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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 hr 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 2 hr 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 hr were associated with improved performance in multiple RIL sets. These experiments translate functional analyses of clock behaviour 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 correlates with performance in nature.

KEYWORDS
Arabidopsis thaliana, circadian clock, fitness, phenotypic plasticity

1 | INTRODUCTION

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 neighbour proximity and elicits competitive elongation responses in plants (Crepy & Casal, 2015; Dorn, Pyle, & Schmitt, 2000; Dudley & Schmitt, 1995, 1996; Schmitt, Dudley, & Pigliucci, 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 time frame with shifts in temperature, light intensity, moisture level and other micrometeorological parameters over the course of a 24-hr 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, Maloof, Straume, Kay, & Harmer, 2008; Duffield, 2003; Farre & Weise, 2012; Lowrey & Takahashi, 2011; Michael et al., 2008). The periodicity of these cycles typically approximates 24 hr (Harmer, 2009; Matsuzaki, Kawahara, & Izawa, 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, namely 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, Devlin, & Kay, 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, Salome, & McClung, 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 multilocus 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-hr cycle is adaptive. In growth chamber studies that resemble classic reciprocal transplant experiments (Clausen, Keck, & Hiesey, 1940), *Arabidopsis thaliana* genotypes harbouring mutations at clock loci that lead to long (28-hr)- or short (20-hr)-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 hr that match their endogenous rhythm, while short-period genotypes perform better under 20-hr diurnal cycles. Notably, a 24-hr environmental cycle may lead to the best performance for all genotypes (Graf, Schlereth, Stitt, & Smith, 2010), perhaps because experimentally altered environmental cycles of 28 or 20 hr 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, Yakir, & Green, 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, Bradshaw, & Holzapfel, 2008; Yan, Andersson, Kondo, Golden, & Johnson, 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, Oakley, Ågren, & Schemske, 2014; Korves et al., 2007; Leinonen, Remington, Leppälä, & Savolainen, 2013; Weinig et al., 2003; Wilczek et al., 2009) can differ across environments. In sum, the fitness consequences of either a functional vs. nonfunctional 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 harbouring 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 hr enable adaptive phase matching to dawn (Hirschie Johnson, Elliott, & Foster,

2003; Johnson & Kondo, 1992), we predicted that near-isogenic lines (NILs) carrying introgressed regions that somewhat shorten periodicity (to 22 hr) would perform less well than NILs with cycles of 24–25 hr. 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 hr would again be associated with enhanced fitness. All of these hypotheses were supported by our field experiments.

2 | MATERIALS AND METHODS

2.1 | 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 hr vs. mutant genotypes with altered cycles near 20 or 28 hr. 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 laboratory 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 hr under free-running conditions, while *ztl-1 and ztl-2* genotypes express a 28-hr cycle under these conditions (Millar, Carre, Strayer, Chua, & Kay, 1995; Somers, Kim, & Geng, 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 genotype, Cvi, of small genomic regions harbouring clock loci into the Landsberg *erecta* genotype (Alonso-Blanco, El-Assal, Coupland, & Koornneef, 1998; Edwards, Lynn, Gyula, Nagy, & Millar, 2005; Ouyang, Andersson, Kondo, Golden, & Johnson, 1998; Swarup et al., 1999). Depending on temperature (either 27°C or 22°C), clock period was ~22–23 hr in one set of NILs vs. ~24–25 hr 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 (Figure 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* had period lengths of 22–23 hr, while NILs 19-2 and 30-2 had period lengths of 25 hr (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 (Figure 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 hr, while the 19-2, 30-2, 26-4 and L*er* had period lengths just over 24 hr (Edwards et al., 2005). There could, nevertheless, be some interday 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 harbour 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, Short, Chua, & Kay, 1992). The two clock markers (leaf movement and gene expression) used to

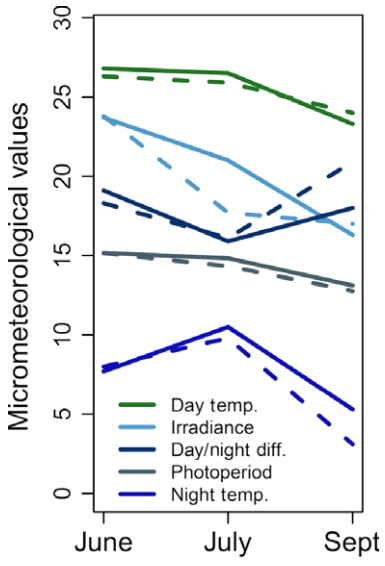

FIGURE 1 Mean values of micrometeorological data during the 5-day entrainment window in June, July and September in the field. Parameters include duration of photoperiod (hours), average daily solar irradiance during entrainment window (MJ/m^2/day), day air temperature (Day Temp.; °C) and night air temperature (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 [Colour figure can be viewed at wileyonlinelibrary.com]

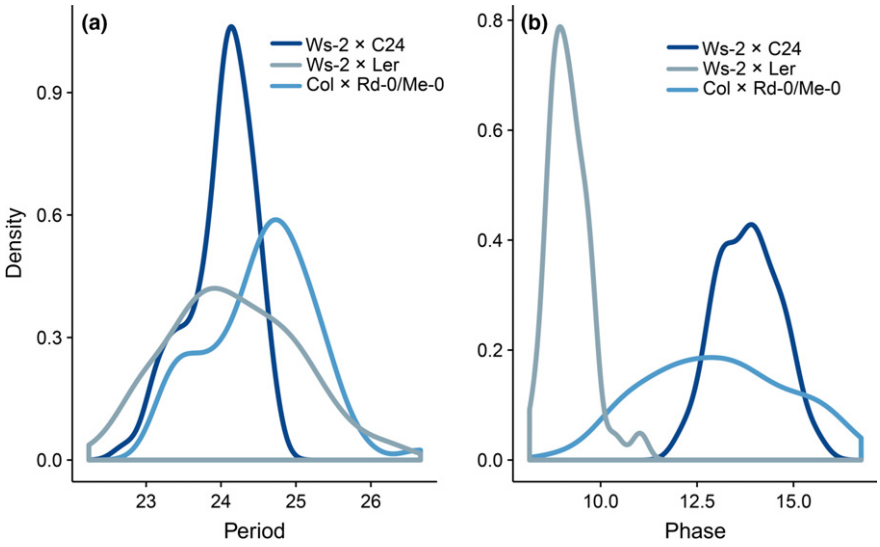

FIGURE 2 Density curves of the genotypic means for circadian period (a) and phase (b) for the Ws-2 × C24, Ws-2 × Ler and Col × Rd-0/Me-0 RIL sets [Colour figure can be viewed at wileyonlinelibrary.com]

estimate genotypic period in the NILs (Edwards et al., 2005) and RILs are strongly correlated (Hall, Kozma-Bognár, Bastow, Nagy, & Millar, 2002; Thain, Hall, & Millar, 2000). Based on the clock markers, RILs expressed a continuous range of clock periods from 21.5 to 26.0 hr (Figure 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 × *Ler*) was the result of a cross between Ws-2 and *Ler* (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 laboratory genotypes and because prior studies showed that 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 of 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 harbour 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 laboratories.

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 (i) 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 (ii) information on performance consequences of diverse clock phenotypes (and not local adaptation per se).

2.2 | 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 × *Ler* 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 4 days at 4°C, transferred to the glasshouse to germinate and thinned to one focal plant per pot. Plants were then transplanted into the field blocks, with 10-cm spacing between adjacent pots.

At the Wyoming field site, 14–16 replicate seeds of each genotype (mutant, NIL and Ws-2 × *Ler* 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 weeks after the initial planting. Planting of the two RIL sets was offset by 1 week in spring (7th May and 14th May) and 12 days in fall (1st September and 12th September). 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 (Figure 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 overwinter 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 weeks after the initial planting. The planting dates within each site (WY and MN) were chosen to ensure that 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.

2.3 | 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 Fig. S1. Having recorded temperature and photoperiod during the field assays, we independently manipulated these factors in a growth chamber experiment to test whether 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 microtitre plates containing Murashige and Skoog mineral plant growth media supplemented with 30 g/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 4 days at 4°C. Plates were then moved to a Percival PGC-9/2 growth chambers set to a 12-hr photoperiod, temperature of 22°C and relative humidity of 50% for 2 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 in 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, for example, 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, 20 μl 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 that 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 min, 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 hr and analysed using fast Fourier transform nonlinear least-squares (FFT-NLLS) analysis from the time-series images using IMAGEPRO/IANDA software (Doyle et al., 2002; McWatters, Bastow, Hall, & Millar, 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, Song, Jackson, Morris, & Carre, 2002) and because phase estimates are commonly made from the first 24-hr 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 jet-lag, 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 (i.e., plate) rarely led to differences in circadian traits (Table 1). We hypothesized that period lengths of or slightly longer than 24 hr 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).

2.4 | 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- to 23-hr vs. longer, 24- to 25-hr 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 whether 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 microtitre 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-squares means for both month and 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. Clock–performance associations were estimated as the genotypic regression of PCA1 on circadian period and phase (PROC GLM) (SAS 1999).

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 × C24 RIL set (a), Ws-2 × Ler RIL set (b) and Col × Rd-0/Me-0 (c)

	Line	Plate
(a) Ws-2 × C24 RILs		
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		
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		
Period	0.12**	0.003
Phase	1.19**	0.09

z-values are reported for random effects.
Significance levels (*p*-value): **** <.0001, *** <.001, ** <.01, * <.05, $^{+}$ <.06.

3 | 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 (Figure 3a) and survival (Figure 3b). In a spring cohort, the two wild-type genotypes produced significantly more fruit than the short-period *toc1* mutants (with ~20-hr endogenous cycles) or the long-period *ztl* mutants (with ~28-hr endogenous cycles) (Figure 3a) (effect of period class, $F = 10.7$, $p < .0001$), and this relationship remained significant after accounting for flowering time variation ($p < .001$). In a fall cohort, period class also affected lifespan ($F = 17.31$, $p < .0001$). 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 (Figure 3b). The *ztl-2*, *toc1-1* and *toc1-2* mutants had lifespans that were between 19% and 38% shorter on average than the cognate wild-type genotype (C24) (Figure 3b), although C24 also showed lower survivorship than Col-0 potentially due to its warm climate provenance. In short, extreme excursions (±4 hr) of circadian period from 24 hr appear to reduce performance.

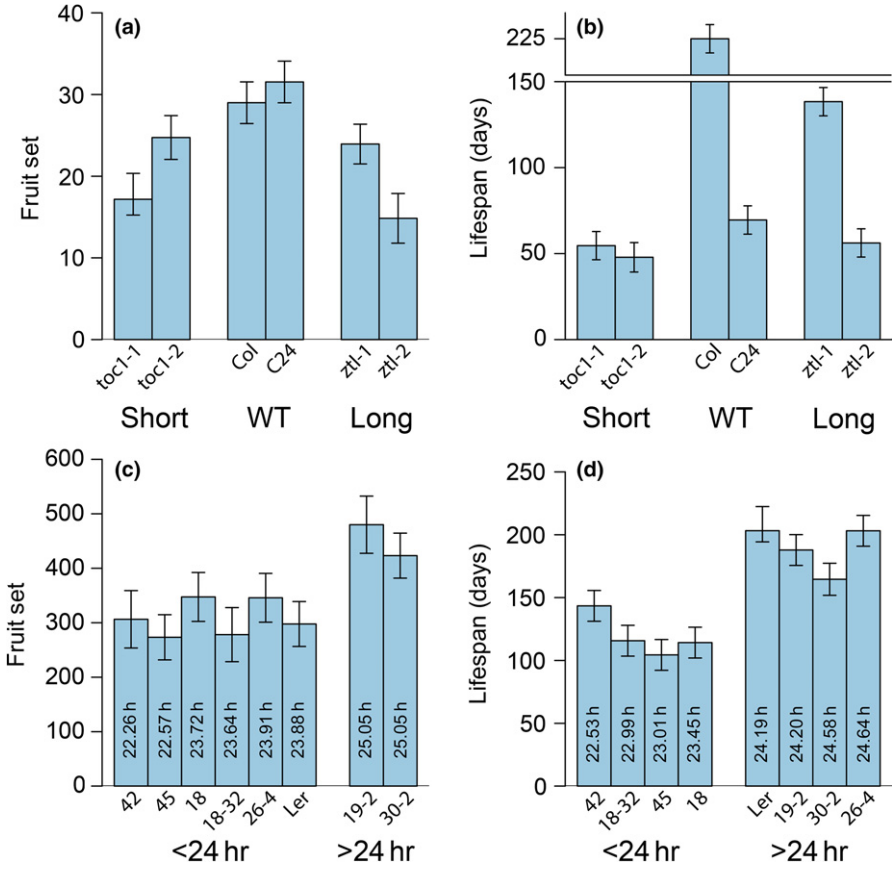

FIGURE 3 Perturbations in the circadian clock affect components of fitness. To understand clock mutation effects, comparisons should be made between the mutant genotype and its cognate wild type; *ztl-1* is in the Col background, and *ztl-2*, *toc1-1* and *toc1-2* are in C24. *ztl*- and *toc1*-mutant genotypes have reduced fruit set (fecundity) in a spring cohort (a) ($F = 10.7$, $p < .0001$, for mean difference between wild-type and mutant classes) and shorter lifespans in a fall cohort (b) ($F = 17.31$, $p < .0001$). Circadian NILs with naturally segregating alleles that result in a circadian period <24 hr have reduced fruit set in a spring cohort (c) ($F = 7.26$, $p = .009$) and reduced survival in a fall cohort (d) ($F = 16.35$, $p = .0001$). 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 [Colour figure can be viewed at wileyonlinelibrary.com]

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 harbouring alternative clock alleles into the L*er* genotype. All genotypes that expressed a circadian period from 22 to 23 hr had reduced fecundity relative to genotypes with circadian periods of ~25 hr (Figure 3c), and this relationship remained significant after accounting for variation in flowering time ($p = .0003$). All NILs that expressed a period length from 22 to 23 hr also failed to survive the winter, while genotypes expressing a circadian period greater than ~24 hr survived (Figure 3d). Thus, a circadian period slightly >24 hr (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–c; Figure 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 hr in the Ws-2 × C24 set on average over all months of the growing season, 9.9 hr in the Ws-2 × L*er* RIL set and 14.3 hr in the Col × Me-o/Rd-0 set in the 1 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 hr in the Ws-2 × C24 set, 23.7 hr 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., $r = .36–.67$, $p < .05$ on average for multiple RIL sets that were measured in multiple months).

Season strongly affected average circadian phase (Table 2a, b, cf month effect; Figure 4a,b) in the two populations where multiple months of circadian data were collected. Compared to both June

TABLE 2 Quantitative genetic models for circadian period and phase under natural entrainment. Two-way ANOVAs of circadian period and phase for Ws-2 × C24 RIL set (a) and Ws-2 × Ler RIL set (b). The effect of month includes the three 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[†]

	Period	Phase
(a) Ws-2 × C24 RILs		
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		
RIL	4.23****	0.29
Month[†]	8.63*	56.19****
RIL × month	2.41**	2.19*
Plate (month)	2.71**	1.91*
	Ws-2 × C24 RILs	**Ws-2 × Ler RILs**
(c) Trait pair		
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): **** <.0001, *** <.001, ** <.01, * <.05, [+] <.06.

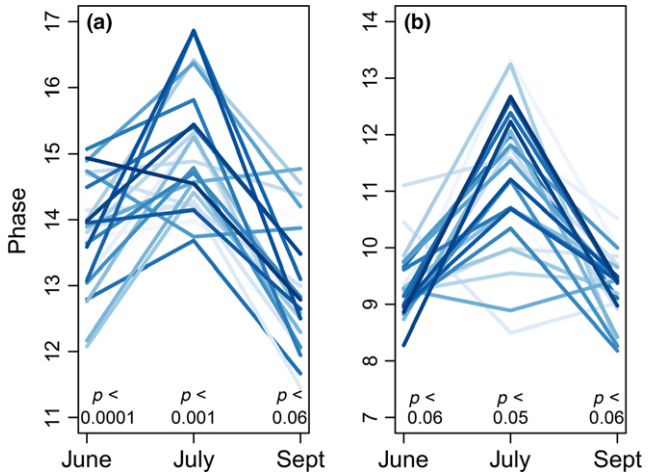

FIGURE 4 Circadian phase varies across monthly sampling points in two RIL sets. Lines on the figures (a and b) connect genotypic values for a single RIL across months, with different genotypes represented by different line shading. Values for 25 randomly selected genotypes within the Ws-2 × C24 RIL set (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 hours 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 [Colour figure can be viewed at wileyonlinelibrary.com]

and September, average phase was delayed in July by approximately 1 hr (Ws-2 × C24 population, Figure 4a) or 2 hr (Ws-2 × *Ler* population, Figure 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 1 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, b, cf month effect for period vs. phase).

A number of known clock inputs varied over the growing season, including temperature, photoperiod and irradiance (Figure 1). It was not possible to test for clock–micrometeorological correlations because each RIL set had only three plantings; as such, there were only three effective data points for comparison. Nevertheless, only mean minimum temperature exhibited a chevron pattern of response similar to the RILs, suggesting that 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) was matched to field conditions during entrainment. Circadian period measured in the growth chamber under either photic cycles ($r = .61$ and $p < .0001$) or thermal cycles ($r = .22$ and $p < .05$) 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 ($r = -.07$ and $p = .54$), nor were phase values estimated under thermal cycles that simulated the field ($r = .13$ and $p = .25$). 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 hr (or delayed phase, which, again, was positively correlated with period) were associated with higher values of performance in the RILs (Figure 5a–c). Specifically, in the Ws-2 × C24 RILs, longer period was associated with increased performance as estimated from PCA1 ($R^2 = .11$, $p = .0031$). Delayed phase was associated with increased values of PCA1 in both the Col × Rd-0/Me-0 ($R^2 = .13$, $p = .0009$) and the Ws-2 × *Ler* RILs ($R^2 = .07$, $p = .037$). 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

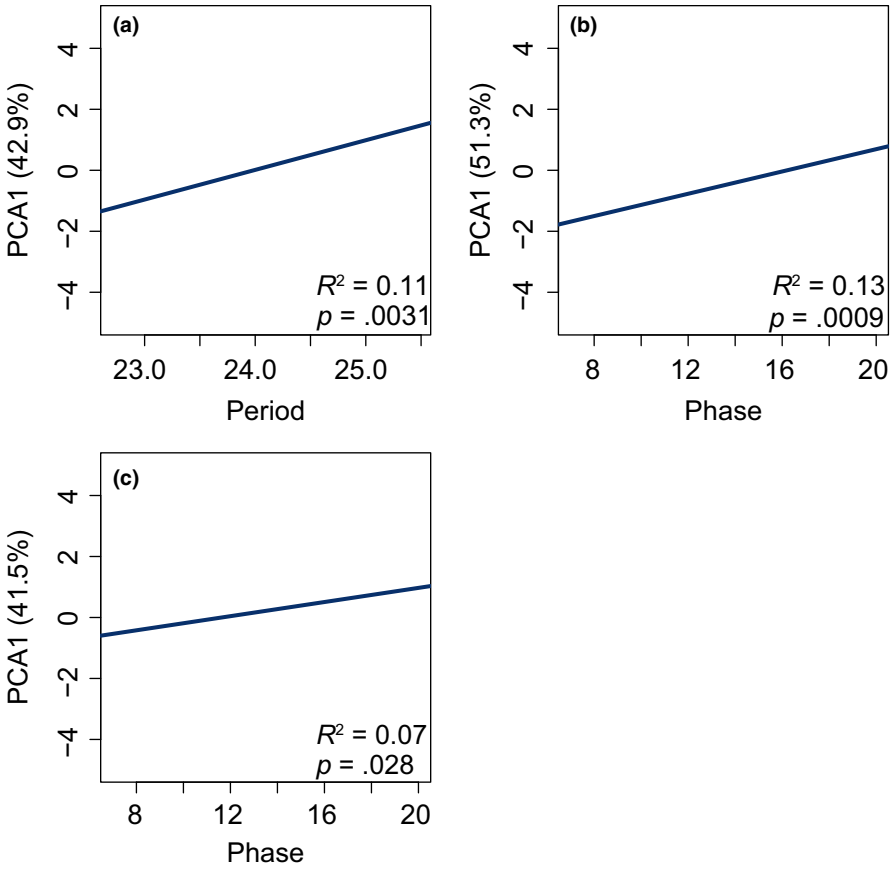

FIGURE 5 Quantitative variation in the circadian clock is associated with PCAs for plant performance loaded with plant size, reproductive timing and fecundity. Each dot represents the mean phenotype for a single RIL. (a) PCA1 is associated with circadian period in the Ws-2 × C24 RIL population. (b) PCA1 is associated with circadian phase in the Col × Rd-0/Me-0 RIL population. (c) PCA1 is associated with circadian phase in the Ws-2 × Ler RIL population [Colour figure can be viewed at wileyonlinelibrary.com]

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 hr is associated with improved performance in comparison with periods closer to 22 hr (Figure 3c,d).

4 | DISCUSSION

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

To test the adaptive significance of the circadian clock, we measured performance both in well-characterized mutants with large-effect clock perturbations and in circadian NILs with comparatively small-effect genomic introgressions. We observed a reduction in two components of fitness, fecundity in a spring cohort and lifespan in a fall cohort, in clock mutants with large differences in period (i.e., ±4-hr differences from 24-hr cycle). We attribute reduced performance of the mutants to clock misfunction, because the mutations are not annotated as acting pleiotropically outside clock pathways. As for the mutants, fitness was reduced among NIL genotypes with 22- to 23-hr period lengths in comparison with genotypes with cycles near 24–25 hr. The results are consistent with adaptive hypotheses that a functional and correctly timed clock enhances fitness in natural settings. Further, clock loci within the introgressed regions include genes from the input pathway (*PHYA*, *PIF3* and *CRY2*), the oscillator (*TOC1*, *ZTL*, *LHY*, *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 hr on average in July relative to June and September. Notably, this pattern was observed in two RIL sets sampled in two successive weeks within each month, suggesting that smaller interweekly 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 (Figure 1). Although it was only a qualitative observation, the advance in phase in the 2 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 1°C can affect clock entrainment (Bohn, Hinderlich, Hütt, Kaiser, & Lüttge, 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, temperature, leading to a strong genotypic association of clock period or phase across environments with similar thermal cycles; alternatively, the clock may be set by a combination of multiple environmental factors. To test for the effect of individual factors on clock parameters, we simulated field temperatures and photoperiods in controlled growth chamber settings and tested for genotypic associations between circadian parameters measured in the field vs. the growth chamber. Circadian period estimated for diverse genotypes in controlled photic or thermal cycles simulating a July field environment was significantly associated with period of those genotypes measured in the field in July. However, neither genotypic phase values estimated in the growth chamber under photic cycles nor those under thermal cycles were correlated with those measured in the field in July. These patterns of association (or lack thereof) require further investigation, but have a few implications. First, the results of prior studies examining period phenotypes (e.g., characterizing genetic loci or QTL affecting period) under controlled photoperiod or temperature settings (Edwards et al., 2005; Lou et al., 2011; Michael et al., 2003b; Swarup et al., 1999) may be directly relevant to clock behaviours in matching field settings. On the other hand, the results suggest either (i) that multiple, simultaneously varying clock inputs may be integrated to yield circadian phase in the field, (ii) that unmeasured factors may disrupt associations between the field and growth chamber or (iii) that some environmental features (such as high irradiance) cannot be adequately replicated in controlled settings, any of which are relevant to studies translating results from controlled to natural settings. While partitioning the contribution of diverse potential input(s) to the clock requires further investigation in the field (Matsuzaki et al., 2015), the current results, nevertheless, provide insights in segregating plant 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., 2016), 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 nonsignificant 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, Higo, & Takano, 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., 2016) 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% to 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 harbouring functionally variable clock alleles such as those sampled here.

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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 (https://doi.org/10.5061/dryad.th8b5).

AUTHOR CONTRIBUTIONS

C.W., S.J.D., J.N.M., S.L.H., M.J.R., Z.G., A.D. and M.T.B. designed research; M.J.R., M.T.B., M.K., Z.G. and S.M.W. performed research; M.J.R., S.L.H. and C.W. analysed data; C.W. and M.J.R. wrote the initial manuscript; and all authors participated in revision.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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