiple tests using sequential Bonferroni (Holm, 1979). We used mixed effect models
295 via the lmer function in the lme4 package in R version 3.0.1. In order to control for the
296 grouping of replicates into two or more environmental chambers, chamber identity was
297 included as a random factor. In cases where chamber replicates were not available, we
298 used linear models (lm base function in R).

299

300 *Days to bolting measurements*

301 Because variance in DTB measures tended to increase with time to bolting, we
302 log-transformed days to bolting data before performing statistical analysis.

303 *Exp. 1—Mixed effect models:* To test if temperature fluctuations influenced the
304 bolting time of each wild type genotype in Exp. 1, we subset the data by average
305 temperature, day length, and vernalization and ran the following model:

306 $$logDTB_{ij}=\mu+Fluc_i + chamber_j \qquad \text{(Eqn. 1)}$$
307 We used a likelihood ratio test of a model with and without the fluctuation term to
308 determine if fluctuation influenced bolting time (Table S2).
309 We were particularly interested in which kinds of allelic variants altered plant
310 responses to fluctuating temperatures. Therefore, we subset the data into each
311 combination of average temperature, day length, and vernalization and ran the following
312 model:
313 $$logDTB_{ijk}=\mu+ genotype_i + fluctuation_j + genotype_i \times fluctuation_i+chamber_k \quad \text{(Eqn. 2)}$$
314 We performed a likelihood ratio test on the interaction term (Table S3).
315 We also tested whether functionality of the *VIN3* gene in the Col *FRI*$_{\text{Sf-2}}$
316 background altered the response within each treatment using a likelihood ratio test for all
317 combinations of average temperature, day length, and fluctuation. We omitted vernalized
318 plants from the analysis (Table S4):
319 $$logDTB_{ij}=\mu+Geno_i + chamber_j \qquad \text{(Eqn. 3)}$$
320 *Exp. 2—Regression analysis:* To test if extremely high constant or variable
321 treatments changed the bolting response of Col or Col *FRI* as compared to warm
322 conditions, we used the following model and performed a likelihood ratio test contrasting
323 22ConLD with 27ConLD and 27VarLD (Table S5).
324 $$logDTB_i=\mu+Treatment_i \qquad \text{(Eqn. 4)}$$
325 *Exp. 3—Mixed effect models* To confirm how certain types of allelic variants
326 altered DTB responses to warm fluctuating temperatures, we ran identical models as
327 those run on Experiment 1 (Eqn. 2) specifically on high floral repression and photoperiod
328 pathway mutants (Table S6).
329 We also tested the effect of various mutations in the Col *FRI*$_{\text{Sf-2}}$ genetic
330 background in each environment using the same method used for Experiment 1 (see Eqn.
331 3). We omitted autonomous mutants in short days because many never bolted (Table S7).
332
333 *Morphology and growth measures*
334 *Exp. 1—Mixed effect models for blade ratios*: To test if petiole elongation
335 changed across treatments we divided the blade length by the total length of the leaf to
336 create a blade ratio. For all factorial combinations of day length, average temperature,

337 and genetic background (Col and L*er*), we analyzed the influence of fluctuations on
338 petiole elongation. We did not test plants that underwent vernalization due to age
339 differences. We controlled for size/age at measurement by using bolting time as a
340 covariate and checked that normality assumptions were met. The model used was:

341 $$\textit{blade ratio}_{ijk} = \mu + \textit{fluctuation}_i + \textit{bolting time}_j + \textit{chamber}_k \quad \text{(Eqn. 5)}$$

342 For each data subset, we conducted a likelihood ratio test for the fluctuation term (Table
343 S8).

344 *Exp. 1—Linear model on hypocotyl measurements:* We used a linear model to
345 discern the effect of fluctuation at warm average temperatures on hypocotyl length. We
346 subset the data by day length and genotype (L*er*, Col, and Ler *phyB-1*) and included leaf
347 number at measurement as a covariate to control for size differences among treatments.
348 The model used was:

349 $$\textit{hypocotyl length}_{ij} = \mu + \textit{leaf number at measurement}_i + \textit{fluctuation}_j \quad \text{(Eqn. 6)}$$

350 We tested for the influence of the fluctuation term using a likelihood ratio test (Table S9).
351 Leaf number was highly correlated with hypocotyl length for L*er phyB-1* in short days so
352 we dropped this test.

353 *Exp.3—Plant size:* To test if fluctuations altered aboveground biomass over time
354 we subset by genotype (Col and Col $FRI_{\text{Sf-2}}$), day length, and plant age and used the
355 following model:

356 $$\textit{weight}_{ij} = \mu + \textit{fluctuation}_i + \textit{chamber}_j \quad \text{(Eqn. 7).}$$

357 For each time point, we used a likelihood ratio test to determine if there were weight
358 differences between plants grown at constant and fluctuating warm treatments (Table
359 S10). We used the same model to test if fluctuations influenced size at bolting, by
360 substituting size at bolting for the dependent variable (Table S11).
361

362 RESULTS

363 *Most genotypes showed little response to temperature fluctuation regardless of*
364 *temperature or day length combination.* Genotype-specific bolting times remained
365 largely consistent across both warm and cool conditions and in both long days and short
366 days (Fig 2a, many points on one to one line). In particular, fluctuating temperatures had

367 no effect on the bolting times of the two early flowering accessions L*er* and Col (Table
368 S2, Fig 2b,c).

369 *However, a set of late flowering genotypes bolted earlier in warm, fluctuating*
370 *temperatures relative to warm constant temperatures* (Fig 2a,b,c). Most of these
371 genotypes had high *FLC* expression due to mutation in the autonomous pathway or
372 introgression of the functional $FRI_{\text{Sf-2}}$ allele into Col; see Fig S2-4 for all genotypes.
373 Relative to the wild type background (L*er* or Col), effects of these genetic perturbations
374 on bolting time were much larger in the 22Con treatments as compared to 22Var. For
375 example, Col $FRI_{\text{Sf-2}}$ bolted 95 days later than Col in 22ConSD but only 30 days later in
376 22VarSD (Table S3). This response to fluctuating temperatures was dependent on the
377 activity of *FLC* as Col $FRI_{\text{Sf-2}}$ *flc* bolted at the same time in fluctuating and constant
378 temperature conditions (Fig 2b,c). Further, when late flowering genotypes were
379 vernalized (a treatment that epigenetically represses *FLC* expression), the effect
380 disappeared (Fig S4; Table S3).

381 To confirm these results, we tested additional late-flowering mutants in the Col
382 genetic background in Experiment 3. Many of these mutant genotypes showed a greater
383 difference in bolting time between variable and constant warm temperatures than Col
384 (genotype x fluctuation interaction). The effects of three in particular—*fca-9, fld-3, ld-1*
385 were significant after correction for multiple tests (sequential Bonferroni method, Fig 3a:
386 Table S6; Fig S5-6 for all genotypes and vernalization states). We examined the effects
387 of further augmentation of floral repressor expression using lines where each autonomous
388 pathway mutation was crossed into the Col $FRI_{\text{Sf-2}}$ background. Median bolting day of
389 each doubly modified genotype was later than the Col $FRI_{\text{Sf-2}}$ allele by itself in both
390 constant and fluctuating conditions, and all bolted earlier in the 22Var treatments than the
391 22Con treatments and some dramatically so (Fig. 3a; Table S7).

392 *Neither high temperatures alone, nor partial vernalization explains the earlier*
393 *bolting time of late flowering genotypes in warm, fluctuating temperatures.* To test if the
394 transient high temperatures of the 22Con treatment (up to 32°C during the day) triggered
395 earlier flowering of Col $FRI_{\text{Sf-2}}$, we measured bolting times of Col and Col $FRI_{\text{Sf-2}}$ in two
396 treatments with higher average temperatures: i) constant 27°C and ii) diurnal fluctuations
397 from 22°C to 32°C with a mean of 27°C, and compared each to constant 22°C. Neither

398 genotype bolted earlier in either of the high-temperature treatments relative to the
399 constant 22°C treatment (Fig 3d; Table S5).

400 Another possible cause of the acceleration could be that vernalization is
401 epigenetically decreasing floral repression during the 12°C nights of the fluctuating
402 treatment (12°C causes partial vernalization in this genotype, Wollenberg & Amasino,
403 2012). However, this explanation is unlikely because the genotype Col *FRI*$_{\text{Sf-2}}$ *vin3-4*
404 that carries a mutated *VIN3* gene and is thus insensitive to vernalization bolted after the
405 same number of days as Col *FRI*$_{\text{Sf-2}}$ (Fig 3b; Table S4) in both the 22Con and 22Var
406 treatments. This result was replicated in Exp. 3 (Fig S5; Table S7).

407 In contrast, in cool treatments regardless of temperature fluctuation treatment,
408 *VIN3* activity is implicated in accelerating the flowering of Col *FRI*$_{Sf\text{-}2}$. Col *FRI*$_{\text{Sf-2}}$ *vin3-4*
409 plants flowered slightly later than Col *FRI*$_{\text{Sf-2}}$ plants in 12°C long days (12Con and
410 12Var: ~4.5 days later) and substantially later in short days (12Con ~45.1 and 12Var: ~30
411 days later; Fig 3c; Table S4). Thus, *VIN3* seems to be involved in accelerating flowering
412 at intermediate temperatures and the effect is most prominent in short days. This result
413 was replicated in Experiment 3 in constant conditions (Fig S5; Table S7 for statistics).
414 Earlier flowering primarily in short days suggests either a photoperiodic gating
415 mechanism or that in long days the process is overshadowed by photoperiodic stimulation
416 of flowering.

417 *In high FLC plants, warm average temperatures caused later bolting under*
418 *constant conditions, but earlier bolting in fluctuating thermal environments.* When high
419 *FLC* genotypes were vernalized (*i.e.* their *FLC* levels were repressed), warmer
420 temperatures led to earlier flowering (Fig 4 solid lines- except *vin3-4 FRI*$_{\text{Sf-2}}$). However,
421 non-vernalized, late flowering genotypes flowered at the same time or later in warm
422 constant conditions as compared to cool conditions (Fig 4 black dashed lines). The
423 introduction of temperature fluctuations reduced this effect (Fig 4 gray dashed lines). The
424 strength of this reversal in plasticity depended on day length. In long days, fluctuating
425 temperatures often led to faster bolting times in warm conditions vs. cool conditions;
426 whereas in short days, fluctuating temperatures lead to similar bolting times in warm and
427 cool conditions.

428 *Flowering acceleration in response to warm temperatures may not occur directly*
429 *through repression of FLC.* Mutants in *GIGANTEA* and *FKF1* in a low *FLC* background
430 also displayed earlier bolting in warm variable treatments but only under long days (Fig
431 5a; Table S3). Experiment 3 confirmed this result (Fig 5c,d; Table S6). Therefore, we
432 looked to see if any mutants downstream of both *FLC* and the photoperiod pathway
433 caused delayed flowering in variable conditions.
434 Bolting time was delayed for an *ft* mutant and a limited number of mutations that
435 influence the expression of *FT* (Fig 5c-f). These effects were dependent on genetic
436 background. *Ft-2* mutants in the L*er* background were delayed in fluctuating conditions
437 while in the Col background there was no difference (Tables S4 and S6). *PhyB* was also
438 delayed in long days by fluctuating temperatures compared to wild type (L*er*), but
439 behaved similarly to wild-type by being accelerated by variable temperatures in short
440 days. This result strongly depended on the measure of flowering time used (Fig S7).
441 Interestingly, *co* mutants did not behave like *ft-2, fkf1-2*, or *gi*. Normally we
442 would expect no phenotypic effect of photoperiod mutants in short days and this is what
443 we see in short day constant conditions. However *co-2* mutants actually have delays in
444 flowering time in the 22VarSD treatment. In contrast, when placed in a Col $FRI_{\text{Sf-2}}$
445 background, the *co* mutant behaved identically to Col $FRI_{\text{Sf-2}}$ in short days. In long days,
446 bolting was extremely delayed compared to Col $FRI_{\text{Sf-2}}$ in constant conditions (~76.6
447 days later) and slightly delayed in fluctuating conditions (~13 days later Table S7). In
448 sum, other genes besides floral repressors could mediate the response to fluctuating
449 temperatures.
450 *Faster growth rate cannot explain the faster bolting in fluctuating temperatures.*
451 Aboveground biomass accumulated similarly in the 22Con and 22Var treatments (Fig.
452 6a). At multiple developmental time points, we found no evidence for differences in plant
453 size for either Col or Col $FRI_{\text{Sf-2}}$ (Table S10). Because growth rates were similar but
454 bolting times differed, the relative effect of variable temperatures on size at bolting
455 differed between Col $FRI_{\text{Sf-2}}$ and Col in long days but not short days. Col $FRI_{\text{Sf-2}}$ plants
456 in the 22VarLD treatments were 88% smaller at bolting than in 22ConLD (.0379 g vs.
457 0.3121 g) while wild type plants were only 25% smaller (~0.003 vs 0.004 grams). In

458 contrast, in short days regardless of genotype variable treatment plants were about 50%
459 smaller than plants grown in constant treatments (Fig 6b,c; Table S10).
460 *Plants displayed extreme shade avoidance morphology in fluctuating treatments*
461 *at warm, but not cool temperatures (Fig 6d)*. In 22Var conditions, we observed a suite of
462 morphological changes associated previously with shade avoidance and exposure to
463 constant high temperature. When controlling for days to bolt, petiole lengths were
464 proportionately longer in 22Var treatments compared to 22Con treatments (Table S8; Fig
465 S8a). This was true in long days for plants with both Col and L*er* backgrounds and in
466 short days for plants with a L*er* background only. Further, in short days, hypocotyls were
467 elongated for both L*er* and Col in the variable warm treatments as compared to constant
468 (see Fig. S9 and Table S9) and leaf angles in Columbia were more than twice as steep in
469 22VarSD (~50 degrees) as compared to 22ConSD (~ 25 degrees). Interestingly, in our
470 experiment *phy-B* mutants, which constitutively display a shade avoidance response, had
471 even more extreme phenotypes in fluctuating warm conditions: each of the three rosette
472 leaves were separated by 1 cm internodes and hypocotyls were further elongated (Fig.
473 S9b, Table S9).
474 In contrast, there was little morphological difference between variable and
475 constant treatments with an average temperature of 12°C. Rosettes were compact and
476 hypocotyls were short: similar in length to those found in 22ConLD conditions, and blade
477 ratios did not differ (Table S8).
478
479 DISCUSSION
480 We tested 59 genetic perturbations known to effect flowering time to genetically
481 dissect the effect of diurnal fluctuations of temperature on growth, morphology, and
482 flowering time. We found that temperature fluctuations specifically at warm average
483 temperatures caused a "shade avoidance" or "high temperature response" morphology.
484 Although bolting of many wild type and mutant genotypes showed little response to
485 temperature variability, a subset of genotypes bolted much faster in warm, fluctuating
486 conditions than in constantly warm conditions. Many of these genotypes were late
487 flowering genotypes (Col $FRI_{\text{Sf-2}}$ and autonomous pathway mutants) that are known to
488 have high *FLC* levels. We found that this acceleration 1) was dependent on a functional

489 *FLC* gene and appeared to be dosage dependent, 2) did not occur because plants were
490 being "vernalized" in a *VIN3*-dependent manner in the fluctuating warm treatment, 3)
491 was not due to plants growing faster in the variable treatment, and 4) was not caused
492 solely by high temperatures in the variable treatment. In addition, for many of these
493 genotypes the standard response of faster flowering in warmer temperatures was reversed
494 so plants actually bolted faster in cool constant conditions than warm constant conditions.
495 In total, these results suggest that the state of the *FLC* pathway modulates a multi-faceted
496 response to fluctuating temperatures. Therefore the large flowering delays documented in
497 the lab for naturally occurring late flowering ecotypes may not adequately reflect the
498 behavior of these genotypes in complex natural environments.
499 We observed a few additional genes not associated with floral repression that
500 when perturbed lead to different responses to fluctuations than wild type (*GIGANTEA,*
501 *FKF1, PHYTOCHROME-B, FLOWERING LOCUS-T, CONSTANS*) hinting that earlier
502 flowering may not be occurring only through modulation of floral repression. Further,
503 some of these effects were background specific—they only were observed in L*er*. These
504 results are consistent with the idea that the relative importance of each upstream gene
505 pathway can vary by genetic background as has been recently shown for germination
506 behavior in these two accessions (Vaistij *et al.*, 2013).
507
508 *High FLC lines and autonomous pathway mutations are not temperature insensitive; they*
509 *reverse plasticity to temperature.*
510 Previous research on the thermal sensitivity of flowering-time mutants suggested
511 that autonomous pathway mutants and high *FLC* lines were "temperature insensitive"
512 because they flowered at similar times in warm and cool conditions (Blazquez *et al.*,
513 2003; Balasubramanian *et al.*, 2006; Lee *et al.*, 2010). In our study we replicated these
514 results but also found that when floral repression was further increased these genotypes
515 were delayed at warm temperatures and are thus not "temperature insensitive". Because
516 previous work on ambient temperature sensing has been done in genotypes that bolt
517 faster in warmer conditions most known ambient temperature mechanisms lead to earlier
518 flowering in warmer conditions: temperature dependent *FLM* splicing (Pose *et al.*, 2013),
519 changes in in repression via *SVP* (Lee *et al.*, 2013), or *PIF4* (Nomoto *et al.*, 2012). It will

520 be worth investigating the mechanisms underlying earlier bolting in cool conditions,
521 because many *A. thaliana* ecotypes have high levels of floral repression and many display
522 no or reversed "thermal sensitivity" (reanalysis Lempe *et al.*, 2005; Fig S9). One
523 possibility is that *VIN3*-dependent vernalization—occurring at higher temperatures than
524 previously suspected (Wollenberg & Amasino, 2012)—explains the faster bolting in cool
525 conditions. However, this process cannot fully explain our data because cool
526 temperatures accelerate *vin3-4 FRI* mutants in short days and in fluctuating warm
527 conditions.
528
529 *Possible mechanisms for the acceleration in warm fluctuating conditions*
530 Later flowering in warm constant temperatures than in fluctuating temperatures
531 was only observed when the network had high levels of *FLC* or had mutations in *GI* or
532 *FKF1*. The fact that vernalization, which reduces the expression of *FLC* and other floral
533 repressors, nullifies the effect supports the notion that floral repression levels are crucial.
534 Because *FLC* levels and autonomous mutations have been shown to lengthen and
535 vernalization shown to shorten the circadian period (Salathia *et al.*, 2006), one possibility
536 is that changes in clock period could delay flowering in warm constant conditions.
537 However, recent work also suggests another possibility. *FLC* directly represses both *FT*
538 and *SOC1* via protein complexes formed with *SVP*, *FLM*, *MAF2*, and *MAF3* (Gu *et al.*,
539 2013). Because expression of *FLM*, *MAF2*, and *MAF3* diurnally cycle and the splice
540 forms of *FLM* and *MAF3* proteins that are present are temperature dependent, it is
541 possible that the composition of floral repressor complexes may shift over the course of
542 the day (Gu *et al.*, 2013) influencing *FT* expression at critical periods (Krzymuski *et al.*,
543 2015).
544 Recently a double coincidence model was suggested for the high temperature
545 triggered architectural responses such as hypocotyl elongation and flowering acceleration
546 (Nomoto *et al.*, 2012). This model suggests that *PIF4* expression levels (a promoter of *FT*
547 expression) increase with temperature—with temperatures at dusk in short days being
548 particularly important. In concordance, we observed the largest morphological changes
549 and floral acceleration occur in short days when high temperatures occur at sunset.

550 However cool nights, not just hot afternoons, were necessary to observe the floral
551 acceleration.
552 Fluctuating temperatures could promote flowering in high floral repression
553 genotypes by 1) indirectly overriding repression via a promotive pathway as occurs with
554 light quality changes (Wollenberg *et al.*, 2008); or 2) actively reducing floral repression
555 in the fluctuating treatment. Preliminary analysis of RNAseq data suggests the fluctuating
556 treatment decreases *FLC* levels by 30-40% in the late afternoon (D. Runcie,
557 unpublished). Additionally, work is needed to identify the particular aspect of the
558 fluctuating profile that promotes flowering: the width of the oscillation, the timing of the
559 fluctuation, or the absolute temperatures in the profile.
560
561 *Contrasting effect sizes in field studies vs. controlled chamber environments*
562 Numerous lab-based experiments have found that variation at the *FRIGIDA* locus
563 can explain ~23-70% of variation in flowering time in non-vernalized plants (Lempe *et*
564 *al.*, 2005; Werner *et al.*, 2005; Shindo *et al.*, 2006). However experiments conducted in
565 chambers simulating seasonal temperature cycles (Scarcelli *et al.*, 2007; Li *et al.*, 2010)
566 and those conducted in the field (Wilczek *et al.*, 2009) found smaller, although
567 significant, effects of *FRI* in warm natural environments despite little vernalization. Our
568 results suggest that constant warm temperatures used in lab experiments may artificially
569 magnify the effect sizes of floral repression genes.
570 Our results also hint that higher irradiance levels could play a role in differences
571 between chamber and field studies. We found that high light levels were able to
572 accelerate flowering, particularly in fluctuating treatments and high *FLC* lines both
573 within and between experiments (Fig S10). Higher light levels increase photosynthetic
574 rates potentially accelerating growth and/or developmental progress (Thornley &
575 Johnson, 1990; Vialet-Chabrand *et al.*, 2013). In sum, introducing fluctuating
576 temperature regimes and increasing light levels in chambers may improve ability to
577 connect genetic effects isolated and studied in the lab to behavior in natural
578 environments.
579
580 *Application of results to understanding plant responses in natural environments*

581 We found that temperature ranges as well as means were crucial for determining
582 phenotype in many but not all genotypes. Interestingly, while many genotypes met our
583 expectation that the transition to flowering would occur faster in warm conditions than
584 cool conditions, we discovered a subset of genotypes for which this expectation is only
585 met in fluctuation conditions and not in constant conditions. These results suggest that
586 once gene networks have been characterized in constant conditions a necessary next step
587 is to examine the consistency of this response to complex environments. In addition,
588 these results demonstrate genotype-specific responses to fluctuating temperatures—
589 adding complexity to the challenge of predicting how organisms will respond to climate
590 change as variability increases.
591

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602 AUTHOR CONTRIBUTIONS

603 L.B., A.W., J.R., S.W., and J.S. designed the experiment, L.B., A.W., and M.C.
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607

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774

775

776 FIGURE LEGENDS

777

778 **Figure 1:** Temperature profiles and summary of treatment abbreviations used in
779 experiments with *Arabidopsis thaliana*. (a) Variable temperature profiles (solid lines) at
780 high temperatures (red) and low temperature (green). Profile shapes differ between long
781 days (lighter line) and short days (darker line) because night lengths differ (8 hours for
782 long day vs. 16 hours for short day). Constant temperatures profiles (dashed lines) were
783 the average temperature of the warm (orange) and cool profiles (blue) and were the same
784 across day lengths. (b) Summary of the factor abbreviations used to characterize the
785 environmental treatments throughout the paper. (c) Summary schematic of the genotypes,
786 treatments, and phenotypes collected in the three experiments reported here. Col and L*er*
787 refer to the Columbia and Landsberg *erecta* accessions respectively; DTB and DTF are
788 days to bolt and days to flower; and RLN and CLN refer to rosette leaf number and
789 cauline leaf number at bolting.

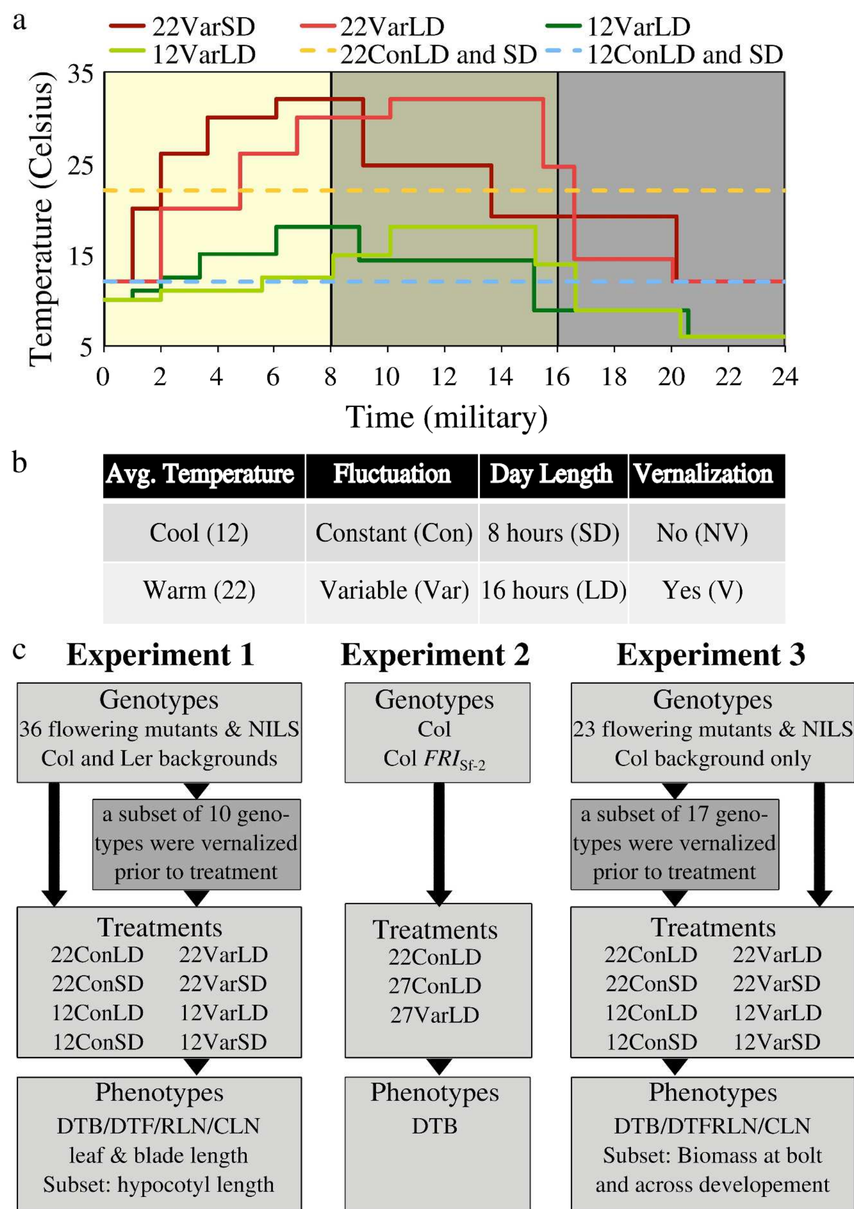
790

791 **Figure 2:** Days to bolting responses of *Arabidopsis thaliana* in constant and variable
792 temperature treatments from Experiment 1. (a) Scatterplot comparing days to bolting of
793 each genotype in constant (x-axis) and variable (y-axis) temperature treatments. Blue,
794 filled symbols indicate 12°C treatments and orange, empty symbols indicate 22°C
795 treatments. Circles and lighter colors denote long days and triangles and darker colors
796 denote short days. Points that fall below the dotted 1:1 line indicate an acceleration of
797 bolting in the variable temperature treatments. (b, c) Median bolting responses of selected
798 genotypes in long (b) and short (c) days in Experiment 1. All graphs were drawn with
799 "ggplot2" package in R. Boxes indicate 25% and 75% quartiles and heavy black line is
800 the median. Whiskers extend to the highest value that is within 1.5 x the interquartile
801 range. Data outside of this range are outliers and visualized as points. Experiment was

802 truncated at 162 days and all unbolted plants were assigned this value as their bolting
803 date. A bold genotype label denotes genotypes that behaved significantly differently from
804 wild type in the two environmental treatments (significant *genotype x fluctuation*
805 interaction) after correction for multiple tests. Results for all genotypes and environments
806 can be found in Fig S2-4.
807
808 **Figure 3:** Median days to bolting responses of selected genotypes of *Arabidopsis*
809 *thaliana*. All graphs were drawn with the "ggplot2" package in R. Boxes indicate 25%
810 and 75% quartiles and heavy black line is the median. Whiskers extend to the highest
811 value that is within 1.5 x the interquartile range. (a) Late flowering, non-vernalized
812 genotypes from long day treatments of Experiment 3. Bolded names denote genotypes
813 that behaved significantly differently from wild type in response to temperature
814 fluctuations (significant *genotype x fluctuation* interaction) after correction for multiple
815 tests. Results for all genotypes and environments can be found in Fig S5-6. (b-c)
816 Response of Col, Col *FRI*, and Col *FRI vin3-4* in long days (b) and short days (c) in
817 Experiment 1. Genotype names with a (V) were vernalized. (d) Behavior of Col and Col
818 *FRI* in Experiment 2. Experiment was truncated at 116 days.
819
820 **Figure 4:** Reaction norms of late flowering *Arabidopsis thaliana* genotypes from
821 Experiment 3 to temperature in long days (a) and short days (b). The behavior of each
822 genotype in 12Con conditions is compared to its behavior in warm constant (black
823 lines/circles) and variable (grey lines/triangles). Solid lines indicate reaction norms of
824 those same late flowering genotypes that were vernalized for 40 days before being placed
825 in their respective treatments. Note these plants bolt faster in warm temperatures than
826 cool temperatures. The genotype Col *FRI vin3-4* is unresponsive to vernalization and thus
827 is labeled on each graph.
828
829 **Figure 5:** Median days to bolting responses of selected genotypes of *Arabidopsis*
830 *thaliana* in warm treatments. For all graphs, boxes indicate 25% and 75% quartiles and
831 heavy black line is the median. Whiskers extend to the highest value that is within 1.5 x
832 the interquartile range. Bolded names denote genotypes that behaved significantly

833 differently from wild type in response to temperature fluctuations (significant *genotype x*
834 *fluctuation* interaction) after correction for multiple tests. (a-b) Photoperiod pathway
835 associated, non-vernalized genotypes from long day (a) and short day (b) treatments of
836 Experiment 1. (c-d) Photoperiod pathway associated, non-vernalized genotypes from
837 long day (c) and short day (d) treatments of Experiment 3. (e-f) Additional genotypes
838 from Experiment 1 in long days (e) and short days (f).
839
840 **Figure 6:** Aboveground growth and morphology of *Arabidopsis thaliana* in
841 environmental treatments. (a) Biomass accumulation of Col (circles) and Col $FRI_{\mathrm{Sf\text{-}2}}$
842 (triangles) in three long day treatments: 12ConLD (blue), 22ConLD (gold), and 22VarLD
843 (red). (b,c) Average plant biomass at bolt in both long (b) and short days (c). For (a-c),
844 error bars indicate standard error of 8 replicate plants spread evenly across 2 replicate
845 chambers of each environment. Note difference in y-axis values between (b) and (c). (d)
846 Pictures of morphology of plants in the warm constant (left) and warm fluctuating (right)
847 treatments in short days. Plants had been in their treatments for 35 days when picture was
848 taken. Some plants pictured had also experienced 28 days of vernalization prior to
849 experiencing the temperature treatments (see methods).
850
851 SUPPLEMENTARY FIGURES
852 **Figure S1:** Scatter plot of the relationship between days from sowing to bolt and days
853 from sowing to flower.
854 **Figure S2:** Summary of results for all non-vernalized Columbia background genotypes
855 from Experiment 1.
856 **Figure S3:** Summary of results for all non-vernalized L*er* background genotypes from
857 Experiment 1.
858 **Figure S4:** Summary of results for all vernalized genotypes from Experiment 1.
859 **Figure S5:** Summary of results for all non-vernalized genotypes from Experiment 3.
860 **Figure S6:** Summary of results for all vernalized genotypes from Experiment 3.
861 **Figure S7:** Number of rosette leaves at bolting for select genotypes.
862 **Figure S8:** Differences in blade morphology and hypocotyl length between
863 environments.

864 **Figure S9:** Reanalysis of data from Lempe (2005) on flowering time of a diverse panel
865 of ecotypes in multiple constant temperatures.
866 **Figure S10:** Comparison of days to bolt data from high irradiance and low irradiance
867 experiments.
868
869
870 SUPPLEMENTARY TABLES
871 **Table S1:** Description of genotypes, sources, and in what experiments they were used.
872 **Table S2:** Likelihood ratio tests to determine if fluctuating temperatures influence
873 bolting times of wild type genotypes (Col and Ler).
874 **Table S3:** Likelihood ratio tests to determine which allelic changes altered plant
875 responses to fluctuating treatments
876 **Table S4:** Likelihood ratio tests to determine whether the bolting time of a *VIN3*
877 mutation in the Col $FRI_{Sf\text{-}2}$ background differed from the Col $FRI_{Sf\text{-}2}$ background.
878 **Table S5:** Likelihood ratio tests to determine if high average temperatures influence
879 bolting time of Col and Columbia $FRI_{\mathrm{Sf\text{-}2}}$ genotypes.
880 **Table S6:** Likelihood ratio tests querying whether each genotype behaves significantly
881 differently to warm fluctuating conditions as compared to Columbia wild type
882 **Table S7:** Likelihood ratio tests to determine whether mutations in the Col $FRI_{\mathrm{Sf\text{-}2}}$
883 background influenced bolting within each treatment.
884 **Table S8:** Likelihood ratio tests to determine if fluctuating temperatures influence blade
885 ratio.
886 **Table S9:** Likelihood ratio tests for the effect of fluctuating temperatures on hypocotyl
887 lengths for Col, L*er*, and L*er phyB-1* in warm treatments.
888 **Table S10:** Likelihood ratio tests to determine if fluctuations at warm temperatures alter
889 above ground biomass at various times throughout development.
890 **Table S11:** Likelihood ratio tests to determine if fluctuations at warm temperatures alter
891 above ground biomass at bolt.
892



Avg. Temperature	Fluctuation	Day Length	Vernalization
Cool (12)	Constant (Con)	8 hours (SD)	No (NV)
Warm (22)	Variable (Var)	16 hours (LD)	Yes (V)

Figure 1
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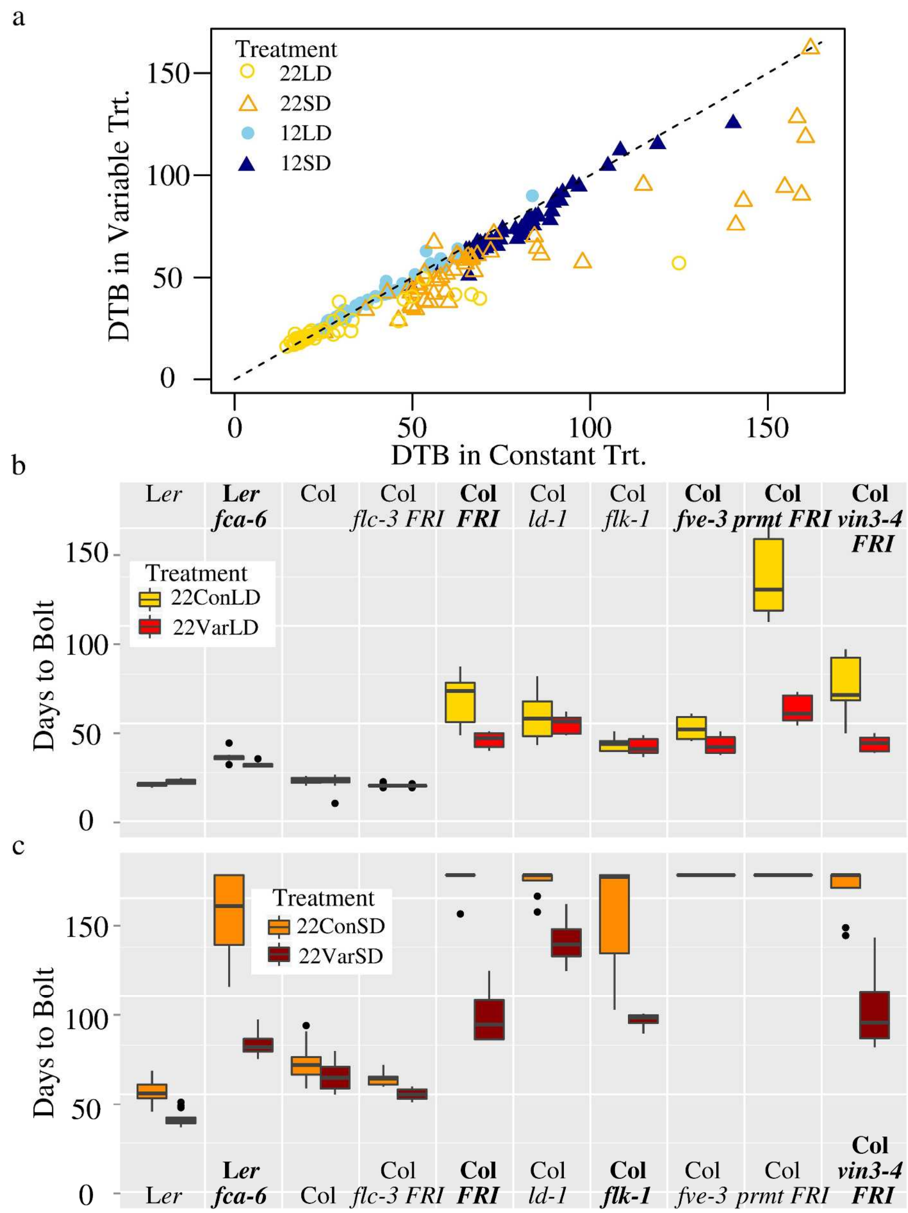

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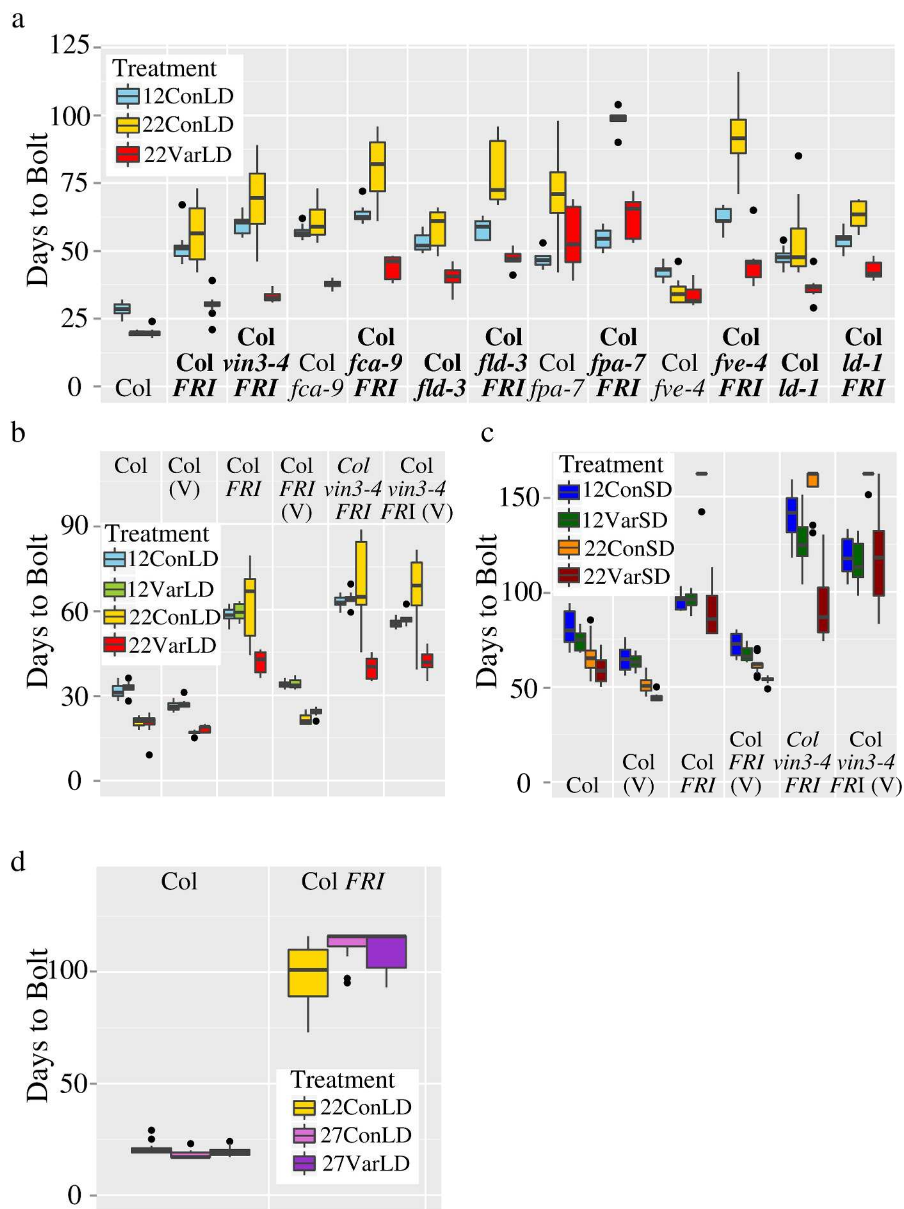

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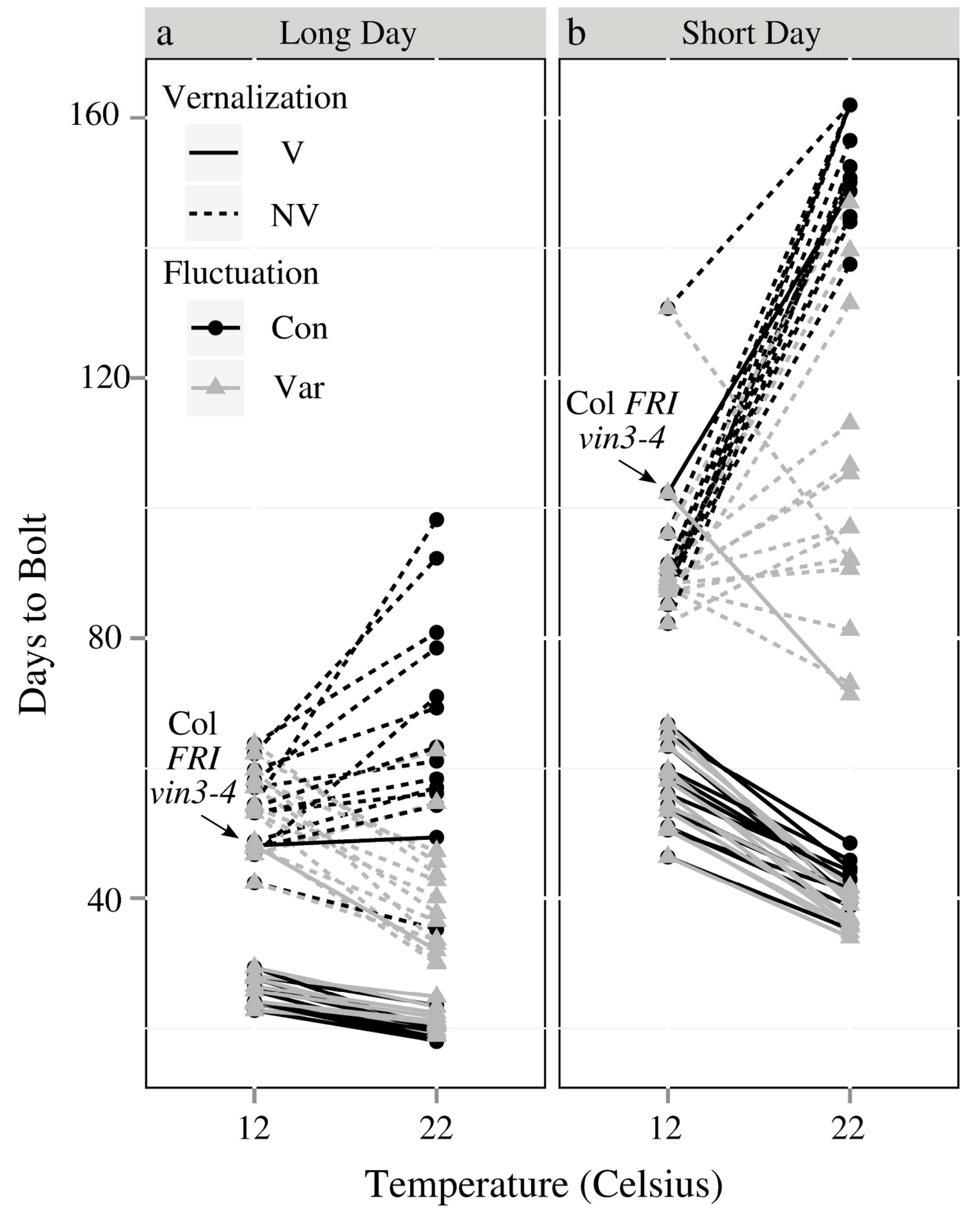

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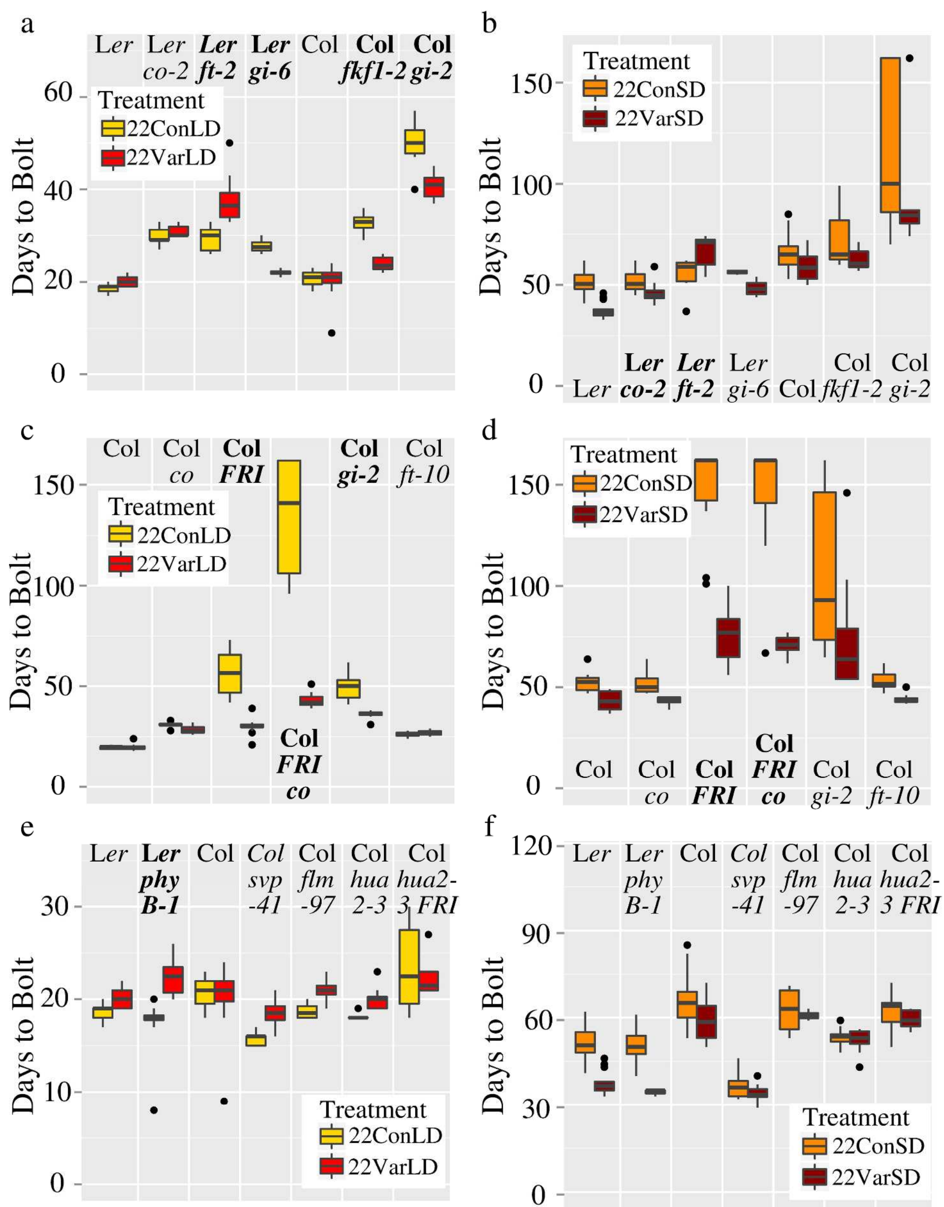

Figure 5
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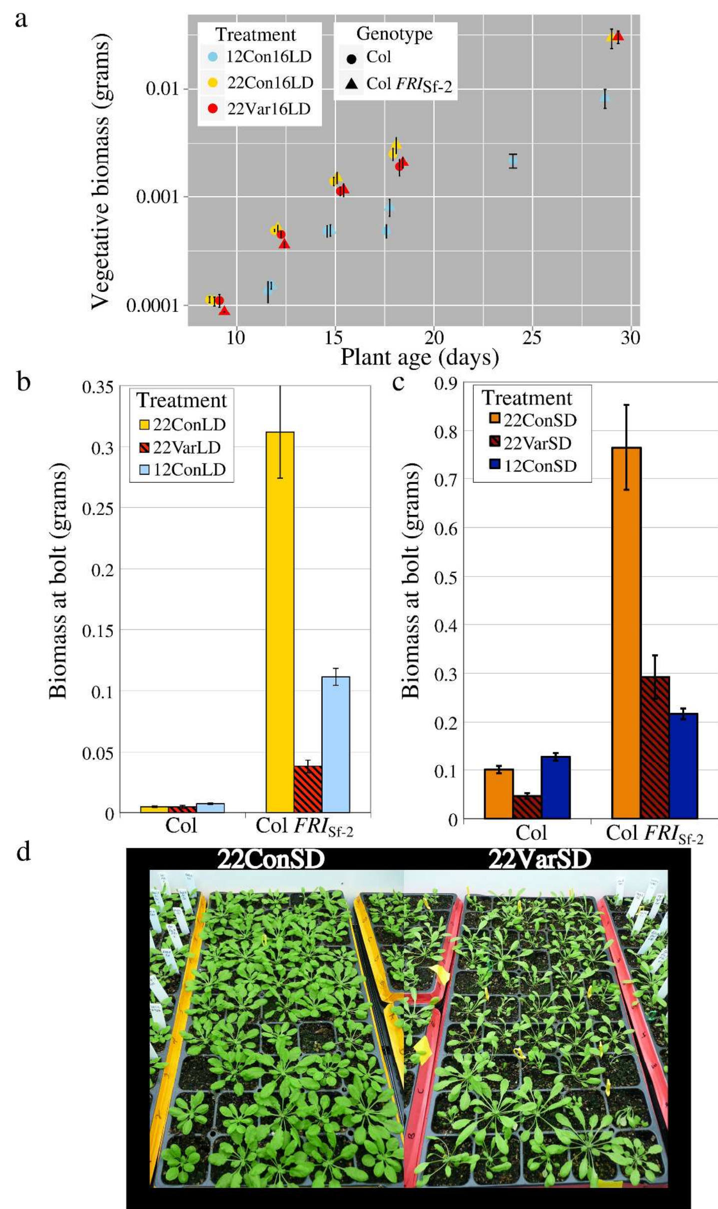

Figure 6
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