Original Article

Variation in circadian rhythms is maintained among and within populations in Boechera stricta

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ABSTRACT

Circadian clocks have evolved independently in all three domains of life, and fitness benefits of a functional clock have been demonstrated in experimental genotypes in controlled conditions. Still, little is known about genetic variation in the clock and its fitness consequences in natural populations from heterogeneous environments. Using Wyoming populations of the Arabidopsis relative Boechera stricta as our study system, we demonstrate that genetic variation in the clock can occur at multiple levels: means of circadian period among populations sampled at different elevations differed by less than 1 h, but means among families sampled within populations varied by as much as 3.5 h. Growth traits also varied among and within populations. Within the population with the most circadian variation, we observed evidence for a positive correlation between period and growth and a negative correlation between period and root-to-shoot ratio. We then tested whether performance tradeoffs existed among families of this population across simulated seasonal settings. Growth rankings of families were similar across seasonal environments, but for root-to-shoot ratio, genotype × environment interactions contributed significantly to total variation. Therefore, further experiments are needed to identify evolutionary mechanisms that preserve substantial quantitative genetic diversity in the clock in this and other species.

Key-words: Adaptation; circadian clock; environmental heterogeneity; genetic differentiation; genotype × environment interaction; maintenance of genetic variation.

INTRODUCTION

Most species are characterized by considerable standing genetic variation in quantitative traits (Barton & Keightley 2002), and one evolutionary force that contributes to its maintenance is adaptation of populations to spatially heterogeneous environments (Kawecki & Ebert 2004). Indeed, population differences in quantitative traits are often associated with environmental variation (Linhart & Grant 1996), and many populations in the wild have been shown to achieve highest fitness in their native environments (Hereford 2009; Leimu & Fischer 2008). For example, spatial variation in growing season length has resulted in populations developing genetic differences in their phenological responses so that they avoid unfavourable growth conditions in spring and autumn (e.g. Savolainen et al. 2007). Environmental conditions also cycle on a shorter temporal scale. Because of the Earth's rotation around its axis, 24 h days with predictable light-dark and temperature cycles characterize most natural ecosystems, resulting in various optimal times for biological processes over the course of a day. The circadian clock is thought to have evolved as a timekeeping system in response to cycling environments in diverse organisms from bacteria to plants and mammals (Bell-Pedersen et al. 2005). Based on stimuli from the surrounding environment, the clock contributes to the regulation of various biological phenomena from gene expression to behaviour and maintains 24 h cycles in these traits. With intact clocks, these rhythms will persist in constant conditions (Pittendrigh 1981), while disruptions of the circadian oscillator may lead to altered rhythms (Millar et al. 1995; Salomé et al. 2002) or even arrhythmia (e.g. Dodd et al. 2005).

Circadian rhythms with periods close to 24 h in free-running conditions are hypothesized to be optimal in the wild (Pittendrigh 1981). Fitness benefits of a circadian clock in diurnal environments have been demonstrated in panels of experimental and natural genotypes of bacteria, plants and insects grown in controlled experimental settings, and performance of these genotypes has been found to be maximized when endogenous rhythms match cycles in the external environment (reviewed in West & Bechtold 2015; Yerushalmi & Green 2009). For instance, in cyanobacteria, mutant genotypes with a longer cycle (period) in constant conditions outcompeted genotypes with a shorter period in unnaturally long days, while the opposite pattern was detected in unnaturally short days (Ouyang et al. 1998). In constant conditions, fitness differences among genotypes were no longer found (Woelfle et al. 2004). Similar findings have been reported in monogenic circadian mutants in Arabidopsis thaliana (L.) Heynh. (Dodd et al. 2005); although in a later study, both short-period and long-period genotypes were found to achieve their highest fitness in 24 h days (Graf et al. 2010).

In the wild, selection is expected to remove non-optimal genetic variation from populations (Falconer & Mackay 1996; Kawecki & Ebert 2004). Consequently, one might expect limited genetic diversity in the circadian clock in organisms in...
natural 24 h environments. Nevertheless, genotypes that have evolved under natural conditions and that have been sampled over a wide geographic range may exhibit circadian rhythms that deviate, sometimes substantially, from 24 h, possibly as a result of latitudinal variation in day length (e.g. de Montaigu et al. 2015; Edwards et al. 2005; Hut et al. 2013; Kusakina et al. 2014; Michael et al. 2003). This suggests genetic variation in the clock is maintained in heterogeneous environments, perhaps owing either to spatially variable selection or to unanticipated performance tradeoffs of variable phenotypes. For example, Michael et al. (2003) hypothesized that the 6.5 h difference in period among natural Arabidopsis accessions could be related to the capability of clocks deviating from 24 h to track seasonal changes in the environment.

In spite of evidence for extensive genetic diversity in the clock in the wild, the relative distribution of naturally segregating quantitative clock genetic variation among versus within populations and its effects on performance in settings that more closely approximate natural conditions remain unknown, particularly in plants. While genetic differentiation among populations is important as a possible indicator of local adaptation, understanding within-population genetic diversity is critical in determining the potential for evolutionary responses to natural selection in changing environments (Hoffmann & Sgrò 2011). Both spatial and temporal heterogeneity in the local environment have been hypothesized to maintain fine-scale quantitative genetic variation within populations by inducing genotype × environment interactions (changes in genotypic rank orders) in fitness-related traits across variable environments (Ellner & Hairston 1994; Gillespie & Turelli 1989). Despite comprehensive theoretical work on the maintenance of genetic diversity and its ubiquity in nature, in general, the mechanisms preserving significant within-population genetic diversity also on small spatial scales remain poorly understood (Kruuk et al. 2014; Salmela 2014). Previous studies on naturally occurring quantitative genetic variation in the clock in plants have sampled Arabidopsis accessions across large geographical gradients and phenotyped these plants generally under standard laboratory conditions, with single genotypes representing specific locations (de Montaigu et al. 2015; Edwards et al. 2005; Kusakina et al. 2014; Michael et al. 2003); no prior plant studies have quantified potential finer-scale among or within-population variation in clock behaviour, despite the potential fitness consequences described earlier and the central importance of genetic variance to evolutionary responses (but see Helm & Visser (2010) for a study on a bird population).

Here, we sampled natural plant populations on a more regional scale in southeastern Wyoming and accounted for the possibility of local within-population genetic diversity in our sampling design. Our study system was Boechera stricta (Graham) Al-Shehbaz, a short-lived perennial and widely distributed North American relative of Arabidopsis that has emerged as one of the model species in adaptation to variable environments (Rushworth et al. 2011). While geographic variation in the clock is often investigated in relation to latitudinal gradients in day length, substantial genetic variability in circadian period may also be found within a given latitude (e.g. Michael et al. 2003). This suggests clocks do not evolve solely in response to natural day length variation. Thus, we hypothesized that if the circadian clock contributed to adaptation also to other environmental factors in addition to day length, significant genetic differences would be observed among populations sampled at the same latitude but at different elevations and that population differences could be related to elevation, a proxy for local temperature regimes. Elevational differences among populations could be expected in various growth traits as well. Further, we hypothesized that genetic variation in quantitative traits could also be found on a very local scale within populations, as in Arabidopsis (Méndez-Vigo et al. 2013). If the circadian clock is linked to performance, we anticipate quantitative clock differences will correlate with various aspects of growth. Finally, we postulated that the occurrence of local genetic variation in the clock within populations could reflect performance tradeoffs among families across seasonal environments within a potential growing season that differ in photoperiod and temperature.

MATERIALS AND METHODS

Study populations

Seeds were collected at four sites in southeastern Wyoming in July and August 2012. Plants from these sites will be referred to as populations. Sampled locations were in the vicinity of SNOTEL weather stations from which long-term climate data were available. Therefore, we were able to simulate natural temperature cycles in controlled growth chamber conditions. The SNOTEL sites at lower elevation were Crow Creek (CC; 41.23°N, 105.38°W; elevation 2593 m) and South Brus Creek (SBC; 41.33°N, 106.50°W; elevation 2572 m). The higher-elevation sites were North French Creek (NFC; 41.33°N, 106.38°W; elevation 3088 m) and Brooklyn Lake (BL; 41.36°N, 106.23°W; elevation 3121 m). The distance of the sampled locations from the nearby weather stations varied from a few metres at BL to about 1.3 km at NFC. Sampling in each population was carried out by maternal family, allowing for the estimation of genetic (among-family) variation within populations in this predominantly self-fertilizing species (Song et al. 2006). At NFC, three families were sampled approximately 600 m away from the site with the rest of the families. The distance between the populations varied from about 10 to 94 km. Wild-collected seeds were used in all experiments; therefore, we cannot exclude the possibility of maternal effects contributing to the observed patterns of differentiation. These effects are likely to be of smaller significance on the within-population scale as phenotypically diverse families were often sampled very close to each other at the sites. Moreover, by using wild-collected seeds, we are likely to capture more natural patterns of variation present within populations at the time of sampling.

Growth chamber environments for measurement of circadian rhythms and whole-plant performance

In order to simulate natural temperature cycles and to match growth conditions between the relatively rapid circadian
screens carried out using small seedlings and growth assays requiring multiple weeks, we used conditions that matched average temperature conditions at the home sites of the populations on 21 June. For each site, we extracted hourly temperature readings on 21 June in different years (2003–2012; http://www.wcc.nrcs.usda.gov/snow/) and calculated hourly means across the sites. During one 24h cycle, temperature varied between about 5 °C at 0400 h and about 17 °C at 1500 h (Supporting Information Fig. S1). Temperature was programmed to change gradually between hourly time points. Photoperiod was set to 15 h, which would be the natural day length at the sampled sites in late June.

We sampled families within the SBC population in two additional growth environments to estimate potential performance tradeoffs across seasonal settings with different photoperiods and temperature cycles. We used families from SBC because this population expressed the greatest level of within-population clock variation, increasing the chances of observing significant family-specific responses to the environment. To maximize among-treatment differences in the photoperiod that the population might experience at its home site during a growing season, these environments simulated average late summer and autumn conditions on 17 August and 21 September when photoperiods are approximately 13.5 h and 12 h, respectively. Twenty-four step temperature cycles were based on climate data (2003–2012) from the site (Supporting Information Fig. S1). In the August treatment, temperature varied between 5.5 °C at 0400 h and 20.3 °C at 1300 h. Chamber restrictions limited minimum temperature in the dark to 4 °C; thus, we could not achieve as low night temperatures in the chamber as at the site in late September. To simulate cool nights in the September treatment, temperature was kept at 4 °C between 2000 h and 0600 h. During the day, temperature peaked at 12.6 °C at 1300 h.

Circadian screens for leaf movement

Leaf movement is a well-known output of the circadian clock that can be scored non-invasively in small seedlings of non-model systems (Tindall et al. 2015). We first examined the patterns of quantitative genetic diversity in circadian period among and within populations in the June environment. Seeds were imbibed in the dark at 4 °C on moist filter paper for 4 d and then transferred to a growth chamber for an additional 4 d under 12 h photoperiod at 20 °C. Following germination, seedlings were transplanted into 0.5″ polyvinyl chloride tubes containing Sunshine Redi-Earth soil (Sungro Horticulture, Agawam, MA, USA) and transferred to the entrainment growth chamber under the average 21 June temperature cycle, as described in the preceding section. After 1 week in the entrainment chamber, 12 seedlings from each family that were growing vertically and symmetrically were transferred to the imaging room under constant light and temperature and arranged into four blocks. To avoid a change in temperature upon transfer from the entrainment to the imaging conditions, the morning temperature of 12 °C was used during the constant imaging. Seedlings were watered once with a 20-20-20 fertilizer and then watered daily to prevent movement due to water loss.

Imaging began 24 h after transfer to constant conditions. Images were taken every 20 min for 5 d. Circadian period was estimated using the newly developed automated analysis programme Tracking Rhythms in Plants (TRiP; Greenham et al. 2015). Individuals with model fit traces outside of the 18–32 h range considered to be circadian were removed.

Growth measurements

Following the circadian screen, we then collected data on growth in additional replicates of the same families within the same average 21 June treatment. Based on the circadian data, families in each population were ranked according to the number of replicates only. Those with the best replication (6–12 replicates) were chosen for the growth measurements in the June environment. Thirteen families were included from BL, CC and SBC, and nine from NFC. Seeds were cold-stratified on moist paper in the dark for 4 d at 4 °C, after which the plates were placed in a growth chamber for 4 d (12 h photoperiod, 21 °C; PGC-9/2 with Percival Advanced Intellus Environmental Controller, Percival Scientific, Perry, IN, USA). Ten germinated seeds per family were sown on moist Sunshine Sungro LP-5 soil (Sungro Horticulture, Agawam, MA, USA) in 2″ net pots. The plants were divided into two growth chamber compartments, with five blocks within each and one replicate per family per block. The order of the plants within the blocks was randomized. Finally, the same growth protocols and new sets of replicates were used for the two additional treatments with families from SBC, which enabled exploring potential performance tradeoffs among genetically diverse families across seasonal environments. Only one growth chamber compartment was used for the simulated August and September environments, with 10 blocks within each and one replicate of each family per block in a randomized order. While chamber conditions (particularly temperature) were closely monitored and were found to match the programmed treatment, the use of one compartment per treatment means we cannot unequivocally attribute family × treatment effects to abiotic factors simulating the home site; this constraint does not affect the primary conclusions. For all environments, 50% of the lights were on during the first and last hour of the photoperiod. Maximum photosynthetic irradiance at the plant level in the chambers, measured with the light meter LI-250 (LI-COR Biosciences, Lincoln, NE, USA), was approximately 250 μmol photons m–2 s–1. Pots were kept moist by watering every 2 d, and flats were rotated within the chamber compartments twice every week.

Measurements started after the plants had been growing in the chambers for 4 weeks. Twelve measures of first-year growth were taken to estimate plant performance in the June environment (Table 2). Similar measures of performance have also been used in earlier circadian studies on A. thaliana (Dodd et al. 2005; Graf et al. 2010; Green et al. 2002; Kusakina et al. 2014). At 4 weeks, plants were measured for the span of the first true leaves and leaf width. At 5 weeks, the number of leaves was recorded. Starting at 5 weeks, one newly emerged leaf was chosen for leaf expansion measurements. The same leaf was measured four times over 20 d. Leaf expansion rate
estimates were obtained by measuring elongation between two successive time points. After 8 weeks of growth, plants were measured for the length and width of the longest leaf, harvested, dried in an oven at 65°C for at least 3 days and measured for above-ground and below-ground biomass, total biomass and root-to-shoot ratio. Eight of the 12 growth traits were measured in families from the SBC population in the August and September environments.

**Statistical analyses**

We used general linear models to analyse variation in the measured traits. Variation in circadian period was analysed using the model $\text{period} = \text{population} + \text{family(population)} + \text{block}$. Analyses were carried out also within each population using the model $\text{period} = \text{family} + \text{block}$. Population was the only fixed factor in the analyses. Interaction terms were non-significant and were thus excluded from the model.

Preliminary analyses indicated significant correlations among most growth traits in the June environment. The number of variables was therefore reduced by a principal component analysis (PCA) with varimax rotation. Genetic differentiation among and within populations was then examined using principal components (PCs). PCA was performed using the whole data set (PCPOP), and separately using data from SBC only (PCSBC). Variation in growth traits in the June environment was analysed using the model $\text{PCPOP} = \text{population} + \text{family(population)} + \text{chamber compartment} + \text{block(chamber compartment)}$. The population was the only fixed factor in the model. The model for PC1POP also included the population × chamber compartment interaction term. Variation in growth traits within populations was analysed using the model $\text{PCSBC} = \text{family} + \text{chamber compartment} + \text{block(chamber compartment)}$. Family × chamber compartment interaction terms were excluded from the models because of their non-significance. For both circadian period and growth traits, variance component estimates due to family (measures of within-population genetic diversity) were obtained using the restricted maximum likelihood approach. Associations between family means of period and growth were examined using Pearson’s correlation in the SBC population, which had the greatest range of genetic variation in circadian period. Performance tradeoffs would be suggested if the associations between period and different metrics of performance (e.g. growth rate and early size) are in opposite directions, that is, the correlation between period and some performance metrics is positive, while the period correlation is negative for other traits.

We used PCA also for growth data for SBC in the June, August, and September treatments (PC1SEASON). To analyse the significance of different factors, we used the following model: $\text{PC1SEASON} = \text{root-to-shoot ratio} = \text{treatment} + \text{family} + \text{family} \times \text{treatment} + \text{block(treatment)}$. Treatment was the only fixed factor in the analysis. Sample size across treatments was very similar (120–127 in each treatment), but variance in PC1SEASON varied almost 19-fold. Therefore, we transformed PC1SEASON by first adding a constant of 2 to all values. After $\log_{10}$ transformation, variance differed only fourfold among treatments. These transformed values were then used in the analysis. For root-to-shoot ratio, variance among treatments differed by about 11-fold. Transformations reduced heteroskedasticity, but did not change the interpretation of the results; we used original values in the analysis. Variance components for the random factors were obtained using the restricted maximum likelihood approach. We used Pearson’s correlation to examine associations between family means in different treatments ($r_{GE}$). In the model, the family × treatment component defines genotype × environment interactions and accounts for both rank shifts of families and differences in among-family variance across treatments. If genotype × environment interactions contributed to the maintenance of diverse families in the population, we would expect a significant interaction term arising from rank order changes and no overall effect of family across environments (Mitchell-Olds 1992). In order to separate the effects of shifts in family rank order versus among-family variance on the magnitude of family × treatment interaction, we used the equation (Cockerham 1963):

$$V_{\text{Family} \times \text{treatment}} = \frac{\sum_{ij} \left[ 2\sigma_i \sigma_j (1 - r_{GE}) + (\sigma_i - \sigma_j)^2 \right]}{t(t-1)},$$

where $V_{\text{Family} \times \text{treatment}}$ is the variance component due to family × treatment interaction, $\sigma_i$ and $\sigma_j$ are the square roots of among-family variance in treatments $i$ and $j$, $r_{GE}$ is the genetic correlation between treatments $i$ and $j$ and $t$ is the number of treatments. The first part of the equation accounts for variance due to changes in rank order among treatments, while the second part accounts for differences in among-family variance among treatments. For genetic correlations in this analysis, we used family variance components estimated within each treatment and across pairs of treatments. Genetic correlations were then estimated as

$$r_{GE} = \frac{V_{\text{Family}}}{\sqrt{(V_{\text{Family}, \text{treatment } i} \times V_{\text{Family}, \text{treatment } j})}},$$

where $V_{\text{Family}}$ is the variance component due to family across the two environments and $V_{\text{Family}, \text{treatment } i}$ and $V_{\text{Family}, \text{treatment } j}$ are the variance components due to family in treatments $i$ and $j$.

All statistical analyses were carried out with IBM SPSS Statistics Version 22.

**RESULTS**

**Genetic variation among and within populations in period in leaf movement**

Families for which circadian period estimates were available for at least five replicates were included in the analysis (the largest number of replicates per family was 12). This resulted in the following sample sizes: 141 in BL (16 families), 257 in CC (30 families), 99 in NFC (14 families) and 154 in SBC (20 families). General linear model results for leaf movement are shown in Table 1. Significant differences in circadian period were observed among populations and among families within populations (Table 1a). Population means were 23.1 h for CC...
and SBC, 22.2 h for NFC and 22.8 h for BL (Fig. 1a and Supporting Information Fig. S2). Highly significant differences among families were found in CC and SBC, with SBC having more among-family variation (Table 1b). In CC, family means ranged from 22.1 to 24.8 h. In SBC, the corresponding range was from 20.6 to 24.2 h (Fig. 1b). Therefore, the range of variation among families within some populations is greater than the mean difference among populations.

Genetic variation among and within populations in growth

Growth was measured in 86–129 samples per population, and the number of replicates per family varied from five (one family) to 10 (37 families). PCA on 12 growth traits resulted in two PCs with eigenvalues above 1. Each PC explained 36% of the variance. Loadings of the traits on the PCs are shown in Table 2. Population means for each trait are shown in Supporting Information Table S1. PC1pop correlated best with early growth, below-ground biomass, and root-to-shoot ratio, while PC2pop was best associated with longest leaf at 8 weeks and above-ground biomass. In both PCs, significant differences were observed among populations and among families within populations (Table 3a). For PC1pop, the populations ranked in the same order as the elevations of the sampled sites, with positive means in BL and NFC and negative in CC and SBC (Fig. 1c). For PC2pop, NFC and SBC had positive values, while for CC and BL, they were negative (Fig. 1c). PC1pop varied among families within populations in BL, CC and SBC (Table 3b), while in PC2pop, among-family variation was found within BL, NFC and SBC (Table 3c). The least variable population in terms of among-family variation in PC1pop was CC, while the most variation was observed in SBC. For PC2pop, fairly similar levels of among-family variation were found in BL, NFC and SBC. Significant differences among the chambers are likely to have arisen from variation in temperature cycles at the early stages of the experiment. A significant population × chamber interaction was found for PC1pop, but the ranking of the populations did not change across the chambers. The variance component due to the interaction was only one-third of that for the family effect.

Correlations between period in leaf movement and growth within South Brush Creek

Preliminary analyses indicated significant associations between the clock and growth within SBC in which circadian period, PC1pop and PC2pop varied among families (Tables 1b, 3b and 3c). PCA performed using data only from SBC resulted in three PCs with eigenvalues above 1. PC1SBC explained 54% of the variation, PC2SBC 18% and PC3SBC 10%. Loadings of the traits on the PCs are shown in Table 2. PC1SBC was highly correlated with early growth rates and final size, PC2SBC correlated best with leaf expansion rates 2 and 3 and PC3SBC was strongly correlated with root-to-shoot ratio. Significant family differences were found in all three PCs (Table 3d). A negative correlation was found between family means of PC1SBC and
PC3SBC ($r = -0.707, P < 0.01$). Family means of circadian period correlated significantly with PC1SBC ($r = 0.657, P < 0.05$; Fig. 2a) and PC3SBC ($r = -0.782, P < 0.01$; Fig. 2a). Associations between period and three individual traits (the span of the first true leaves at 4 weeks, the number of leaves at 5 weeks and above-ground biomass) that correlated strongly with PC1SBC are shown in Fig. 2b, c and d. General linear model results for these traits are shown in Supporting Information Table S2. The correlation between family means of period and PC2SBC was negative ($r = -0.328$) but non-significant ($P = 0.274$).

Growth in South Brush Creek in different seasonal environments

Principal component analysis performed on growth data from June, August and September environments resulted in one PC with an eigenvalue above 1. The PC1SEASON explained 83% of the variation. Loadings of the traits on the PC are shown in Table 2. Seven out of the eight growth traits were strongly correlated with PC1SEASON. The only variable that did not correlate strongly was root-to-shoot ratio, which was used as a response variable in the analysis of variance without modifications. For PC1SEASON, we observed significant differences among treatments and families and family × treatment interactions (Table 4 and Fig. 3a). Mean overall PC1SEASON was highest in June, with the August and September treatments having very similar means that were noticeably smaller than that in June. The variance component due to family (0.000712) accounted for 13% of the total variation, while the variance component due to family × treatment interaction (0.000324) accounted for only 6% (of which approximately 66% was estimated to be due to changes in family rank order among the treatments). Family means of PC1SEASON in June correlated significantly with those in August ($r_{GE} = 0.629$, Table 2.

### Table 2. Loadings of the growth traits on different PCs for the four populations in the average 21 June environment (PC1POP), SBC only in the June environment (PC3SBC) and SBC in three seasonal treatments (PC1SEASON)

<table>
<thead>
<tr>
<th>Trait</th>
<th>All populations – June Component</th>
<th>SBC only – June Component</th>
<th>SBC – three seasons Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>Span of first true leaves at 4 weeks</td>
<td>0.786</td>
<td>0.932</td>
<td>0.938</td>
</tr>
<tr>
<td>Leaf width at 4 weeks</td>
<td>0.725</td>
<td>0.928</td>
<td>0.947</td>
</tr>
<tr>
<td>No of leaves at 5 weeks</td>
<td>0.618</td>
<td>0.800</td>
<td>Na</td>
</tr>
<tr>
<td>Leaf expansion rate 1</td>
<td>0.660</td>
<td>0.890</td>
<td>Na</td>
</tr>
<tr>
<td>Leaf expansion rate 2</td>
<td>-0.214</td>
<td>0.272</td>
<td>Na</td>
</tr>
<tr>
<td>Leaf expansion rate 3</td>
<td>-0.694</td>
<td>-0.492</td>
<td>Na</td>
</tr>
<tr>
<td>Longest leaf length at 8 weeks</td>
<td>0.243</td>
<td>0.696</td>
<td>0.973</td>
</tr>
<tr>
<td>Longest leaf width at 8 weeks</td>
<td>0.0934</td>
<td>0.248</td>
<td>0.925</td>
</tr>
<tr>
<td>Above-ground biomass</td>
<td>0.482</td>
<td>0.927</td>
<td>0.984</td>
</tr>
<tr>
<td>Below-ground biomass</td>
<td>0.790</td>
<td>0.842</td>
<td>0.957</td>
</tr>
<tr>
<td>Total biomass</td>
<td>0.605</td>
<td>0.940</td>
<td>0.987</td>
</tr>
<tr>
<td>Root-to-shoot ratio</td>
<td>0.735</td>
<td>0.0766</td>
<td>-0.472</td>
</tr>
</tbody>
</table>

PC, principal component; SBC, South Brush Creek.

Leaf expansion rate 1 = leaf expansion between the first and second measurement; leaf expansion rate 2 = leaf expansion between the second and third measurement; and leaf expansion rate 3 = leaf expansion between the third and fourth measurement.
The correlation between means in June and September was similar in direction but non-significant ($r_{GE} = 0.456$, $P = 0.117$). Family means of PC1_{SEASON} in August were significantly correlated with those in September ($r_{GE} = 0.653$, $P < 0.05$). Therefore, families with longer periods and larger size in the June environment remained larger across the seasonal environments (Fig. 3b). Similar results were obtained when examining individual traits (data not shown).

Treatments varied significantly in root-to-shoot ratio (Table 4 and Fig. 3c), with the highest mean in August (0.613) and the lowest in June (0.254). The family effect was not significant across the three treatments, although a positive correlation was detected between family means in June and September ($r_{GE} = 0.630$, $P < 0.05$). We also detected significant family × treatment interactions; approximately 55% of the family × treatment interaction variance was estimated to be due to changes in family rank order among the treatments. A negative correlation was found between family means of PC1_{SEASON} and root-to-shoot ratio in August ($r = -0.667$, $P < 0.05$).

### Table 3. General linear model results for (a) PC1_{POP} and PC2_{POP} with all populations, (b) PC1_{POP} within populations, (c) PC2_{POP} within populations and (d) PC1_{SBC}, PC2_{SBC} and PC3_{SBC} within SBC

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>MS</th>
<th>F-ratio</th>
<th>d.f.</th>
<th>MS</th>
<th>F-ratio</th>
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<td><strong>PC1_{POP}</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Population</td>
<td>3</td>
<td>27.4</td>
<td>9.09 *</td>
<td>3</td>
<td>14.5</td>
<td>7.46 ***</td>
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<td>Family (population)</td>
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<td>1.29</td>
<td>2.63 *****</td>
<td>44</td>
<td>1.96</td>
<td>2.78 *****</td>
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<td>Chamber</td>
<td>1</td>
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<td>31.1 **</td>
<td>1</td>
<td>21.5</td>
<td>7.44 *</td>
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<td>P × C interaction</td>
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<td>4.57 **</td>
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<tr>
<td>Block (chamber)</td>
<td>8</td>
<td>1.65</td>
<td>3.36 ***</td>
<td>8</td>
<td>2.89</td>
<td>4.10 ***</td>
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<td><strong>PC2_{POP}</strong></td>
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<thead>
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<th>Source of variation</th>
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Loadings of the traits on the PCs are shown in Table 2.

PC, principal component; CC, Crow Creek; SBC, South Brush Creek; NFC, North French Creek; BL, Brooklyn Lake; P × C = population × chamber; $V_{Family}$ = among-family variance component.

* $P > 0.05$,
* $P < 0.05$,
** $P < 0.01$,
*** $P < 0.001$,
**** $P < 0.0001$.
DISCUSSION

Genetic differentiation in circadian rhythms and growth among and within populations

Naturally occurring genetic variation in the clock in plants has previously been examined using accessions of *A. thaliana* sampled across vast geographic areas (de Montaigu et al. 2015; Edwards et al. 2005; Kusakina et al. 2014; Michael et al. 2003), but no studies have examined such patterns of variation at the population level. Further, the exact environmental factors that drive genetic differentiation in the clock remain unknown. Here, we document significant differences in circadian period among populations of *B. stricta* sampled at different elevations within a relatively small area in southeastern Wyoming. Period lengths were shorter in the two higher-elevation populations, a pattern similar to that reported by Edwards et al. (2005) on a...
larger geographic scale; because photoperiods at the sampled sites are very similar, differentiation in the clock could be related to lower overall temperatures at higher elevations and variation in daily temperature cycles among the sites, potentially resulting in different optimal times for various biological processes at various locations. This is consistent with the observation of temperature being one of the inputs of the circadian clock (McClung & Davis 2010). However, the relationships among elevation, temperature and photoperiod are complex. At higher elevation with cooler temperatures, the growing season will likely start later and finish earlier in the calendar year and so be subject to a different photoperiodic environment than that encountered at lower elevations. This is likely to have consequences for growth, reproduction, growth cessation and the induction of dormancy in perennials. Because only four populations were sampled, additional populations will need to be sampled in the same region and across a wider gradient in elevation to specifically test for a cline in the clock across elevations. Future research will address the potential patterns of co-variation between the clock, temperature and photoperiodic responses in populations from this region.

In contrast to earlier studies on natural variation in circadian rhythms in plants (de Montaigu et al. 2015; Edwards et al. 2005; Kusakina et al. 2014; Michael et al. 2003), we sampled multiple families per location in order to investigate possible within-population genetic variation in the clock and based our chamber treatments on temperature data from the home sites of the populations. We found that the magnitude of population differences (less than 1 h) was small compared with the within-population genetic diversity at the two lower-elevation sites: period varied by 2.7–3.5 h among families sampled within a few hundred metres. Thus, with our within-population sampling, we captured significant proportions of circadian period variation previously documented among A. thaliana accessions sampled across much larger geographic areas; for example, the range of 3.5 h at SBC accounted for 54% of the variation in period of leaf movement among 150 globally sampled accessions (Michael et al. 2003), or up to 81% of the variation in periodicity of gene expression among 76 mostly Eurasian accessions (de Montaigu et al. 2015). In the sole other study screening for within-population variation in the clock, genetic variation in period of locomotor activity was found in a sample of one Dutch population of the bird Parus major, with family means varying by less than 1 h (Helm & Visser 2010). Thus, the magnitude of fine-scale genetic variation in the current study is high in comparison with past studies.

In growth, populations from higher elevations and with shorter circadian periods were found to have more rapid early growth, larger below-ground biomass and higher root-to-shoot ratio. These features may be beneficial at sites with shorter growing seasons and colder winters (e.g. Chapin & Chapin 1981; Poorter et al. 2012). The ranking of populations in terms of final size was somewhat different, with no clear grouping in terms of elevation. Among-family variation was also found in the two higher-elevation populations (BL and NFC) with no significant period variation. For PC2POP these populations were more variable than CC. These findings indicate that the lack of variation in the clock at higher elevations is not due to genetic drift, which would be expected to reduce all aspects of genetic diversity (Falconer & Mackay 1996). Further experiments on additional populations will help in determining whether lack of variation in the clock is a general feature of higher-elevation populations in the region and whether this pattern could be related for instance to the very short growing season at their home sites.

**Associations between the clock and growth in South Brush Creek**

Even though both global and local genetic diversity in the circadian clock can be significant (e.g. Helm & Visser 2010; Michael et al. 2003), it is not clear how such variation is associated with other quantitative traits. We detected evidence for a positive period–growth association in the SBC population where the most circadian variation was found and where families with longer periods generally grew more rapidly and were larger in size after 8 weeks of growth. At the population level, populations with shorter periods had more rapid early growth but were not necessarily largest in terms of final size, suggesting that patterns of co-variation between the clock and other traits may differ when measured among versus within populations. How variation in period, a trait that is typically not expressed in cycling conditions, could be physiologically linked to growth is not known, but period is expected to correlate with the peak timing.
(phase) of biological activities in cycling conditions (Pittendrigh 1981). Indeed, many experiments indicate a positive association between period and phase (Brown et al. 2008; Fleury et al. 2000; Lankinen 1993; Zhang et al. 2013), although this pattern is not always observed (de Montaigu et al. 2015; Michael et al. 2003).

It is important to note that in our wild study system, the observed significant associations do not necessarily reflect causal relationships between period and growth as parallel, but independent selection on clock and growth traits might have occurred in diverse environments. However, it has been shown in A. thaliana that single mutations in clock genes can affect not just circadian period but also other quantitative traits such as growth and gas exchange (e.g. Dodd et al. 2005). Mapping studies on diverse quantitative traits in natural variants of B. stricta will allow for exploring the shared genetic basis of the clock and other quantitative traits.

Maintenance of quantitative genetic variation in South Brush Creek

Genetic diversity in quantitative traits may be maintained within populations if fitness rankings of genotypes vary among different environmental conditions (Mitchell-Olds 1992). Classically, fitness consequences of variation in circadian period have been tested in experiments carried out in unnatural day lengths (Dodd et al. 2005; Ouyang et al. 1998; Woelfle et al. 2004). In the case of B. stricta, however, the extensive local variation in the clock found among families within SBC must have evolved in natural 24h environments, and therefore, possible tradeoffs among families should be investigated in conditions that resemble the population’s home site conditions. Because the circadian clock is generally assumed to contribute to adaptation to light–dark cycles (e.g. Bell-Pedersen et al. 2005; Hut et al. 2013; Michael et al. 2003), we tested the hypothesis that performance tradeoffs among genetically distinct families in seasonal environments could explain the maintenance of significant fine-scale diversity in SBC so that shorter-period families would grow better in late summer and autumn environments with shorter photoperiods (cf. Dodd et al. 2005; Ouyang et al. 1998; Woelfle et al. 2004). Growth in these environments was lower than in June, suggesting that early summer conditions are the most optimal for growth. Yet, in spite of different photoperiods and temperatures used, the significant family effect and positive across-treatment genetic correlations indicated that the longer-period families that were generally larger in the June environment remained larger across months of the potential growing season and that contrary to our expectations, genotype × environment interactions contribute only little to the maintenance of genetic variation in the amount of growth.

Contrasting patterns of variation were observed in root-to-shoot ratio, which was highest in the August treatment with the warmest temperatures and lowest in June. No significant family effect on average was found across the treatments, but families responded differently to the seasonal environments, resulting in rank changes across the treatments. Further, while period and growth were positively correlated, these two traits were both negatively correlated with root-to-shoot ratio in the June environment; evidence for a negative correlation between growth and root-to-shoot ratio was found also in the August treatment. These patterns suggest a within-population tradeoff that might be an important factor in preserving genetic diversity at this site. In particular, increased growth may enhance performance (reproductive output) of some genotypes in some years, while increased allocation to roots in other genotypes may enhance survival in dry or cold conditions (Larsen et al. 1986; Lloret et al. 1999; Poorter et al. 2012); this latter point is also suggested by the higher ratios observed in the two higher-elevation B. stricta populations that experience colder and longer winters in their native environments. Taken together, our results indicate that the fitness consequences of the naturally occurring fine-grained genetic diversity in the circadian clock and the causes behind its maintenance in this perennial species need to be examined in natural conditions, in order that variation in the clock, growth and its allocation can be associated with over-winter survival, timing of reproduction and reproductive fitness in the spatially and temporally variable high-elevation environments in the Rocky Mountains.

ACKNOWLEDGMENTS

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COMPETING INTERESTS

We have no competing interests.

REFERENCES


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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Table S1. Population means (± standard errors) for all growth traits in the average June 21 environment. Populations are ranked according to the elevation of the nearby weather station, from CC at 2,539 m to BL at 3,121 m.

Table S2. General linear model results for three individual traits that were strongly correlated with PC1sbc. NS = p > 0.05, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

Figure S1. Temperature treatments used in this experiment. The average June 21 environment (15-hour photoperiod) was based on annual temperature data from all four sampled sites, while the August (15.5-hour photoperiod) and September (12-hour photoperiod) treatments were based on data from SBC only. Minimum temperature used in the chambers was 4 °C due to chamber restrictions.

Figure S2. An alternative version of Fig. 1a, with population means and standard deviations for circadian period in leaf movement.