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MOLECULAR ECOLOGY

Circadian rhythms vary over the growing season and correlate with fitness components

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Circadian rhythms vary over the growing season and correlate with fitness components

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24

25 **Abstract**

26 Circadian clocks have evolved independently in all three domains of life, suggesting that internal
27 mechanisms of time-keeping are adaptive in contemporary populations. However, the
28 performance consequences of either discrete or quantitative clock variation have rarely been
29 tested in field settings. Clock sensitivity of diverse segregating lines to the environment remains
30 uncharacterized as do the statistical genetic parameters that determine evolutionary potential. In
31 field studies with *Arabidopsis thaliana*, we found that major perturbations to circadian cycle
32 length (referred to as clock period) via mutation reduce both survival and fecundity. Subtler
33 adjustments via genomic introgression of naturally occurring alleles indicated that clock periods
34 slightly >24 hrs were adaptive, consistent with prior models describing how well the timing of
35 biological processes is adjusted within a diurnal cycle (referred to as phase). In segregating
36 recombinant inbred lines (RILs), circadian phase varied up to two hours across months of the
37 growing season, and both period and phase expressed significant genetic variances. Performance
38 metrics including developmental rate, size, and fruit set were described by principal components
39 (PC) analyses and circadian parameters correlated with the first PC, such that period lengths
40 slightly >24 hrs were associated with improved performance in multiple RIL sets. These
41 experiments translate functional analyses of clock behavior performed in controlled settings to
42 natural ones, demonstrating that quantitative variation in circadian phase is highly responsive to
43 seasonally variable abiotic factors. The results expand upon prior studies in controlled settings,
44 showing that discrete and quantitative variation in clock phenotypes correlate with performance
45 in nature.

46

47 The natural environment is complex, and the ability to respond to reliable cues of local
48 environmental conditions often enhances performance, a response pattern referred to as adaptive
49 plasticity (Getty 1996; Peirson 2015; Scheiner & Holt 2012). In some cases, changes in the
50 environment may occur over many days or weeks (for instance, as a consequence of increasing
51 competition as the season progresses) or even months (for instance, as a consequence of changes
52 in abiotic conditions over the growing season). Well-documented cues exist for the preceding
53 examples of microsite variation; light quality provides a reliable indication of neighbor proximity
54 and elicits competitive elongation responses in plants (Crepey & Casal 2015; Dorn *et al.* 2000;
55 Dudley & Schmitt 1995; Dudley & Schmitt 1996; Schmitt *et al.* 1999; Smith 2000; Weinig
56 2000), while photoperiod predicts seasonal changes within a latitude (Johansson *et al.* 2015). The
57 physical environment also changes on a shorter diurnal timeframe with shifts in temperature,
58 light intensity, moisture level and other micrometeorological parameters over the course of a 24-
59 hour day. Circadian clocks, which have evolved in all three domains of life (Dunlap 1999; Edgar
60 *et al.* 2012; McClung 2013), respond to many environmental factors and drive oscillations (or
61 cycles) in developmental, morphological, and physiological outputs (Covington *et al.* 2008;
62 Duffield 2003; Farre & Weise 2012; Lowrey & Takahashi 2011; Michael *et al.* 2008). The
63 periodicity of these cycles typically approximates 24 hours (Harmer 2009; Matsuzaki *et al.*
64 2015), and clock function is therefore hypothesized to adaptively coordinate biological activities
65 with changes in diurnal conditions in contemporary natural settings.

66 The clock consists of three connected components, including an input pathway, a core
67 oscillator, and an output pathway. The coordinated action of these pathways enables organisms
68 to reliably detect and respond to local dawn/dusk timing. More specifically, the input pathway
69 detects changes in many environmental factors, including light and temperature (Anwer & Davis

70 2013; Boikoglou *et al.* 2011; Somers *et al.* 1998), which set or entrain the clock to local time. If
71 inputs are removed following entrainment (a setting referred to as free-running conditions), the
72 core oscillator of the circadian clock regulates continued cycling of phenotypic outputs. Through
73 the use of experimental genetic materials and controlled settings, significant progress has been
74 made in elucidating input loci contributing to both photic and thermal entrainment as well as loci
75 participating in the oscillator and output pathways (Boikoglou *et al.* 2011; Kim *et al.* 2012;
76 Michael *et al.* 2003a). Two recent studies in rice examined the extent to which diverse inputs
77 entrained the clock in a wild-type and clock mutant (*GIGANTEA*) genotype grown in the field.
78 Temperature was shown to play a predominant role (Izawa *et al.* 2011; Matsuzaki *et al.* 2015).
79 Clock responses to simultaneously varying abiotic inputs in field environments have not been
80 measured in genetic lines segregating at multiple clock loci, although such multi-locus variation
81 will likely lead to variable clock phenotypes among genotypes in natural populations. Further,
82 genetic variances and covariances that are estimated in segregating populations and determine
83 the potential for clock evolution in a quantitative-genetic framework (Falconer & Mackay 1996;
84 Lynch & Walsh 1998) remain uncharacterized in the field.

85 Studies in controlled settings suggest that clock regulation of biological processes
86 expressed on a 24-hour cycle is adaptive. In growth-chamber studies that resemble classic
87 reciprocal transplant experiments (Clausen *et al.* 1940), *Arabidopsis thaliana* genotypes
88 harboring mutations at clock loci that lead to long- (28-hour) or short- (20-hour) cycle
89 phenotypes accumulate more biomass when grown in their simulated “home” environment
90 (Dodd *et al.* 2005). That is, long-period mutants accumulate more biomass than short-period
91 genotypes under experimental diurnal cycles of 28 hours that match their endogenous rhythm,
92 while short-period genotypes perform better under 20-hr diurnal cycles. Notably, a 24-hour

93 environmental cycle may lead to the best performance for all genotypes (Graf *et al.* 2010),
94 perhaps because experimentally altered environmental cycles of 28 or 20 hours detrimentally
95 affect many functions. In an experimental population segregating for null alleles at clock loci,
96 alleles that conferred a match between endogenous and experimental cycles appeared to evolve
97 to higher frequency (Yerushalmi *et al.* 2011). Aside from major mutations, natural variation
98 among *A. thaliana* accessions in the relative timing (or phase) of clock gene (*GIGANTEA*)
99 expression within a cycle affects the expression of downstream genes (*PHYTOCHROME*
100 *INTERACTING FACTOR 4*) that in turn influence growth in growth-chamber studies (de
101 Montaigu *et al.* 2015). The match between endogenous and environmental cycles also affects
102 performance in cyanobacteria, *Drosophila*, and mosquito under controlled conditions (Beaver *et*
103 *al.* 2002; Emerson *et al.* 2008; Yan *et al.* 1998). In the limited field studies to date, a genotype
104 with a loss-of-function mutation at the rice clock gene, *OsGIGANTEA*, did not differ in
105 performance from the wild-type genotype (Izawa *et al.* 2011), while circadian-controlled solar
106 tracking was recently shown to confer increased pollinator visitation and biomass accumulation
107 in one sunflower genotype (Atamian *et al.* 2016). Further field studies comparing the
108 performance of genotypes expressing either discrete or quantitative clock phenotypes are
109 necessary to understand the adaptive significance of the clock, because the fitness consequences
110 of even large-effect (e.g., flowering-time) mutations (Brachi *et al.* 2010; Dittmar *et al.* 2014;
111 Korves *et al.* 2007; Leinonen *et al.* 2013; Weinig *et al.* 2003; Wilczek *et al.* 2009) can differ
112 across environments. In sum, the fitness consequences of either a functional *vs.* non-functional
113 clock or of extant quantitative variation remain largely unresolved in field environments, despite
114 the extensive transcriptomic and phenotypic effects in controlled settings (Covington *et al.*
115 2008).

116 To test for performance effects of the clock, we compared survival and fecundity between
117 wild-type genotypes and clock mutants with large-effect perturbations of clock function, between
118 near-isogenic lines (NILs) with small-effect introgressions of genomic regions carrying naturally
119 occurring, alternative clock alleles, and among a panel of recombinant inbred lines that express
120 quantitative clock variation. Based on functional hypotheses regarding clock sensitivity to abiotic
121 inputs and the adaptive significance of the clock, we tested several predictions. First, we
122 anticipated that genotypes harboring large-effect clock mutations and showing substantial
123 endogenous period deviations (20- or 28-hr endogenous cycles) would have reduced
124 performance relative to wild-type genotypes (with nearly 24-hr endogenous cycles) in the field.
125 Second, because circadian periods equal to or slightly longer than 24 hrs enable adaptive phase
126 matching to dawn (Hirschie Johnson *et al.* 2003; Johnson & Kondo 1992), we predicted that
127 near-isogenic lines (NILs) carrying introgressed regions that somewhat shorten periodicity (to 22
128 hrs) would perform less well than NILs with cycles of 24-25 hrs. Third, we anticipated that RILs
129 would vary in the expression of circadian phase across months of the growing season, that clock
130 plasticity would reflect an integrated response to multiple environmental inputs, and that
131 quantitative clock variation in RILs would be associated with performance such that period
132 lengths near 24-25 hrs would again be associated with enhanced fitness. All of these hypotheses
133 were supported by our field experiments.

134

135 **Materials and Methods**

136

137 **Genetic lines**

138 We grew clock mutant genotypes and their cognate wild types in the field to test the
139 performance effects of discrete clock phenotypes, that is, we compared survival and fecundity of
140 wild-type genotypes with circadian cycles near 24 hrs *vs.* mutant genotypes with altered cycles
141 near 20 or 28 hrs. We chose to use null mutant genotypes of the clock genes, *TIMING OF CAB*
142 *EXPRESSION 1* and *ZEITLUPE*, to test the performance effects of clock malfunction, as these
143 were used previously in lab experiments testing growth consequences of the clock (Dodd *et al.*
144 2005). The *toc1-1* and *toc1-2* mutant genotypes express a shortened clock cycle of 20 hrs under
145 free-running conditions, while *ztl-1* and *ztl-2* genotypes express a 28 hr cycle under these
146 conditions (Millar *et al.* 1995; Somers *et al.* 2004; Strayer *et al.* 2000). The mutant alleles used
147 here were all developed in the C24 background, with *ztl-1* later introgressed into the Col
148 background. To account for genetic background and test performance effects, *toc1-1*, *toc1-2* and
149 *ztl-2* should therefore be compared to C24, whereas *ztl-1* should be compared to Col. We used
150 multiple mutant alleles at each locus to account for variation in allele strength. Based on
151 functional hypotheses for the circadian clock, we would expect wild-type genotypes to have
152 higher fitness than the clock mutants expressing extreme clock phenotypes, if a match between
153 endogenous period length and environmental cycles confers a fitness advantage.

154 To test the adaptive consequences of subtler discrete clock phenotypes, we measured
155 fecundity and survival in a panel of near isogenic lines (NILs) that contain introgressions from
156 the genotype, Cvi, of small genomic regions harboring clock loci into the Landsberg *erecta*
157 genotype (Alonso-Blanco *et al.* 1998; Edwards *et al.* 2005; Ouyang *et al.* 1998; Swarup *et al.*
158 1999). Depending on temperature (either 27° or 22°C), clock period was ~22-23 hrs in one set of
159 NILs *vs.* ~24-25 hrs in another set (Edwards *et al.* 2005). The experimental temperature of 27°C
160 used by Edwards *et al.* 2005 closely approximates daytime temperatures in our June and July

161 cohorts (Fig. 1), and was accordingly used to estimate period length in the NIL cohort planted
162 early in the season. Specifically, under summer daytime temperatures of $\sim 27^{\circ}\text{C}$, we anticipate
163 that NILs 18, 18-32, 26-4, 42, 45, and *Ler* have period lengths of 22-23 hrs while NILs 19-2 and
164 30-2 have period lengths have period lengths of 25 hrs (Edwards *et al.* 2005). The experimental
165 temperature of 22°C used by Edwards *et al.* 2005 closely approximates daytime temperatures at
166 the time of the September planting (Fig. 1) and during end-of-season plant growth through mid-
167 October (when daytime temperatures recorded at the micrometeorological station within the field
168 site averaged 21.7°C). Period length of some NILs was sensitive to temperature, and at 22°C , we
169 anticipate that NILs 18, 18-32, 42 and 45 had period lengths of 22-23 hrs while the 19-2, 30-2 as
170 well as 26-4 and *Ler* had period lengths just over 24 hrs (Edwards *et al.* 2005). There could,
171 nevertheless, be some inter-day fluctuations around these NIL period lengths.

172 While NILs are effective for testing the performance consequences of discrete clock
173 phenotypes arising from introgression of alternative clock alleles in small genomic regions, RILs
174 may express quantitative clock variation that more closely resembles that observed in natural
175 populations (Michael *et al.* 2003b). We used experimental segregating progenies to test clock
176 sensitivity to complex field inputs, to estimate genetic (co)variances, and to evaluate associations
177 between clock phenotypes and performance. More specifically, we developed multiple
178 segregating progenies of *A. thaliana*, each of which harbor the reporter gene *LUCIFERASE*
179 (*LUC*) linked to the promoter of the clock output gene, *COLD-CIRCADIAN RHYTHM-RNA*
180 *BINDING 2* (*CCR2*), allowing for quantification of circadian period and phase (Millar *et al.*
181 1992). The two clock markers (leaf movement and gene expression) used to estimate genotypic
182 period in the NILs (Edwards *et al.* 2005) and RILs are strongly correlated (Hall *et al.* 2002;
183 Thain *et al.* 2000). Based on the clock markers, RILs expressed a continuous range of clock

184 periods from 21.5-26.0 hrs (Fig. 2) that closely approximated the values of the NILs described
185 above.

186 One set of 84 RILs ($Ws-2 \times C24$) was the result of a cross between the natural accessions
187 *Ws-2* (Wassilewskija, Russia) and *C24* (Coimbra, Portugal, and genetically indistinguishable
188 from *Co-1*). The second set of 92 RILs ($Ws-2 \times Ler$) was the result of a cross between *Ws-2* and
189 *Ler* (*Landsberg-erecta*, Landsberg, Germany), with both sets having *Ws-2* as the maternal
190 parent. The third RIL set results from a cross between *Col* (Columbia, Missouri, USA, possibly
191 derived from Germany) \times *Rd-0* (Rodenbach, Germany)/*Me-0* (Mechtshausen, Germany). The
192 parental genotypes were chosen in part because they are commonly used lab genotypes, and
193 because prior studies showed they differed in clock phenotypes (Dowson-Day & Millar 1999;
194 Michael *et al.* 2003b). The crossing design of two RIL sets crossed to *Ws-2* is described in
195 greater detail elsewhere (Boikoglou 2008). In brief, the parental genotypes were crossed to create
196 a heterozygous F_1 , and the resulting F_1 was backcrossed to the maternal parent, because it carried
197 the reporter construct. The BC_1F_2 genotypes were then selfed to the BC_1F_6 generation through
198 single-seed descent. The last RIL set, $Col \times Rd-0/Me-0$, was developed by a standard crossing
199 design; homozygous parental genotypes (albeit where the second parent appears to be a genomic
200 hybrid of two German accessions) were crossed to obtain a heterozygous F_1 , which was selfed to
201 produce a segregating F_2 and each F_2 was advanced by single-seed descent to homozygosity at
202 the F_8 . The *Col* parent carries the reporter construct, such that half the F_8 offspring carried the
203 transgene and only these offspring were used in the experiment. As a result of a single parent
204 contributing the construct, all RILs within a set harbor the *CCR2::LUC* reporter construct in the
205 same position within the genome, meaning that any possible insertion effects are common to all

206 lines. The difference in RIL crossing derives from the fact that the populations were developed in
207 different labs.

208 The NIL and RIL genotypes are not locally adapted, as the parental genotypes did not
209 evolve in the location where the field experiments were performed. Thus, the results provide 1)
210 mechanistic insights as to clock responses to multiple abiotic factors that may vary
211 simultaneously (or may as yet be unknown as clock inputs) and cannot be exactly simulated in a
212 growth chamber and 2) information on performance consequences of diverse clock phenotypes
213 (and not local adaptation *per se*).

214

215 **Field Experiments**

216 To measure components of fitness, genotypes were planted in randomized blocks in
217 spring and fall at the University of Wyoming Agriculture Experiment Station (clock mutants,
218 NILs, Ws-2 × *Ler* RILs, and Ws-2 × C24 RILs) or at the University of Minnesota Agriculture
219 Experiment Station (Col × Rd-0/Me-0 RILs). For all plantings, seeds were planted on the surface
220 in 5 cm diameter baskets filled with Sunshine Sungro LP-5 soil (Sungro Horticulture, Agawam,
221 MA, USA), cold stratified for four days at 4°C, transferred to the greenhouse to germinate, and
222 thinned to one focal plant per pot. Plants were then transplanted into the field blocks, with 10cm-
223 spacing between adjacent pots.

224 At the Wyoming field site, 14-16 replicate seeds of each genotype (mutant, NIL, and Ws-
225 2 × *Ler* and Ws-2 × C24 RIL sets) were planted either in early May as a spring cohort, or in
226 early September as a fall cohort; seedlings were transplanted to the field 2.5 wks after the initial
227 planting. Planting of the two RIL sets was offset by one week in spring (May 7th and May 14th)
228 and 12 days in fall (September 1st and September 12th). Replicates planted in May *vs.* September

229 experienced different day and night temperatures, photoperiod lengths, and irradiance levels
230 during the growing season, and staggered RIL plantings within May and September also sampled
231 slightly different conditions (Fig. 1). Notably, the preceding three abiotic factors have been
232 described as the primary inputs to the circadian clock (McClung 2006; Millar 2004; Nohales &
233 Kay 2016). Other measured micrometeorological features, such as humidity, did not vary across
234 months. Experimental plots were irrigated at 5 a.m. daily to field capacity, such that plants never
235 experienced water stress. At the Minnesota field site, due to poor over-winter survival in a pilot
236 experiment, only a spring cohort of the Col \times Rd-0/Me-0 RIL set was planted, in which 12
237 replicate seeds were planted in the first week of April and then transplanted to the field 3 wks
238 after the initial planting. The planting dates within each site (WY and MN) were chosen to
239 ensure abiotic conditions (primarily temperature) were suitable for germination and growth of *A.*
240 *thaliana*.

241 The following traits were measured in spring cohorts: vegetative size, as estimated by the
242 length of the longest leaf prior to reproduction, date of first flowering, and fecundity, as
243 estimated by total fruit number. For the fall cohorts, lifespan, the number of days a plant was
244 alive following germination, was estimated by visually inspecting plants for the presence or
245 absence of green tissue throughout the winter and subsequent spring. Plants that lived for greater
246 than 180 days were considered to have survived the winter, because this duration meant that
247 plants had lived beyond the date of the last hard frost. Plantings and phenotyping followed
248 protocols described under APHIS Biotechnology Regulatory Services notifications 06-100-101n
249 and 12-101-102n for RILs.

250

251 **Circadian assays**

252 We screened the RILs for circadian parameters under two sets of conditions, first in the
253 field and then under growth-chamber conditions that simulated the temperature and photoperiod
254 cycles in the field. The *Ws-2* × *Ler* and *Ws-2* × *C24* populations were entrained under June,
255 July, and September conditions in WY to estimate period and phase and to assess clock
256 sensitivity to the growing season. The *Col* × *Rd-0/Me-0* RILs were entrained under May
257 conditions in MN to estimate genotypic values in period and phase. Temperature and irradiance
258 values during the June, July, and September entrainment windows for one RIL set (*Ws-2* × *Ler*)
259 are provided as supplemental figure S1. Having recorded temperature and photoperiod during the
260 field assays, we independently manipulated these factors in a growth-chamber experiment to test
261 if one abiotic factor could induce circadian phenotypes similar to those measured in the month of
262 July in the field using the *Ws-2* × *C24* population.

263 For each experiment, six-to-eight replicates of each RIL were planted into 96-well
264 microtiter plates containing Murashige and Skoog mineral plant growth media supplemented
265 with 30g/L sucrose (Murashige & Skoog 1962). Plates were covered by sealing tape to retain
266 adequate moisture; notably, the tape filters UV wavelengths, and as such the effects of UV as a
267 clock input can be excluded. Seeds were dark-stratified for four days at 4°C. Plates were then
268 moved to a Percival PGC-9/2 growth chambers set to a 12-hour photoperiod, temperature of
269 22°C and relative humidity of 50% for two days to synchronize germination. Following
270 germination, plates of seedlings were moved into the field and entrained under natural conditions
271 for 5-day windows, a period of time sufficient for clock entrainment. Seedlings within the two
272 *Ws-2* RIL sets were entrained in windows starting in mid-June, mid-July, and mid-September.
273 Plants within the *Col* × *Rd-0/Me-0* set were entrained in mid-May. Although the seedlings were
274 not planted in soil, field entrainment reflects an improvement over controlled conditions, because

275 light levels are higher in the field than growth chamber and because light levels, light quality,
276 photoperiod, and temperature vary dynamically over the course of the day and among days in a
277 way not matched by growth-chamber settings.

278 For the follow-up growth chamber entrainment experiments, we used the same
279 germination conditions described above and used entrainment conditions that matched field
280 temperatures or photoperiods in July with D26.5°C/N10.5°C temperature cycle and 14h50m
281 photoperiods. We attempted to otherwise match the growth-chamber and field entrainment and
282 measurement conditions, *e.g.*, similar plate production, similar timing of plate transfer to the
283 imaging camera, and similar conditions in the incubator with the imaging camera, in order to
284 provide the best basis for comparisons between the growth chamber and field environments.

285 After entrainment, 20µl of a 100 mM D-luciferin monopotassium salt and 0.01% Triton
286 X-100 solution was added to each well, to elicit bioluminescence. Plates were moved to a
287 Percival 141NL incubator set to darkness and a stable temperature of 22°C to enable collection
288 of bioluminescence data and to ensure experimental plants expressed circadian phenotypes
289 resulting from field entrainment conditions and not the chamber assay conditions. Within the
290 incubator, plates were placed under an ORCA-II ER digital camera (Hamamatsu Photonics
291 C4742-98-24ER). Long-exposure images, 30 minutes, of the seedlings were collected every hour
292 for 4 days to quantify bioluminescence. Period and phase values were extracted from the
293 imaging window between 10 and 60 hours and analyzed using Fast Fourier Transform-Nonlinear
294 Least Squares (FFT-NLLS) analysis from the time-series images using ImagePro / IandA
295 software (Doyle *et al.* 2002; McWatters *et al.* 2000; Plautz *et al.* 1997). We used this window,
296 because rhythms entrained by different conditions persist for several cycles after plants are
297 transferred to free-running conditions (Anwer *et al.* 2014; Boikoglou *et al.* 2011; Roden *et al.*

298 2002) and because phase estimates are commonly made from the first 24-hour cycle (de
299 Montaigu *et al.* 2015). The trait “period” estimates average cycle length, and the trait “phase”
300 estimates the timing of peak expression. Because we were most interested in the endogenous
301 phase in relation to diurnal cycles in the natural environment, we used “sidereal phase”, which is
302 phase expression patterns relative to dawn and not adjusted for genotypic period length.

303 We attribute differences in circadian phenotypes (period and phase) to entrainment
304 conditions in the field for two reasons. First, as described, using luciferase bioluminescence as a
305 proxy for the circadian clock, plants express a “memory” of entrainment akin to jetlag, in which
306 endogenous cycles report the entraining environment for several cycles after transfer to free-
307 running conditions (Anwer *et al.* 2014; Boikoglou *et al.* 2011). Second, microenvironmental
308 noise among spatial measurement blocks (that is, plate) rarely led to differences in circadian
309 traits (Table 1). We hypothesized that period lengths of or slightly longer than 24 hrs in RILs and
310 NILs would be associated with improved performance (as this duration would ensure resonance
311 between endogenous and environmental cycles), and that phase might also be associated with
312 performance (as the timing of biological activities relative to dawn could optimize function, for
313 instance, the upregulation of photosynthetic proteins).

314

315 **Statistical Analyses**

316 For clock mutant genotypes, we used two-way ANOVA to partition variance attributable
317 to circadian class (*i.e.*, wild-type, short-, or long-period), genotype nested within circadian class
318 (e.g., *toc1-1* nested within short-period), and field spatial block. In these analyses, genotype
319 nested within circadian class tests for differences between the mutant alleles at a locus, while
320 circadian class tests for differences attributable to clock phenotype. In a related analysis, we

321 tested for differences between mutants in a specific background (i.e., *ztl-1* vs. Col, and *ztl-2*,
322 *toc1-1*, *toc1-2* vs. C24).

323 For clock NILs, we used two-way ANOVA to partition variance attributable to circadian
324 class (i.e., shorter, 22-23hr vs. longer, 24-25hr circadian period), genotype nested within
325 circadian class, and field spatial block. In these analyses, genotype nested within circadian class
326 tests for differences between the introgressed genomic regions, while circadian class tests for
327 differences attributable to clock phenotype. For both mutants and NILs in spring cohorts, we
328 performed analysis of covariance, including flowering time as a covariate in the original models,
329 to test if flowering time could explain circadian class effects on fruit set. Plants in fall cohorts did
330 not flower before winter, and thus differences in flowering time could not explain variation in
331 survivorship.

332 For RIL phenotypic traits and components of fitness, we first used two-way ANOVA
333 within each month to partition variance attributable to genotype and block (effect of microtiter
334 plate for circadian parameters or field spatial block for other traits). We then used ANOVA to
335 estimate the fixed effect of season and the random effects of genotype, genotype \times season
336 interaction, and plate nested within season for the circadian traits using restricted maximum
337 likelihood methods (PROC MIXED) (SAS 1999).

338 From the preceding analyses, we estimated least-square means for both month and for
339 genotype within each month. Genotypic values were used to test for across-environment
340 correlations (r_{GE}) and associations between circadian traits and components of fitness (PROC
341 GLM) (SAS 1999). Specifically, the across-environment correlations were estimated as the
342 bivariate correlation between the genotypic value of a trait (period or phase) in, for instance,
343 June and July (PROC CORR). We performed Principal Components Analysis (PCA) on the

344 genotypic values, to compress traits (size, reproductive timing, and fecundity) into one
345 performance metric (PROC PRINCOMP) (SAS 1999). PCA loadings are shown in Table S1,
346 Supporting Information. Clock-performance associations were estimated as the genotypic
347 regression of PCA1 on circadian period and phase (PROC GLM) (SAS 1999).

348

349 **Results**

350

351 We grew *A. thaliana* clock mutants, *ztl-1*, *ztl-2*, *toc1-1*, and *toc1-2*, and their cognate
352 wild-type genotypes, C24 (Coimbra, Portugal) and Col (Columbia), in spring and fall seasonal
353 settings to test the fitness consequences of a match (or mismatch) between endogenous circadian
354 and natural diurnal cycles. Clock phenotype significantly affected both fecundity (Fig. 3A) and
355 survival (Fig. 3B). In a spring cohort, the two wild-type genotypes produced significantly more
356 fruit than the short-period *toc1* mutants (with ~20-hour endogenous cycles) or the long-period *ztl*
357 mutants (with ~28-hour endogenous cycles) (Fig. 3A) (effect of period class, $F = 10.7$, $p <$
358 0.0001), and this relationship remained significant after accounting for flowering time variation
359 ($p < 0.001$). In a fall cohort, period class also affected lifespan ($F = 17.31$, $p < 0.0001$). The long-
360 period mutant, *ztl-1*, had a short lifespan compared to its isogenic wild-type control (Col), and
361 unlike Col it failed to survive the winter (Fig. 3B). The *ztl-2*, *toc1-1*, and *toc1-2* mutants had
362 lifespans that were between 19 -38% shorter on average than the cognate wild-type genotype
363 (C24) (Fig. 3B), although C24 also showed lower survivorship than Col-0 potentially due to its
364 warm climate provenance. In short, extreme excursions (± 4 hrs) of circadian period from 24 hrs
365 appear to reduce performance.

366 To test the adaptive consequences of subtler clock adjustments, we also measured
367 fecundity and survival in near isogenic lines (NILs) developed by introgression of small genomic
368 regions harboring alternative clock alleles into the *Ler* genotype. All genotypes that expressed a
369 circadian period from 22-23 hrs had reduced fecundity relative to genotypes with circadian
370 periods of ~25 hrs (Fig. 3C), and this relationship remained significant after accounting for
371 variation in flowering time ($p = 0.0003$). All NILs that expressed a period length from 22-23 hrs
372 also failed to survive the winter, while genotypes expressing a circadian period greater than ~24
373 hrs survived (Fig. 3D). Thus, a circadian period slightly >24 hrs (but presumably less than the
374 extreme 28-hr cycles of the long-period *ztl* mutants) appears as a performance threshold in lines
375 with clocks modified by introgression of natural alleles. Annotated clock loci within the
376 introgressed regions include *CRY2*, *GI*, *LHY*, *PHYA*, *PIF3*, *PRR3*, *SRR1*, *TOC1*, and *ZTL*.

377 Based on the RIL measurements, genotypic variance components for phase and period
378 were significantly greater than zero or marginally so within each month (Table 1A, B and C, Fig.
379 2), and either decreased in magnitude over the course of the growing season (Ws-2 \times C24) or
380 remained of similar low magnitude over the season for phase or comparatively high magnitude
381 for period (Ws-2 \times *Ler*). Peak phase was 13.9 hrs in the Ws-2 \times C24 set on average over all
382 months of the growing season, 9.9 hrs in the Ws-2 \times *Ler* RIL set, and 14.3 hrs in the Col \times Me-
383 o/Rd-0 set in the one month it was measured; the 4-hr delay conferred by C24 *vs.* *Ler* (when
384 crossed to Ws-2) is consistent with previously documented effects of C24 *vs.* *Ler* alleles on clock
385 phenotypes (see Discussion). Mean period length was similar among all RIL sets, namely 23.9
386 hrs in the Ws-2 \times C24 set, 23.7 hrs in the Ws-2 \times *Ler* RIL set and 24.5 in the Me-o/Rd-0 \times Col
387 RIL. Period and phase were always positively correlated (e.g., $r = 0.36-0.67$, $p < 0.05$ on average
388 for multiple RIL sets that were measured in multiple months).

389 Season strongly affected average circadian phase (Fig. 4A, B; Table 2A and B, cf month
390 effect) in the two populations where multiple months of circadian data were collected. Compared
391 to both June and September, average phase was delayed in July by approximately 1 hour (Ws-2
392 × C24 population, Fig. 4A) or 2 hours (Ws-2 × *Ler* population, Fig. 4B); the different
393 populations thus responded to monthly abiotic differences in a parallel way. Because the
394 plantings of the two RIL populations were offset by approximately one week, the results suggest
395 that slight environmental differences between sequential weeks were outweighed by larger
396 differences among the months of June, July, and September. In both populations, average
397 differences in circadian period length across months were of smaller magnitude than differences
398 for phase (Table 2A and B, cf month effect for period vs. phase).

399 A number of known clock inputs varied over the growing season, including temperature,
400 photoperiod and irradiance (Fig. 1). It was not possible to test for clock-micrometeorological
401 correlations because each RIL set had only three plantings, as such there were only 3 effective
402 data points for comparison. Nevertheless, only mean minimum temperature exhibited a chevron-
403 pattern of response similar to the RILs, suggesting this environmental variable could be an
404 important input. We used growth-chamber experiments that manipulated one abiotic factor to
405 further evaluate the specific role of field temperatures and photoperiods in determining clock
406 phenotypes. In these experiments, either day / night temperature cycles (with constant light) or
407 photoperiod duration (with constant temperatures) were matched to field conditions during
408 entrainment. Circadian period measured in the growth chamber under either photic cycles ($r =$
409 0.61 and $p < 0.0001$) or thermal cycles ($r = 0.22$ and $p < 0.05$) was significantly correlated with
410 period measured in the field. Phase values measured in the growth chamber under photic cycles
411 were not correlated with those measured in the field ($r = -0.07$ and $p = 0.54$), nor were phase

412 values estimated under thermal cycles that simulated the field ($r = 0.13$ and $p = 0.25$). Thus,
413 while circadian period in the field could be predicted from controlled photic or thermal
414 treatments, phase could not be.

415 Genotype \times month interactions were significant (Table 2A and B), indicating that the
416 rank order of genotypes (or variance among genotypes) shifted across months. Pairwise
417 correlations between months (r_{GE}) were often not significantly different from 0 for phase,
418 indicating that genotypic phase values in June were unrelated to phase as measured in other
419 months of the growing season. r_{GE} for period, by contrast, were significant in the majority of
420 cases (Table 2C).

421 With regard to performance effects of quantitative clock variation, period lengths closer
422 to 24.5-25 hrs (or delayed phase, which, again, was positively correlated with period) were
423 associated with higher values of performance in the RILs (Fig. 5A, B, C). Specifically, in the
424 Ws-2 \times C24 RILs, longer period was associated with increased performance as estimated from
425 PCA1 ($R^2 = 0.11$, $p = 0.0031$). Delayed phase was associated with increased values of PCA1 in
426 both the Col \times Rd-0/Me-0 ($R^2 = 0.13$, $p = 0.0009$) and the Ws-2 \times Ler RILs ($R^2 = 0.07$, $p =$
427 0.037). The consistent pattern of longer period or delayed phase being associated with
428 performance despite the genetic heterogeneity of the RILs, the environmental heterogeneity
429 within a field site, and the differences across geographic regions, suggests a biologically
430 meaningful performance association with the clock. These results also parallel those obtained in
431 the NILs indicating that a circadian period slightly longer than 24 hrs is associated with
432 improved performance in comparison to periods closer to 22 hrs (Fig. 3C and D).

433

434 **Discussion**

435 The environment changes rapidly on a diurnal basis, and the circadian clock may provide
436 a means to perceive these changes and adaptively time biological processes across the 24-hr day.
437 Yet, little is known about how the clock affects performance in natural settings. In the current
438 study, we raised diverse experimental genetic lines in seasonal field settings. Mutants and NILs
439 used here are effective tools for testing the fitness consequences of discrete phenotypes, while
440 experimental crosses segregating for naturally occurring alleles display a quantitative distribution
441 of phenotypes more representative of natural populations. The experimental design adopted here
442 enables estimation of clock sensitivity to season, of statistical genetic parameters that determine
443 adaptive evolution, and of associations between quantitative clock parameters and components of
444 fitness.

445 To test the adaptive significance of the circadian clock, we measured performance both in
446 well-characterized mutants with large-effect clock perturbations as well as in circadian NILs
447 with comparatively small-effect genomic introgressions. We observed a reduction in two
448 components of fitness, fecundity in a spring cohort and lifespan in a fall cohort, in clock mutants
449 with large differences in period (i.e., ± 4 hr differences from 24-hr cycle). We attribute reduced
450 performance of the mutants to clock malfunction, because the mutations are not annotated as
451 acting pleiotropically outside clock pathways. As for the mutants, fitness was reduced among
452 NIL genotypes with 22-23 hr period lengths in comparison to genotypes with cycles near 24-25
453 hrs. The results are consistent with adaptive hypotheses that a functional and correctly-timed
454 clock enhances fitness in natural settings. Further, clock loci within the introgressed regions
455 include genes from the input pathway (*PHYA*, *PIF3* and *CRY2*), the oscillator (*TOC1*, *ZTL*, *LHY*,
456 *SRR1* and *PRR3*), and an output pathway (*GI*) of the circadian clock (Edwards *et al.* 2005),

457 suggesting that allelic substitutions at a handful of loci in any of the three clock components can
458 have dramatic fitness effects.

459 Results from the RILs indicate how the circadian clock responds to the environment in
460 lines segregating at multiple clock loci and provide information about the quantitative-genetic
461 architecture of the clock. Circadian phase on average over all genotypes was sensitive to
462 environmental inputs that varied over the growing season, such that phase was delayed 1-2 hours
463 on average in July relative to June and September. Notably, this pattern was observed in 2 RIL
464 sets sampled in two successive weeks within each month, suggesting that smaller inter-weekly
465 abiotic changes are outweighed by larger changes across months and demonstrating that
466 genetically distinct lines respond in a similar manner to seasonal changes. A number of known
467 clock inputs varied over the growing season, including temperature, photoperiod and irradiance
468 (Fig. 1). Although it was only a qualitative observation, the advance in phase in the two months
469 with cooler overnight temperatures (June and September) is consistent with the observation that
470 low night-time temperatures can advance phase and shorten period (Anwer *et al.* 2014;
471 Boikoglou *et al.* 2011) and that temperature differentials as low as 1°C can affect clock
472 entrainment (Bohn *et al.* 2003). Although additional years of data are needed to diagnose the
473 causal environmental input(s), the potential association of phase with temperature (or
474 temperature in combination with other factors) is consistent with the recent observation in rice
475 that temperature more so than photoperiod affected expression patterns of clock-related genes in
476 the field (Matsuzaki *et al.* 2015).

477 The preceding results describe how the *environment* affects circadian period or phase on
478 average, but it is also important to predict *genotypic* values within an environment. The circadian
479 clock of diverse genotypes in the field may be entrained primarily by one factor, for instance,

480 temperature, leading to a strong genotypic association of clock period or phase across
481 environments with similar thermal cycles; alternatively, the clock may be set by a combination
482 of multiple environmental factors. To test for the effect of individual factors on clock parameters,
483 we simulated field temperatures and photoperiods in controlled growth-chamber settings and
484 tested for genotypic associations between circadian parameters measured in the field *vs.* the
485 growth chamber. Circadian period estimated for diverse genotypes in controlled photic or
486 thermal cycles simulating a July field environment was significantly associated with period of
487 those genotypes measured in the field in July. However, neither genotypic phase values
488 estimated in the growth chamber under photic cycles nor under thermal cycles were correlated
489 with those measured in the field in July. These patterns of association (or lack thereof) require
490 further investigation, but have a few implications. First, the results of prior studies examining
491 period phenotypes (*e.g.*, characterizing genetic loci or QTL affecting period) under controlled
492 photoperiod or temperature settings (Edwards *et al.* 2005; Lou *et al.* 2011; Michael *et al.* 2003b;
493 Swarup *et al.* 1999) may be directly relevant to clock behaviors in matching field settings. On the
494 other hand, the results suggest either 1) that multiple, simultaneously varying clock inputs may
495 be integrated to yield circadian phase in the field, 2) that unmeasured factors may disrupt
496 associations between the field and growth chamber, or 3) that some environmental features (such
497 as high irradiance) cannot be adequately replicated in controlled settings, any of which are
498 relevant to studies translating results from controlled to natural settings. While partitioning the
499 contribution of diverse potential input(s) to the clock requires further investigation in the field
500 (Matsuzaki *et al.* 2015), the current results nevertheless provide insights in segregating plant
501 populations as to the magnitude of quantitative variation in period and phase that may be

502 expressed over the growing season, including average differences across months of the growing
503 season, average differences among genotypes, and genotype \times month interactions.

504 The evolutionary potential of a trait is determined in part by its quantitative-genetic
505 architecture, including the relative magnitude of genetic variances and covariances with other
506 traits (Falconer & Mackay 1996). The pattern observed here, of significant line variances in all
507 months of the growing season, is consistent with prior studies mapping QTL for clock
508 parameters in controlled settings in *A. thaliana* (Edwards *et al.* 2005; Lou *et al.* 2011; Michael *et*
509 *al.* 2003b; Swarup *et al.* 1999), with significant variance components estimated for period in the
510 wild relative of *A. thaliana*, *Boecheera stricta* (Salmela *et al.* 2015), and with significant
511 variances estimated in a population of great tits (*Parus major*) (Helm & Visser 2010). With
512 regard to phenotypic differences between RIL sets, the observation that phase was advanced by
513 several hours in each month in the Ws-2 \times *Ler* relative to the Ws-2 \times C24 cross is consistent
514 with the past observation that alleles derived from *Ler* lead to faster cycling of the clock than do
515 C24 alleles (Dowson-Day & Millar 1999). Finally, the consistent observation of non-significant
516 r_{GE} (for phase) also suggests the potential for adaptive evolutionary responses of the circadian
517 clock to selection in different months of the growing season in wild populations segregating for
518 functionally similar alleles to those sampled in our experimental populations.

519 Phenotypic evolution is also influenced by the strength of selection. The possibility that
520 quantitative clock variation will affect performance is supported by the observation that altered
521 expression of the *A. thaliana* circadian gene *BBX32* leads to increased seed weight, flower
522 number and pod number in *Glycine max* (Preuss *et al.* 2012), and that altered expression of
523 another circadian gene *RDD1* in *Oryza sativa* causes decreased grain size (Iwamoto *et al.* 2009).
524 Further, quantitative clock variation is associated with gas-exchange in *B. rapa* (Edwards *et al.*

2011) and with growth and allocation in *Boechera stricta* (Salmela *et al.* 2015) grown in controlled settings. Here, we observe that quantitative clock variation correlates with size, reproductive timing and survival in the field. The proportion of variation explained by circadian period or phase ranged from 7-13% of the performance PCAs, which may be considered substantial for quantitative traits with many contributing genetic and environmental factors.

Extending beyond studies in controlled settings, the current results show that genotypes with discrete clock phenotypes differ in performance, including discrete phenotypes that reflect major perturbations in clock function arising from mutation as well as more subtle phenotypic differences arising from genomic introgression of alternative natural alleles. Further, quantitative clock variation is highly sensitive to and is associated with performance in complex field environments. The quantitative-genetic features estimated here indicate the potential for evolutionary responses to natural selection in heterogeneous wild populations harboring functionally variable clock alleles such as those sampled here.

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845 MTB, MK, ZG and SMW performed research; MJR, SLH, and CW analyzed data; CW and MJR
846 wrote the initial manuscript, and all authors participated in revision.

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848 **Data Accessibility** Genotypic means for each reported trait for the RIL sets, NILs and mutants have
849 been deposited at the Dryad Digital Repository (www.dx.doi.org/dryadXXXX).

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885 **Table 1.** Within-month ANOVAs partitioning variance between the main effects of RIL and
 886 microenvironmental effect of plate for circadian period and phase for Ws-2 x C24 RIL set (A),
 887 Ws-2 x Ler RIL set (B) and Col x Rd-0/Me-0 (C). z-values are reported for random effects.
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| A. Ws-2 × C24 RILs | | |
|-------------------------|-------------------|-------|
| | Line | Plate |
| June Period | 4.15**** | 1.32 |
| June Phase | 4.51**** | 1.68* |
| July Period | 3.04** | 0.61 |
| July Phase | 3.19*** | 1.01 |
| Sept. Period | 2.04* | 0.86 |
| Sept. Phase | 1.53 [□] | 0.66 |
| B. Ws-2 × Ler RILs | | |
| | Line | Plate |
| June Period | 2.93** | 0.65 |
| June Phase | 1.57 [□] | 0.82 |
| July Period | 3.94**** | 0.81 |
| July Phase | 1.87* | 1.27 |
| Sept. Period | 4.05**** | 1.75* |
| Sept. Phase | 1.54 [□] | 0.81 |
| C. Col × Rd-0/Me-0 RILs | | |
| | Line | Plate |
| Period | 0.12** | 0.003 |
| Phase | 1.19** | 0.09 |

889 Significance levels (p-value): **** < 0.0001, *** < 0.001, ** < 0.01, * < 0.05, [□] < 0.06
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911 **Table 2.** Quantitative genetic models for circadian period and phase under natural entrainment.
 912 Two-way ANOVAs of circadian period and phase for Ws-2 x C24 RIL set (A) and Ws-2 x Ler
 913 RIL set (B). The effect of month includes the 3 levels of June, July, and September. Across-
 914 month correlations are more consistently observed for circadian period than circadian phase (C).
 915 z-values are reported for random effects and f-values for fixed effects†.

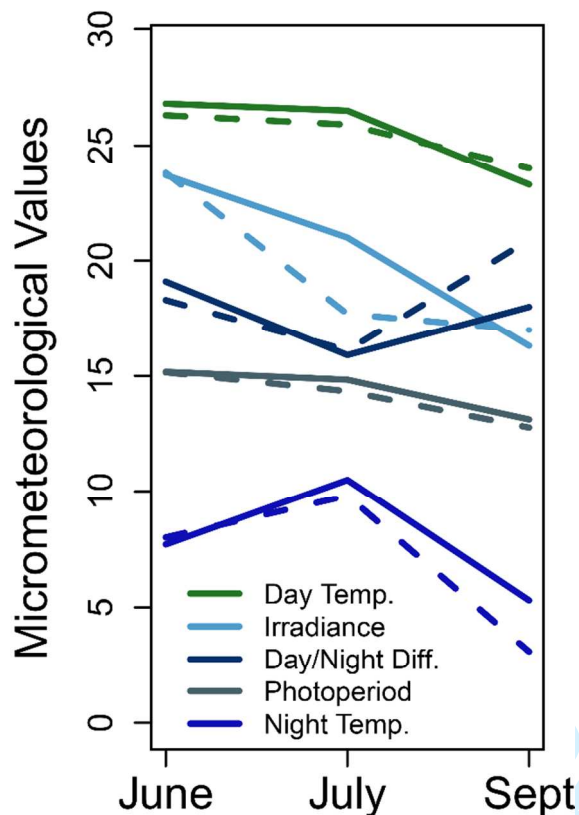
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| A. Ws-2 × C24 RILs | | |
|-----------------------|-------------------------|-----------------|
| | Period | Phase |
| RIL | 2.32* | 2.72** |
| Month† | 2.55 | 38.59***** |
| RIL × Month | 3.32*** | 3.43*** |
| Plate (Month) | 1.63 [‡] | 2.36** |
| B. Ws-2 × Ler RILs | | |
| | Period | Phase |
| RIL | 4.23***** | 0.29 |
| Month† | 8.63* | 56.19***** |
| RIL × Month | 2.41** | 2.19* |
| Plate (Month) | 2.71** | 1.91* |
| C. Trait Pair | | |
| | Ws-2 × C24 RILs | Ws-2 × Ler RILs |
| June and July Period | 0.55***** | 0.39** |
| June and Sept. Period | 0.10 | 0.43*** |
| July and Sept. Period | 0.06 | 0.42*** |
| June and July Phase | 0.20[‡] | -0.18 |
| June and Sept. Phase | 0.18 | -0.05 |
| July and Sept. Phase | -0.08 | -0.06 |

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921 Significance levels (p-value): ***** < 0.0001, *** < 0.001, ** < 0.01, * < 0.05, [‡] < 0.06

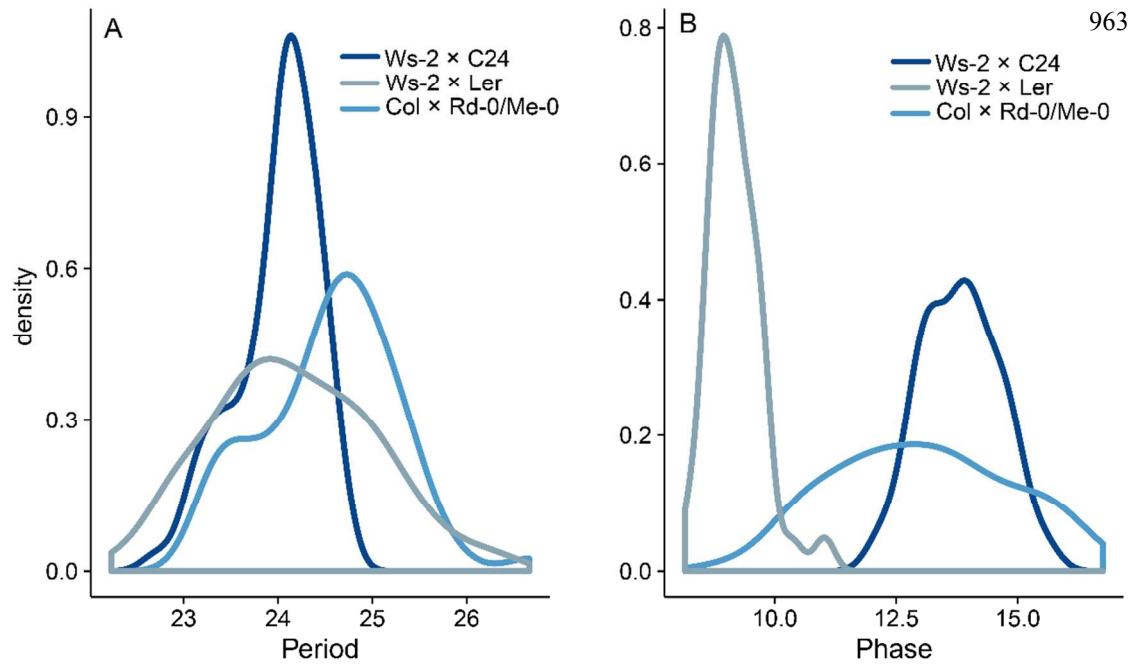
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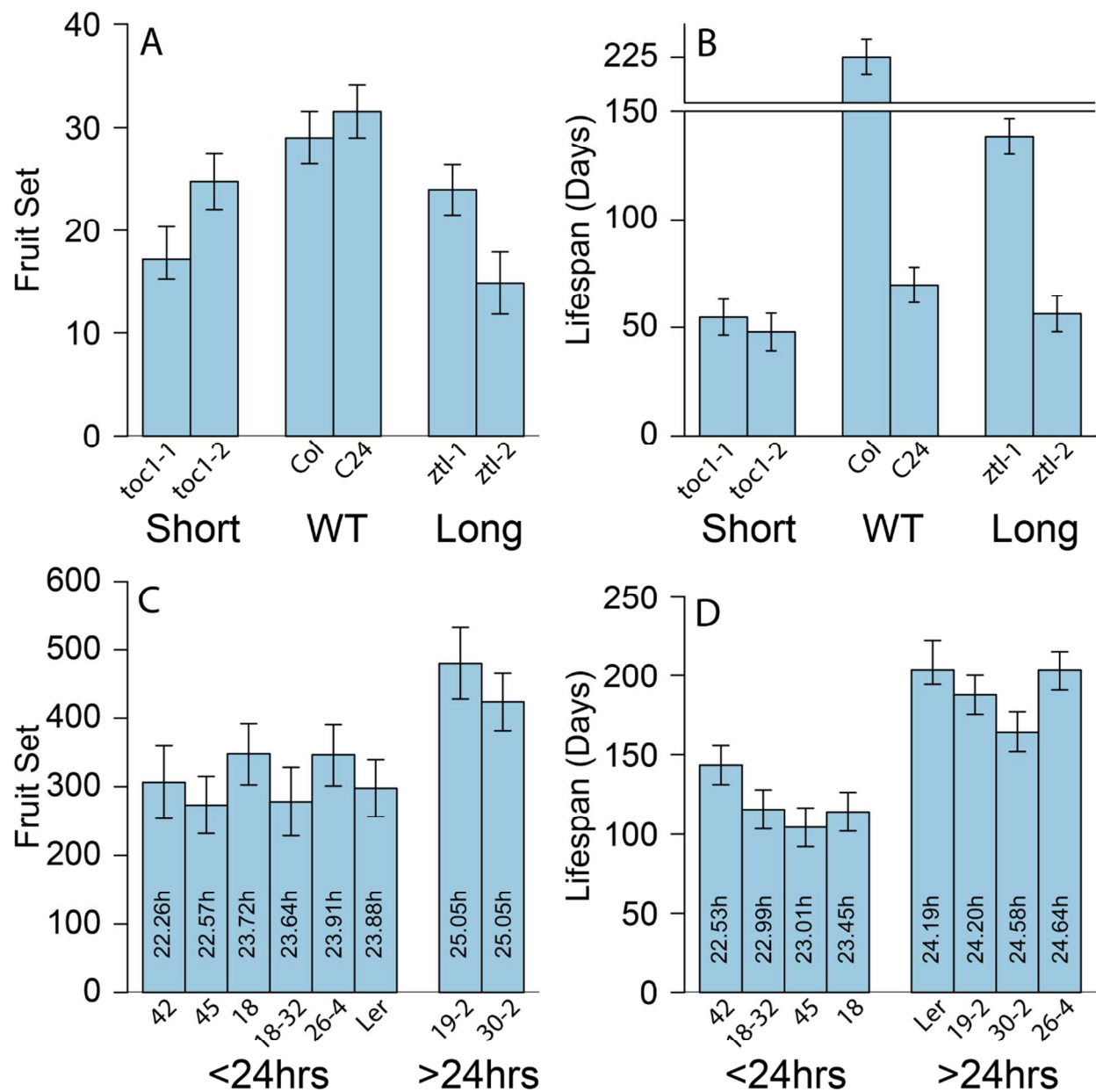
940 **Figure 1.** Mean values of micrometeorological data during the 5-day entrainment window in
941 June, July, and September in the field. Parameters include duration of photoperiod (hours),
942 average daily solar irradiance during entrainment window (MJ/m²/day), day air temperature (Day
943 Temp.; °C) and night air temperature (Night Temp.; °C) obtained during entrainment, and the
944 difference between the day and night temperatures (Day/Night Diff.; °C). Solid lines show
945 micrometeorological data for the Ws-2 × C24 RIL set, and dashed lines show data for the Ws-2
946 × Ler RIL set.

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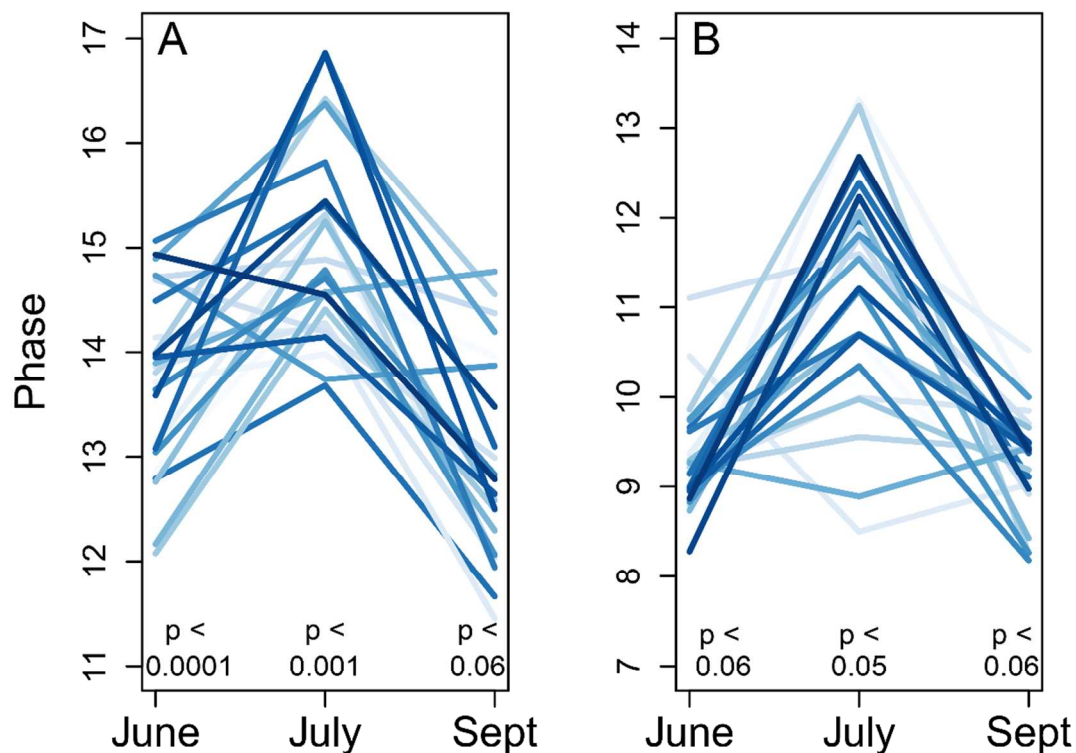


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Figure 2. Density curves of the genotypic means for circadian period (A) and phase (B) for the Ws-2 x C24, Ws-2 x Ler, and Col x Rd-0/Me-0 RIL sets.



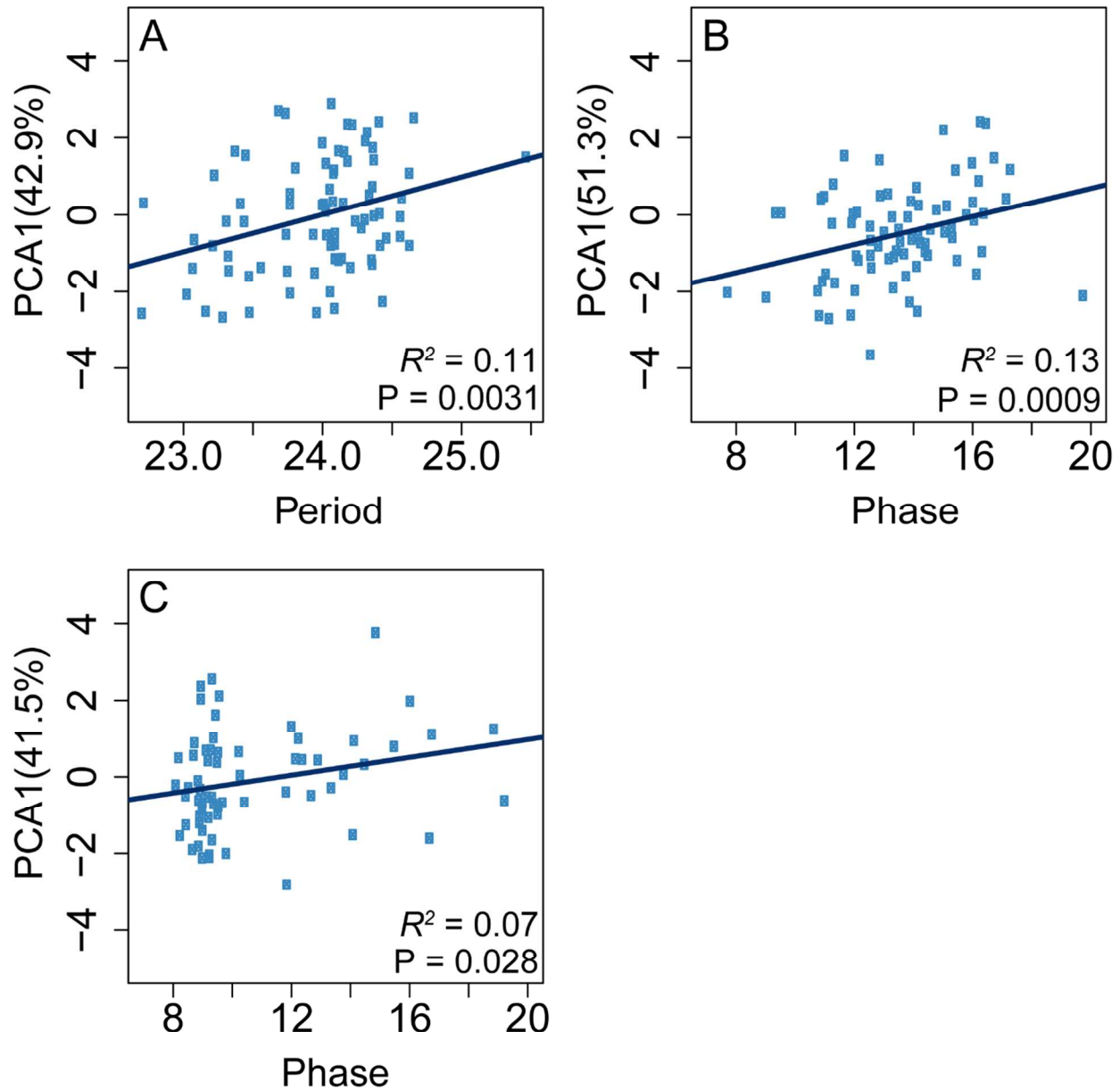
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 982 **Figure 3.** Perturbations in the circadian clock affect components of fitness. To understand clock
 983 mutation effects, comparisons should be made between the mutant genotype and its cognate
 984 wild-type; *ztl-1* is in the Col background, and *ztl-2*, *toc1-1*, and *toc1-2* are in C24. *ztl* and *toc1*
 985 mutant genotypes have reduced fruit set (fecundity) in a spring cohort (A) ($F = 10.7$, $p < 0.0001$,
 986 for mean difference between wild-type and mutant classes) and shorter lifespans in a fall cohort
 987 (B) ($F = 17.31$, $p < 0.0001$). Circadian NILs with naturally segregating alleles that result in a
 988 circadian period <24 hours have reduced fruit set in a spring cohort (C) ($F = 7.26$, $p = 0.009$) and
 989 reduced survival in a fall cohort (D) ($F = 16.35$, $p = 0.0001$). For (C), NIL period lengths were
 990 estimated by leaf movement measurements at 27°C , which approximates maximum daytime
 991 temperatures mid-season in the spring/summer in the field, while for (D), NIL period lengths
 992 were measured at 22°C , which approximates maximum daytime temperatures as plants are
 993 germinating and establishing in fall in the field.



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996 **Figure 4.** Circadian phase varies across monthly sampling points in two RIL sets. Lines on the
997 figures (A and B) connect genotypic values for a single RIL across months, with different
998 genotypes represented by different line shading. Values for 25 randomly selected genotypes
999 within the $W_s\text{-}2 \times C24$ RIL set (A) and the $W_s\text{-}2 \times Ler$ RIL set (B) are shown. P-values for the
1000 genotype effects are shown within each month. Phase was advanced by several hrs in each month
1001 in the $W_s\text{-}2 \times Ler$ cross relative to the $W_s\text{-}2 \times C24$ cross, consistent with the past observation
1002 that alleles derived from Ler lead to faster cycling of the clock than do C24 alleles.

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1022 **Figure 5.** Quantitative variation in the circadian clock is associated with PCAs for plant
 1023 performance loaded with plant size, reproductive timing and fecundity. Each dot represents the
 1024 mean phenotype for a single RIL. (A) PCA1 is associated with circadian period in the Ws-2 \times
 1025 C24 RIL population. (B) PCA1 is associated circadian phase in the Col \times Rd-0/Me-0 RIL
 1026 population. (C) PCA1 is associated circadian phase in the Ws-2 \times Ler RIL population.

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Data Accessibility

Genotypic means for each reported trait from each of the RIL sets and the mutants will be made publicly available on Dryad following publication of results.

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