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

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Abstract

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

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

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

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

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

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

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

Materials and Methods

Genetic lines

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

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

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

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

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

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

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

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

Field Experiments

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

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

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

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

Circadian assays

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

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

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

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

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

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

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

Statistical Analyses

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

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

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

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

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

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

Results

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

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

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

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

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

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

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

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

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 misfunction, 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),

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

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

The preceding results describe how the *environment* affects circadian period or phase on average, but it is also important to predict *genotypic* values within an environment. The circadian 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

expressed over the growing season, including average differences across months of the growing season, average differences among genotypes, and genotype × month interactions.

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

Phenotypic evolution is also influenced by the strength of selection. The possibility that quantitative clock variation will affect performance is supported by the observation that altered expression of the *A. thaliana* circadian gene *BBX32* leads to increased seed weight, flower number and pod number in *Glycine max* (Preuss *et al.* 2012), and that altered expression of another circadian gene *RDD1* in *Oryza sativa* causes decreased grain size (Iwamoto *et al.* 2009). 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.

References and Notes

Alonso-Blanco C, El-Assal SE-D, Coupland G, Koornneef M (1998) Analysis of natural allelic variation at flowering time loci in the Landsberg erecta and Cape Verde Islands ecotypes of *Arabidopsis thaliana*. *Genetics* **149**, 749-764.

Anwer MU, Boikoglou E, Herrero E*, et al.* (2014) Natural variation reveals that intracellular distribution of *ELF3* protein is associated with function in the circadian clock. *Elife* **3**, e02206.

Anwer MU, Davis SJ (2013) An overview of natural variation studies in the *Arabidopsis thaliana* circadian clock **24**, 422-429.

Atamian HS, Creux NM, Brown EA*, et al.* (2016) Circadian regulation of sunflower heliotropism, floral orientation, and pollinator visits. *Science* **353**, 587-590.

Beaver L, Gvakharia B, Vollintine T*, et al.* (2002) Loss of circadian clock function decreases reproductive fitness in males of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences* **99**, 2134-2139.

Bohn A, Hinderlich S, Hütt M-T, Kaiser F, Lüttge U (2003) Identification of rhythmic subsystems in the circadian cycle of crassulacean acid metabolism under thermoperiodic perturbations. *Biological Chemistry* **384**, 721-728.

Boikoglou E (2008) *Quantitative genetic analysis of temperature entrainment in the Arabidopsis thaliana circadian clock*, Max-Planck-Institut für Pflanzenzüchtungsforschung.

Boikoglou E, Ma ZS, von Korff M*, et al.* (2011) Environmental memory from a circadian oscillator: The *Arabidopsis thaliana* clock differentially integrates perception of photic vs. thermal entrainment. *Genetics* **189**, 655-U796.

Brachi B, Faure N, Horton M, *et al.* (2010) Linkage and association mapping of *Arabidopsis thaliana* flowering time in nature. *PLoS Genetics* **6**, e1000940.
Clausen J, Keck D, Hiesey W (1940) Experimental studies on the nature of species I. Effects of varied environments on western North American plants. Carnegie Institution of Washington. *Washington DC., USA*.
Covington MF, Maloof JN, Straume M, Kay SA, Harmer SL (2008) Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. *Genome Biology* **9**.
Crepy MA, Casal JJ (2015) Photoreceptor mediated kin recognition in plants. *New Phytologist* **205**, 329-338.
de Montaigu A, Giakountis A, Rubin M, *et al.* (2015) Natural diversity in daily rhythms of gene expression contributes to phenotypic variation. *Proceedings of the National Academy of Sciences* **112**, 905-910.
Dittmar EL, Oakley CG, Ågren J, Schemske DW (2014) Flowering time QTL in natural populations of *Arabidopsis thaliana* and implications for their adaptive value. *Molecular Ecology* **23**, 4291-4303.
Dodd AN, Salathia N, Hall A, *et al.* (2005) Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science* **309**, 630-633.
Dorn LA, Pyle EH, Schmitt J (2000) Plasticity to light cues and resources in *Arabidopsis thaliana*: testing for adaptve value and costs *Evolution* **54**, 1982-1994.
Dowson-Day MJ, Millar AJ (1999) Circadian dysfunction causes aberrant hypocotyl elongation patterns in *Arabidopsis*. *Plant Journal* **17**, 63-71.
Doyle MR, Davis SJ, Bastow RM, *et al.* (2002) The *ELF4* gene controls circadian rhythms and flowering time in *Arabidopsis thaliana*. *Nature* **419**, 74-77.
Dudley S, Schmitt J (1995) Genetic differentiation in morphological responses to simulated foliage shade between populations of *Impatiens capensis* from open and woodland sites. *Functional Ecology*, 655-666.
Dudley SA, Schmitt J (1996) Testing the adaptive plasticity hypothesis: density-dependent selection on manipulated stem length in *Impatiens capensis*. *American Naturalist*, 445-465.
Duffield GE (2003) DNA microarray analyses of circadian timing: The genomic basis of biological time. *Journal of Neuroendocrinology* **15**, 991-1002.
Dunlap JC (1999) Molecular bases for circadian clocks. *Cell* **96**, 271-290.
Edgar RS, Green EW, Zhao Y, *et al.* (2012) Peroxiredoxins are conserved markers of circadian rhythms. *Nature* **485**, 459-464.
Edwards CE, Ewers BE, Williams DG, *et al.* (2011) The genetic architecture of ecophysiological and circadian traits in *Brassica rapa*. *Genetics* **189**, 375-390.
Edwards KD, Lynn JR, Gyula P, Nagy F, Millar AJ (2005) Natural allelic variation in the temperature-compensation mechanisms of the *Arabidopsis thaliana* circadian clock. *Genetics* **170**, 387-400.
Emerson KJ, Bradshaw WE, Holzapfel CM (2008) Concordance of the circadian clock with the environment is necessary to maximize fitness in natural populations. *Evolution* **62**, 979-983.
Falconer DS, Mackay TFC (1996) *Introduction to Quantitative Genetics*, 4th edn.
Farre EM, Weise SE (2012) The interactions between the circadian clock and primary metabolism. *Current Opinion in Plant Biology* **15**, 293-300.
Getty T (1996) The maintenance of phenotypic plasticity as a signal detection problem. *American Naturalist*, 378-385.
Graf A, Schlereth A, Stitt M, Smith AM (2010) Circadian control of carbohydrate availability for growth in *Arabidopsis* plants at night. *Proceedings of the National Academy of Sciences* **107**, 9458-9463.
Hall A, Kozma‐Bognár L, Bastow RM, Nagy F, Millar AJ (2002) Distinct regulation of *CAB* and *PHYB* gene expression by similar circadian clocks. *The Plant Journal* **32**, 529-537.
Harmer SL (2009) The circadian system in higher plants. *Annual review of plant biology* **60**, 357-377.
Helm B, Visser ME (2010) Heritable circadian period length in a wild bird population. *Proceedings of the Royal Society B: Biological Sciences* **277**, 3335-3342.
Hirschie Johnson C, Elliott JA, Foster R (2003) Entrainment of circadian programs. *Chronobiology International* **20**, 741-774.
Iwamoto M, Higo K, Takano M (2009) Circadian clock- and phytochrome-regulated *Dof*-like gene, *Rdd1*, is associated with grain size in rice. *Plant, Cell & Environment* **32**, 592-603.
Izawa T, Mihara M, Suzuki Y, *et al.* (2011) *Os-GIGANTEA* confers robust diurnal rhythms on the global transcriptome of rice in the field. *The Plant Cell* **23**, 1741-1755.
Johansson M, Ramos-Sánchez JM, Conde D, *et al.* (2015) Role of the circadian clock in cold acclimation and winter dormancy in perennial plants. In: *Advances in Plant Dormancy*, pp. 51-74. Springer.
Johnson CH, Kondo T (1992) Light pulses induce" singular" behavior and shorten the period of the circadian phototaxis rhythm in the CW15 strain of *Chlamydomonas*. *Journal of Biological Rhythms* **7**, 313-327.

Kim Y, Yeom M, Kim H, *et al.* (2012) *GIGANTEA* and *EARLY FLOWERING 4* in *Arabidopsis* exhibit differential phase-specific genetic influences over a diurnal cycle. *Molecular Plant* **5**, 678-687.
Korves TM, Schmid KJ, Caicedo AL, *et al.* (2007) Fitness effects associated with the major flowering time gene *FRIGIDA* in *Arabidopsis thaliana* in the field. *The American Naturalist* **169**, E141-E157.
Leinonen PH, Remington DL, Leppälä J, Savolainen O (2013) Genetic basis of local adaptation and flowering time variation in *Arabidopsis lyrata*. *Molecular Ecology* **22**, 709-723.
Lou P, Xie Q, Xu X, *et al.* (2011) Genetic architecture of the circadian clock and flowering time in *Brassica rapa*. *Theoretical and Applied Genetics* **123**, 397-409.
Lowrey PL, Takahashi JS (2011) Genetics of circadian rhythms in mammalian model organisms. *Genetics of Circadian Rhythms* **74**, 175-230.
Lynch M, Walsh B (1998) *Genetics and analysis of quantitative traits* Sinauer Sunderland, MA.
Matsuzaki J, Kawahara Y, Izawa T (2015) Punctual transcriptional regulation by the rice circadian clock under fluctuating field conditions. *The Plant Cell* **27**, 633-648.
McClung CR (2006) Plant circadian rhythms. *The Plant Cell* **18**, 792-803.
McClung CR (2013) Beyond *Arabidopsis*: the circadian clock in non-model plant species **24**, 430-436.
McWatters HG, Bastow RM, Hall A, Millar AJ (2000) The *ELF3* zeitnehmer regulates light signalling to the circadian clock. *Nature* **408**, 716-720.
Michael TP, Mockler TC, Breton G, *et al.* (2008) Network discovery pipeline elucidates conserved time-of-day-specific cis-regulatory modules. *PLoS Genetics* **4**, 1-17.
Michael TP, Salome PA, McClung CR (2003a) Two *Arabidopsis* circadian oscillators can be distinguished by differential temperature sensitivity. *Proceedings of the National Academy of Sciences* **100**, 6878-6883.
Michael TP, Salomé PA, Yu HJ, *et al.* (2003b) Enhanced fitness conferred by naturally occurring variation in the circadian clock. *Science* **302**, 1049-1053.
Millar AJ (2004) Input signals to the plant circadian clock. *Journal of Experimental Botany* **55**, 277-283.
Millar AJ, Carre IA, Strayer CA, Chua NH, Kay SA (1995) Circadian clock mutants in *Arabidopsis* identified by luciferase imaging. *Science* **267**, 1161-1163.
Millar AJ, Short SR, Chua NH, Kay SA (1992) A novel circadian phenotype based on firefly luciferase expression in transgenic plants. *Plant Cell* **4**, 1075-1087.
Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497.
Nohales MA, Kay SA (2016) Molecular mechanisms at the core of the plant circadian oscillator. *Nature Structural & Molecular Biology* **23**, 1061-1069.
Ouyang Y, Andersson CR, Kondo T, Golden SS, Johnson CH (1998) Resonating circadian clocks enhance fitness in cyanobacteria. *Proceedings of the National Academy of Sciences* **95**, 8660-8664.
Peirson BE (2015) Plasticity, stability, and yield: The origins of Anthony David Bradshaw's model of adaptive phenotypic plasticity. *Studies in History and Philosophy of Science Part C: Studies in History and Philosophy of Biological and Biomedical Sciences* **50**, 51-66.
Plautz JD, Straume M, Stanewsky R, *et al.* (1997) Quantitative analysis of *Drosophila* period gene transcription in living animals. *Journal of Biological Rhythms* **12**, 204-217.
Preuss SB, Meister R, Xu Q, *et al.* (2012) Expression of the *Arabidopsis thaliana BBX32* gene in soybean increases grain yield. *PLoS One* **7**, e30717.
Roden LC, Song HR, Jackson S, Morris K, Carre IA (2002) Floral responses to photoperiod are correlated with the timing of rhythmic expression relative to dawn and dusk in *Arabidopsis*. *Proceedings of the National Academy of Sciences* **99**, 13313-13318.
Salmela MJ, Greenham K, Lou P, *et al.* (2015) Variation in circadian rhythms is maintained among and within populations in *Boechera stricta*. *Plant, Cell & Environment*.
SAS (1999) *SAS/STAT User's Guide* SAS Institute, Cary, NC.
Scheiner SM, Holt RD (2012) The genetics of phenotypic plasticity. X. Variation versus uncertainty. *Ecology and Evolution* **2**, 751-767.
Schmitt J, Dudley SA, Pigliucci M (1999) Manipulative approaches to testing adaptive plasticity: phytochrome-mediated shade-avoidance responses in plants. *The American Naturalist* **154**, S43-S54.
Smith H (2000) Phytochromes and light signal perception by plants—an emerging synthesis. *Nature* **407**, 585-591.
Somers DE, Devlin PF, Kay SA (1998) Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock. *Science* **282**, 1488-1490.
Somers DE, Kim W-Y, Geng R (2004) The F-box protein *ZEITLUPE* confers dosage-dependent control on the circadian clock, photomorphogenesis, and flowering time. *The Plant Cell* **16**, 769-782.

Strayer C, Oyama T, Schultz TF, *et al.* (2000) Cloning of the *Arabidopsis* clock gene *TOC1*, an autoregulatory response regulator homolog. *Science* **289**, 768-771.
Swarup K, Alonso‐Blanco C, Lynn JR, *et al.* (1999) Natural allelic variation identifies new genes in the *Arabidopsis* circadian system. *The Plant Journal* **20**, 67-77.
Thain SC, Hall A, Millar AJ (2000) Functional independence of circadian clocks that regulate plant gene expression. *Current Biology* **10**, 951-956.
Weinig C (2000) Differing selection in alternative competitive environments: shade-avoidance responses and germination timing. *Evolution* **54**, 124-136.
Weinig C, Dorn LA, Kane NC, *et al.* (2003) Heterogeneous selection at specific loci in natural environments in *Arabidopsis thaliana*. *Genetics* **165**, 321-329.
Wilczek AM, Roe JL, Knapp MC, *et al.* (2009) Effects of genetic perturbation on seasonal life history plasticity. *Science* **323**, 930-934.
Yan OY, Andersson CR, Kondo T, Golden SS, Johnson CH (1998) Resonating circadian clocks enhance fitness in cyanobacteria. *Proceedings of the National Academy of Sciences* **95**, 8660-8664.
Yerushalmi S, Yakir E, Green RM (2011) Circadian clocks and adaptation in *Arabidopsis*. *Molecular Ecology* **20**, 1155-1165.
Alonso-Blanco C, El-Assal SE-D, Coupland G, Koornneef M (1998) Analysis of natural allelic variation at flowering time loci in the Landsberg erecta and Cape Verde Islands ecotypes of *Arabidopsis thaliana*. *Genetics* **149**, 749-764.
Anwer MU, Boikoglou E, Herrero E, *et al.* (2014) Natural variation reveals that intracellular distribution of *ELF3* protein is associated with function in the circadian clock. *Elife* **3**, e02206.
Anwer MU, Davis SJ (2013) An overview of natural variation studies in the *Arabidopsis thaliana* circadian clock **24**, 422-429.
Atamian HS, Creux NM, Brown EA, *et al.* (2016) Circadian regulation of sunflower heliotropism, floral orientation, and pollinator visits. *Science* **353**, 587-590.
Beaver L, Gvakharia B, Vollintine T, *et al.* (2002) Loss of circadian clock function decreases reproductive fitness in males of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences* **99**, 2134-2139.
Bohn A, Hinderlich S, Hütt M-T, Kaiser F, Lüttge U (2003) Identification of rhythmic subsystems in the circadian cycle of crassulacean acid metabolism under thermoperiodic perturbations. *Biological Chemistry* **384**, 721-728.
Boikoglou E (2008) *Quantitative genetic analysis of temperature entrainment in the Arabidopsis thaliana circadian clock*, Max-Planck-Institut für Pflanzenzüchtungsforschung.
Boikoglou E, Ma ZS, von Korff M, *et al.* (2011) Environmental memory from a circadian oscillator: The *Arabidopsis thaliana* clock differentially integrates perception of photic vs. thermal entrainment. *Genetics* **189**, 655-U796.
Brachi B, Faure N, Horton M, *et al.* (2010) Linkage and association mapping of *Arabidopsis thaliana* flowering time in nature. *PLoS Genetics* **6**, e1000940.
Clausen J, Keck D, Hiesey W (1940) Experimental studies on the nature of species I. Effects of varied environments on western North American plants. Carnegie Institution of Washington. *Washington DC., USA*.
Covington MF, Maloof JN, Straume M, Kay SA, Harmer SL (2008) Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. *Genome Biology* **9**.
Crepy MA, Casal JJ (2015) Photoreceptor mediated kin recognition in plants. *New Phytologist* **205**, 329-338.
de Montaigu A, Giakountis A, Rubin M, *et al.* (2015) Natural diversity in daily rhythms of gene expression contributes to phenotypic variation. *Proceedings of the National Academy of Sciences* **112**, 905-910.
Dittmar EL, Oakley CG, Ågren J, Schemske DW (2014) Flowering time QTL in natural populations of *Arabidopsis thaliana* and implications for their adaptive value. *Molecular Ecology* **23**, 4291-4303.
Dodd AN, Salathia N, Hall A, *et al.* (2005) Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science* **309**, 630-633.
Dorn LA, Pyle EH, Schmitt J (2000) Plasticity to light cues and resources in *Arabidopsis thaliana*: testing for adaptve value and costs *Evolution* **54**, 1982-1994.
Dowson-Day MJ, Millar AJ (1999) Circadian dysfunction causes aberrant hypocotyl elongation patterns in *Arabidopsis*. *Plant Journal* **17**, 63-71.
Doyle MR, Davis SJ, Bastow RM, *et al.* (2002) The *ELF4* gene controls circadian rhythms and flowering time in *Arabidopsis thaliana*. *Nature* **419**, 74-77.
Dudley S, Schmitt J (1995) Genetic differentiation in morphological responses to simulated foliage shade between populations of *Impatiens capensis* from open and woodland sites. *Functional Ecology*, 655-666.

Dudley SA, Schmitt J (1996) Testing the adaptive plasticity hypothesis: density-dependent selection on manipulated stem length in *Impatiens capensis*. *American Naturalist*, 445-465.

Duffield GE (2003) DNA microarray analyses of circadian timing: The genomic basis of biological time. *Journal of Neuroendocrinology* **15**, 991-1002.

Dunlap JC (1999) Molecular bases for circadian clocks. *Cell* **96**, 271-290.

Edgar RS, Green EW, Zhao Y*, et al.* (2012) Peroxiredoxins are conserved markers of circadian rhythms. *Nature* **485**, 459-464.

Edwards CE, Ewers BE, Williams DG*, et al.* (2011) The genetic architecture of ecophysiological and circadian traits in *Brassica rapa*. *Genetics* **189**, 375-390.

Edwards KD, Lynn JR, Gyula P, Nagy F, Millar AJ (2005) Natural allelic variation in the temperature-compensation mechanisms of the *Arabidopsis thaliana* circadian clock. *Genetics* **170**, 387-400.

Emerson KJ, Bradshaw WE, Holzapfel CM (2008) Concordance of the circadian clock with the environment is necessary to maximize fitness in natural populations. *Evolution* **62**, 979-983.

Falconer DS, Mackay TFC (1996) *Introduction to Quantitative Genetics*, 4th edn.

Farre EM, Weise SE (2012) The interactions between the circadian clock and primary metabolism. *Current Opinion in Plant Biology* **15**, 293-300.

Getty T (1996) The maintenance of phenotypic plasticity as a signal detection problem. *American Naturalist*, 378-385.

Graf A, Schlereth A, Stitt M, Smith AM (2010) Circadian control of carbohydrate availability for growth in *Arabidopsis* plants at night. *Proceedings of the National Academy of Sciences* **107**, 9458-9463.

Hall A, Kozma-Bognár L, Bastow RM, Nagy F, Millar AJ (2002) Distinct regulation of *CAB* and *PHYB* gene expression by similar circadian clocks. *The Plant Journal* **32**, 529-537.

Harmer SL (2009) The circadian system in higher plants. *Annual review of plant biology* **60**, 357-377.

Helm B, Visser ME (2010) Heritable circadian period length in a wild bird population. *Proceedings of the Royal Society B: Biological Sciences* **277**, 3335-3342.

Hirschie Johnson C, Elliott JA, Foster R (2003) Entrainment of circadian programs. *Chronobiology International* **20**, 741-774.

Iwamoto M, Higo K, Takano M (2009) Circadian clock- and phytochrome-regulated *Dof*-like gene, *Rdd1*, is associated with grain size in rice. *Plant, Cell & Environment* **32**, 592-603.

Izawa T, Mihara M, Suzuki Y*, et al.* (2011) *Os-GIGANTEA* confers robust diurnal rhythms on the global transcriptome of rice in the field. *The Plant Cell* **23**, 1741-1755.

Johansson M, Ramos-Sánchez JM, Conde D*, et al.* (2015) Role of the circadian clock in cold acclimation and winter dormancy in perennial plants. In: *Advances in Plant Dormancy*, pp. 51-74. Springer.

Johnson CH, Kondo T (1992) Light pulses induce" singular" behavior and shorten the period of the circadian phototaxis rhythm in the CW15 strain of *Chlamydomonas*. *Journal of Biological Rhythms* **7**, 313-327.

Kim Y, Yeom M, Kim H*, et al.* (2012) *GIGANTEA* and *EARLY FLOWERING 4* in *Arabidopsis* exhibit differential phase-specific genetic influences over a diurnal cycle. *Molecular Plant* **5**, 678-687.

Korves TM, Schmid KJ, Caicedo AL*, et al.* (2007) Fitness effects associated with the major flowering time gene *FRIGIDA* in *Arabidopsis thaliana* in the field. *The American Naturalist* **169**, E141-E157.

Leinonen PH, Remington DL, Leppälä J, Savolainen O (2013) Genetic basis of local adaptation and flowering time variation in *Arabidopsis lyrata*. *Molecular Ecology* **22**, 709-723.

Lou P, Xie Q, Xu X*, et al.* (2011) Genetic architecture of the circadian clock and flowering time in *Brassica rapa*. *Theoretical and Applied Genetics* **123**, 397-409.

Lowrey PL, Takahashi JS (2011) Genetics of circadian rhythms in mammalian model organisms. *Genetics of Circadian Rhythms* **74**, 175-230.

Lynch M, Walsh B (1998) *Genetics and analysis of quantitative traits* Sinauer Sunderland, MA.

Matsuzaki J, Kawahara Y, Izawa T (2015) Punctual transcriptional regulation by the rice circadian clock under fluctuating field conditions. *The Plant Cell* **27**, 633-648.

McClung CR (2006) Plant circadian rhythms. *The Plant Cell* **18**, 792-803.

McClung CR (2013) Beyond *Arabidopsis*: the circadian clock in non-model plant species **24**, 430-436.

McWatters HG, Bastow RM, Hall A, Millar AJ (2000) The *ELF3* zeitnehmer regulates light signalling to the circadian clock. *Nature* **408**, 716-720.

Michael TP, Mockler TC, Breton G*, et al.* (2008) Network discovery pipeline elucidates conserved time-of-day-specific cis-regulatory modules. *PLoS Genetics* **4**, 1-17.

Michael TP, Salome PA, McClung CR (2003a) Two *Arabidopsis* circadian oscillators can be distinguished by differential temperature sensitivity. *Proceedings of the National Academy of Sciences* **100**, 6878-6883.

Michael TP, Salomé PA, Yu HJ, *et al.* (2003b) Enhanced fitness conferred by naturally occurring variation in the circadian clock. *Science* **302**, 1049-1053.
Millar AJ (2004) Input signals to the plant circadian clock. *Journal of Experimental Botany* **55**, 277-283.
Millar AJ, Carre IA, Strayer CA, Chua NH, Kay SA (1995) Circadian clock mutants in *Arabidopsis* identified by luciferase imaging. *Science* **267**, 1161-1163.
Millar AJ, Short SR, Chua NH, Kay SA (1992) A novel circadian phenotype based on firefly luciferase expression in transgenic plants. *Plant Cell* **4**, 1075-1087.
Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497.
Nohales MA, Kay SA (2016) Molecular mechanisms at the core of the plant circadian oscillator. *Nature Structural & Molecular Biology* **23**, 1061-1069.
Ouyang Y, Andersson CR, Kondo T, Golden SS, Johnson CH (1998) Resonating circadian clocks enhance fitness in cyanobacteria. *Proceedings of the National Academy of Sciences* **95**, 8660-8664.
Peirson BE (2015) Plasticity, stability, and yield: The origins of Anthony David Bradshaw's model of adaptive phenotypic plasticity. *Studies in History and Philosophy of Science Part C: Studies in History and Philosophy of Biological and Biomedical Sciences* **50**, 51-66.
Plautz JD, Straume M, Stanewsky R, *et al.* (1997) Quantitative analysis of *Drosophila* period gene transcription in living animals. *Journal of Biological Rhythms* **12**, 204-217.
Preuss SB, Meister R, Xu Q, *et al.* (2012) Expression of the *Arabidopsis thaliana BBX32* gene in soybean increases grain yield. *PLoS One* **7**, e30717.
Roden LC, Song HR, Jackson S, Morris K, Carre IA (2002) Floral responses to photoperiod are correlated with the timing of rhythmic expression relative to dawn and dusk in *Arabidopsis*. *Proceedings of the National Academy of Sciences* **99**, 13313-13318.
Salmela MJ, Greenham K, Lou P, *et al.* (2015) Variation in circadian rhythms is maintained among and within populations in *Boechera stricta*. *Plant, Cell & Environment*.
SAS (1999) *SAS/STAT User's Guide* SAS Institute, Cary, NC.
Scheiner SM, Holt RD (2012) The genetics of phenotypic plasticity. X. Variation versus uncertainty. *Ecology and Evolution* **2**, 751-767.
Schmitt J, Dudley SA, Pigliucci M (1999) Manipulative approaches to testing adaptive plasticity: phytochrome-mediated shade-avoidance responses in plants. *The American Naturalist* **154**, S43-S54.
Smith H (2000) Phytochromes and light signal perception by plants—an emerging synthesis. *Nature* **407**, 585-591.
Somers DE, Devlin PF, Kay SA (1998) Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock. *Science* **282**, 1488-1490.
Somers DE, Kim W-Y, Geng R (2004) The F-box protein *ZEITLUPE* confers dosage-dependent control on the circadian clock, photomorphogenesis, and flowering time. *The Plant Cell* **16**, 769-782.
Strayer C, Oyama T, Schultz TF, *et al.* (2000) Cloning of the *Arabidopsis* clock gene *TOC1*, an autoregulatory response regulator homolog. *Science* **289**, 768-771.
Swarup K, Alonso-Blanco C, Lynn JR, *et al.* (1999) Natural allelic variation identifies new genes in the *Arabidopsis* circadian system. *The Plant Journal* **20**, 67-77.
Thain SC, Hall A, Millar AJ (2000) Functional independence of circadian clocks that regulate plant gene expression. *Current Biology* **10**, 951-956.
Weinig C (2000) Differing selection in alternative competitive environments: shade-avoidance responses and germination timing. *Evolution* **54**, 124-136.
Weinig C, Dorn LA, Kane NC, *et al.* (2003) Heterogeneous selection at specific loci in natural environments in *Arabidopsis thaliana*. *Genetics* **165**, 321-329.
Wilczek AM, Roe JL, Knapp MC, *et al.* (2009) Effects of genetic perturbation on seasonal life history plasticity. *Science* **323**, 930-934.
Yan OY, Andersson CR, Kondo T, Golden SS, Johnson CH (1998) Resonating circadian clocks enhance fitness in cyanobacteria. *Proceedings of the National Academy of Sciences* **95**, 8660-8664.
Yerushalmi S, Yakir E, Green RM (2011) Circadian clocks and adaptation in *Arabidopsis*. *Molecular Ecology* **20**, 1155-1165.

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

Table 1. Within-month ANOVAs partitioning variance between the main effects of RIL and microenvironmental effect of plate for circadian period and phase for Ws-2 x C24 RIL set (A), Ws-2 x Ler RIL set (B) and Col x Rd-0/Me-0 (C). z-values are reported for random effects.

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

Significance levels (p-value): **** < 0.0001, *** < 0.001, ** < 0.01, * < 0.05, ⍰ < 0.06

Table 2. Quantitative genetic models for circadian period and phase under natural entrainment. Two-way ANOVAs of circadian period and phase for Ws-2 x C24 RIL set (A) and Ws-2 x Ler RIL set (B). The effect of month includes the 3 levels of June, July, and September. Across-month correlations are more consistently observed for circadian period than circadian phase (C). z-values are reported for random effects and f-values for fixed effects†.

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

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

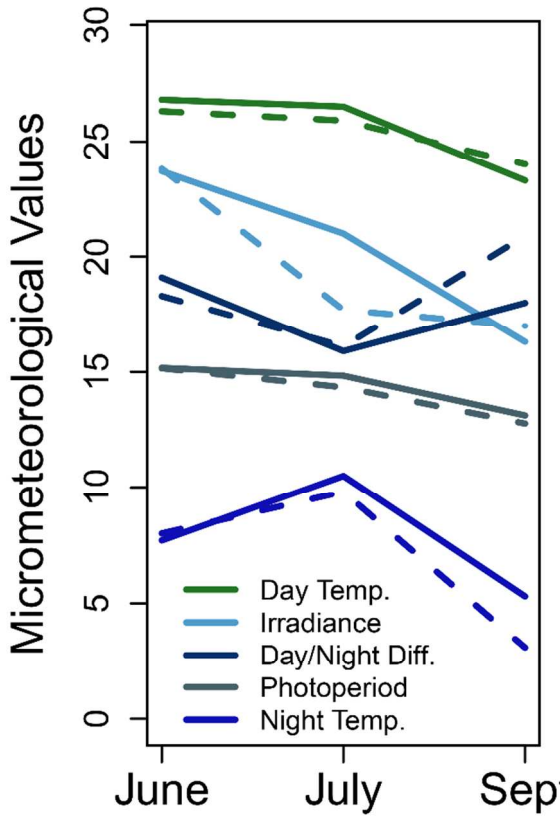
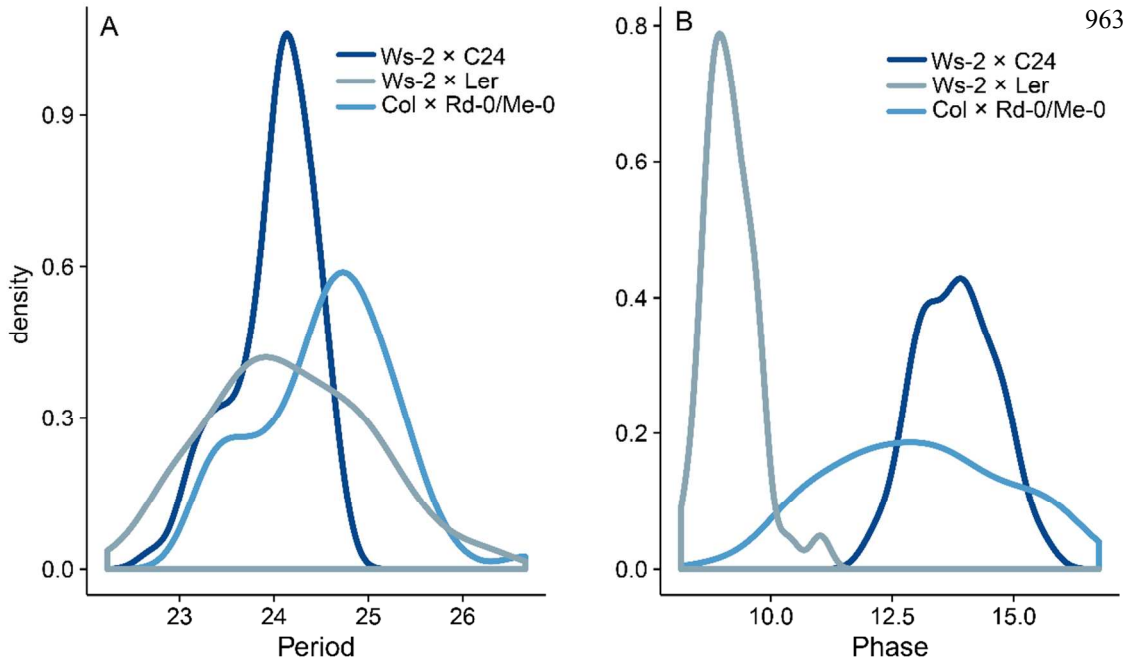


Figure 1. Mean values of micrometeorological data during the 5-day entrainment window in June, July, and September in the field. Parameters include duration of photoperiod (hours), average daily solar irradiance during entrainment window (MJ/m^2/day), day air temperature (Day Temp.; °C) and night air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



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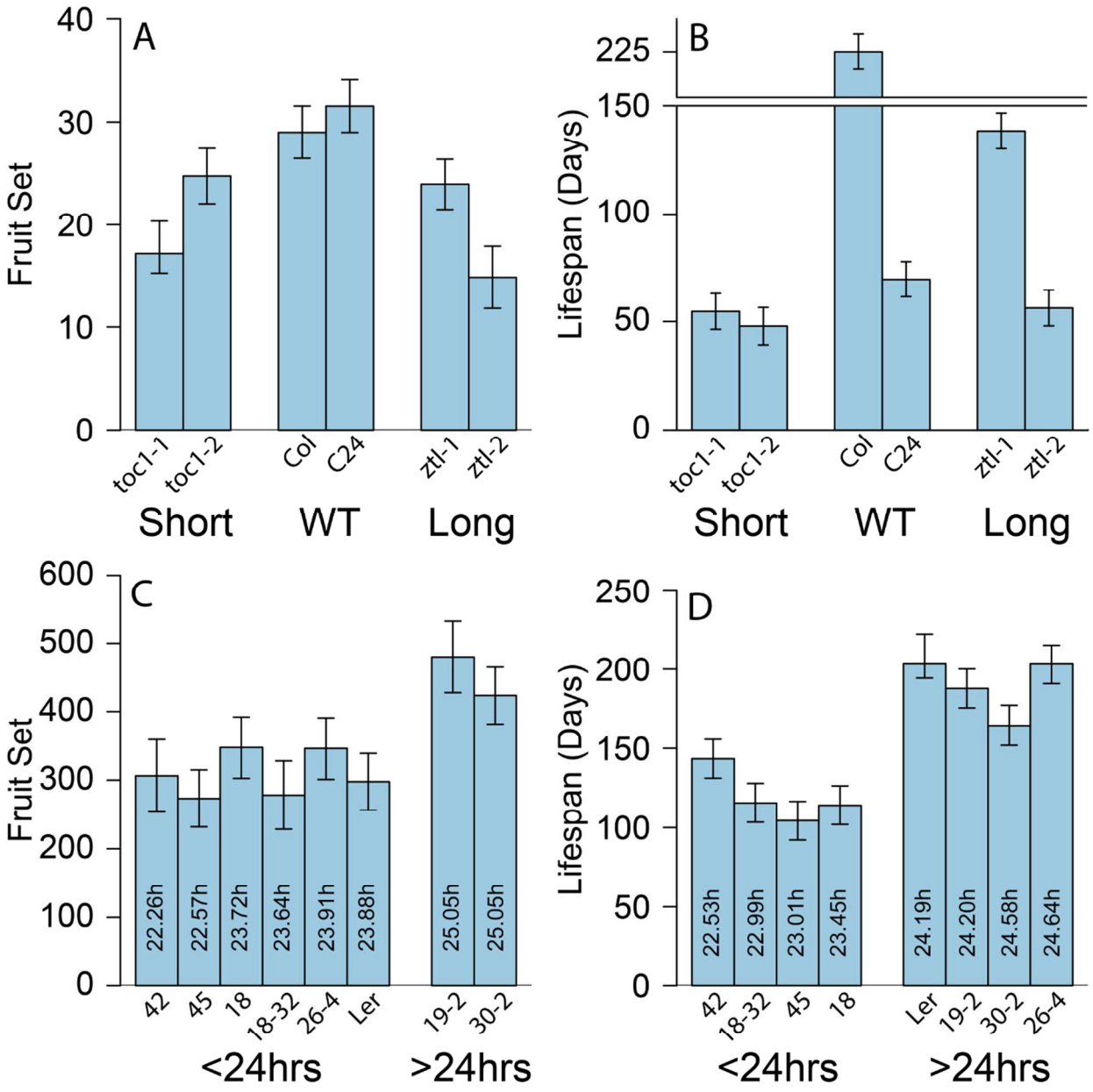

Figure 3. Perturbations in the circadian clock affect components of fitness. To understand clock mutation effects, comparisons should be made between the mutant genotype and its cognate wild-type; *ztl-1* is in the Col background, and *ztl-2*, *toc1-1*, and *toc1-2* are in C24. *ztl* and *toc1* mutant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

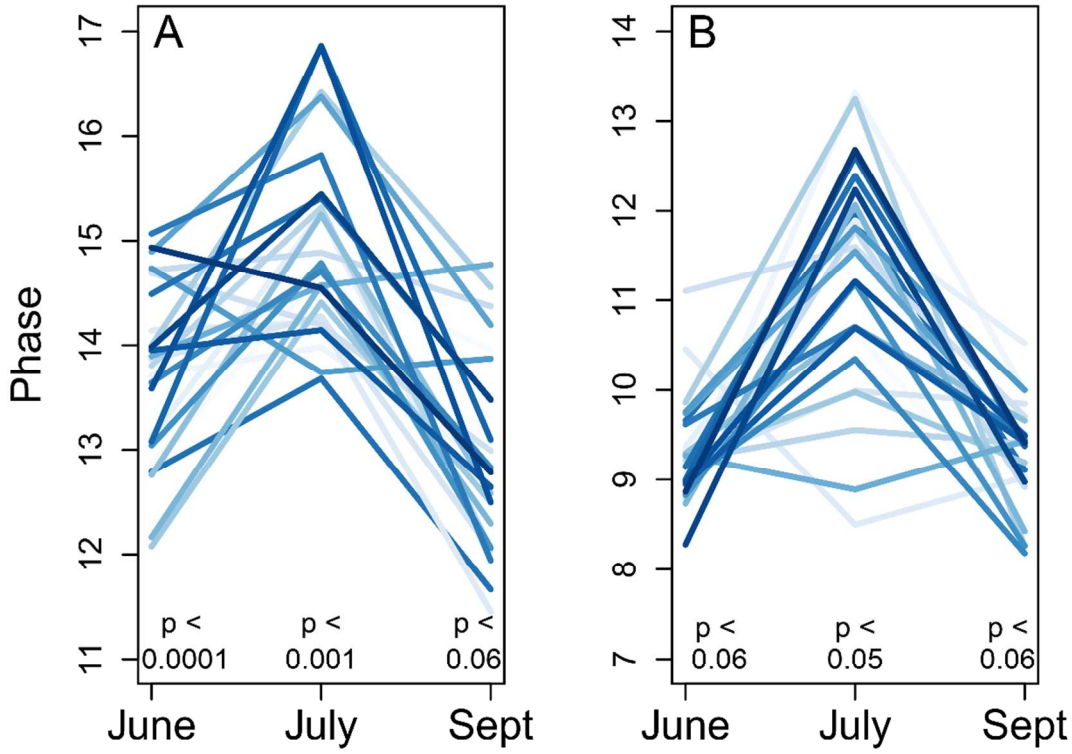

Figure 4. Circadian phase varies across monthly sampling points in two RIL sets. Lines on the figures (A and B) connect genotypic values for a single RIL across months, with different genotypes represented by different line shading. Values for 25 randomly selected genotypes within the Ws-2 × C24 RIL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

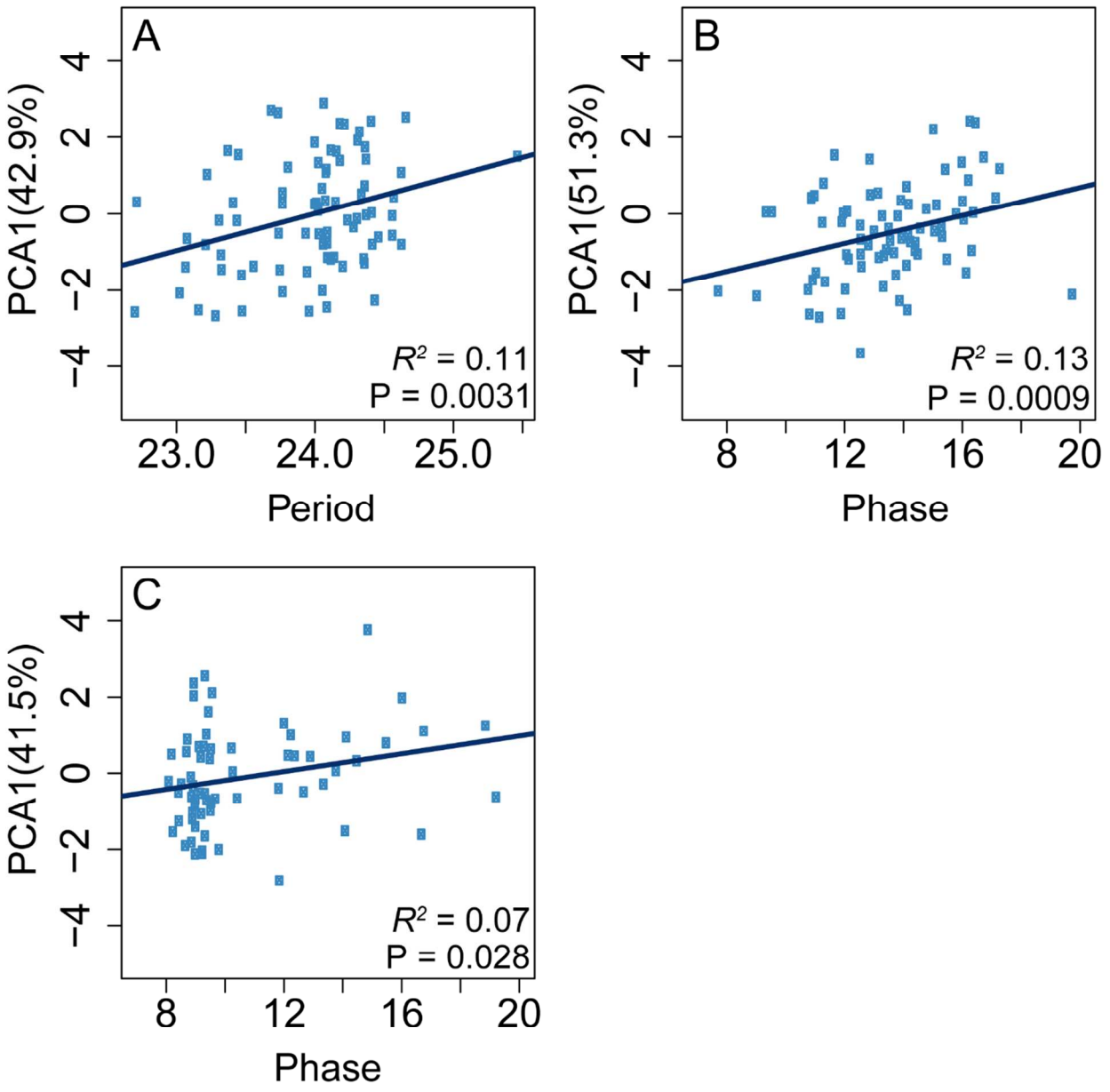

Figure 5. Quantitative variation in the circadian clock is associated with PCAs for plant performance loaded with plant size, reproductive timing and fecundity. Each dot represents the mean phenotype for a single RIL. (A) PCA1 is associated with circadian period in the Ws-2 × C24 RIL population. (B)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

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.