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Source: Zoological Science, 36(2) : 95-104

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zs180148

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ZOOLOGICAL SCIENCE **36**: 95–104 (2019)
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Temperature Entrainment of Circadian Locomotor and Transcriptional Rhythms in the Cricket, *Gryllus bimaculatus*

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Most animals exhibit circadian rhythms in various physiological and behavioral functions regulated by circadian clock that resides in brain and in many peripheral tissues. Temperature cycle is an important time cue for entrainment, even in mammals, since the daily change in body temperature is thought to be used for phase regulation of clocks in peripheral tissues. However, little is known about the mechanisms by which temperature resets the clock. In the present study, we investigated the effect of temperature on circadian activity rhythm and clock gene transcription by using the cricket, *Gryllus bimaculatus*. We show that temperature cycle can entrain both behavioral and transcriptional rhythms of clock genes, such as *period*, *timeless*, *cryptochrome2* and *cycle* in the circadian pacemaker tissue, optic lobe. Under temperature cycle, phase of evening peak of locomotor activity occurred 1 h before the warm-to-cold phase transition, which is associated with earlier peaks of mRNA expression rhythm of the clock genes than that under light/dark cycles. When the temperature cycle was advanced by 6 h, behavioral rhythms re-entrained to newly phased temperature cycle after ~16 transient cycles. The mRNA oscillation of *period* and *timeless* gained stable rhythm under phase advanced temperature cycles with a lesser number of transient cycles than *cryptochrome2* and *cycle*. These results suggest that temperature cycle can entrain behavioral and molecular rhythms in cricket and clock genes vary in sensitivity to temperature. It is thus likely that clock genes play differential roles in resetting the clock with environmental temperature changes.

Key words: circadian rhythm, clock genes, cricket, mRNA oscillation, optic lobe, temperature entrainment

INTRODUCTION

The circadian clock that governs circadian rhythms in various physiological functions in animals exhibits an endogenous period of about 24 hour under constant conditions, and is largely unaffected by changes in ambient temperature (Bruce and Pittendrigh, 1956; Hastings and Sweeney, 1957). Although the endogenous period of the circadian clock is temperature compensated, temperature cycles can entrain the circadian clock. While it has been reported that light/dark cycles and temperature cycles can entrain locomotor activity (Tomioka et al., 1998; Wheeler et al., 1993) and molecular rhythms in the clock neurons of *Drosophila* (Yoshii et al., 2005; Yoshii et al., 2009), preliminary studies have suggested fundamental mechanistic differences between photic and thermal entrainment pathways (Boothroyd et al., 2007). For instance, temperature cycles can drive robust clock gene oscillations in clock neurons as well as entrain locomotor activity behaviour under constant light, a condition which is known to cause arrhythmicity in both molecular and behavioural rhythms (Yoshii et al., 2005). In addition, among the approximately 150 neurons of clock circuitry in *Drosophila*, lateral neurons (LNs) appear to be more light-entrainable, whereas lateral posterior neurons (LPNs) and dorsal neuron 2 (DN2) are more sensitive to temperature (Miyasako et al., 2007). These temperature-entrainable neurons do not express circadian photoreceptor cryptochrome (CRY), indicating that CRY negative neurons mainly mediate temperature inputs whereas CRY positive neurons are more essential for light entrainment (Gentile et al., 2013; Yoshii et al., 2010). The molecular mechanism by which light resets the circadian clock is relatively well understood: CRY leads to degradation of TIM protein in a light-dependent manner (Koh et al., 2006). However, this is not the case for temperature entrainment.

Although our understanding of the molecular mechanism by which temperature resets a running clock is limited, previous studies have reported that temperature can alter key aspects such as transcription (Boothroyd et al., 2007), mRNA splicing (Majercak et al., 2004; Majercak et al., 1999),

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doi:10.2108/zs180148

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protein stability (Sidote et al., 1998) and protein-protein interaction (Gekakis et al., 1995) of circadian clock components in *Drosophila*. In addition, *period* (*per*)-null and *timeless* (*tim*)-null mutant flies retain remnant temperature-entrainable oscillatory mechanism to drive activity rhythm, whereas this temperature-dependent oscillatory mechanism does not operate in cyc^{o} and Clk^{Jrk} fruit flies, indicating the involvement of *Clk* and *cyc* in temperature entrainment mechanism (Yoshii et al., 2002). These findings notwithstanding, the role of clock genes in resetting the running clock with changes in temperature remains to be elucidated.

Molecular oscillatory mechanisms of the circadian clock have been studied extensively in fruit flies. Besides *Drosophila*, functional analysis of clock genes has been performed in only a few insect species. The molecular oscillation of circadian clock in the cricket, *Gryllus bimaculatus* has been substantially clarified: it is based on cyclical expression of *per* and *tim*. Transcription of these genes is activated by transcription factors CLOCK (CLK) (Moriyama et al., 2012) and CYCLE (CYC) (Uryu et al., 2013) during the late day to early night. The product proteins PER (Moriyama et al., 2008) and TIM (Danbara et al., 2010) most likely accumulate and feedback to inhibit their transcription through inactivation of CLK and CYC during the night. CYC is also under circadian control. Thus key components of the circadian clock in the cricket is featured by both *Drosophila*-type and mammalian-type oscillatory components (Reviewed in Tomioka, 2014; Uryu et al., 2013). Furthermore, considering the behavioural aspects under light/dark cycles, crickets are predominantly nocturnally active at higher ambient temperature (25°C) whereas they become diurnal at lower ambient temperature (20°C) under light/dark cycles (Ikeda and Tomioka, 1993) indicating the crucial temperature dependency of the phase of activity rhythm in crickets. The evolutionary aspects of molecular mechanism underlying temperature entrainment have received less empirical attention. The cricket serves as a useful model organism for exploring the molecular changes incorporated into the temperature entrainment mechanism of the circadian timing system during evolution.

The aim of the present study was to explore the molecular oscillatory mechanism through which the temperature resets the circadian clock in the cricket, *Gryllus bimaculatus*. The results of this study show that the temperature cycle can entrain both the molecular rhythm of clock genes and behavioural output rhythm. In addition, molecular rhythms of circadian oscillatory components exhibit differential sensitivity to changes in temperature.

MATERIALS AND METHODS

Intact crickets for experiments

Adult male crickets, *Gryllus bimaculatus*, were used for all experiments. Nymphal/adult crickets purchased or obtained from laboratory colony were maintained under standard laboratory conditions of 12:12 h light/dark cycles (LD) where light came on at Japan Standard Time (JST) 06:00, designated as Zeitgeber time 00 (ZT00), and was switched off at JST 18:00 (ZT12). The light regimens were provided by a white fluorescent lamp (FL15N, Panasonic, Osaka, Japan), and the light intensity was 1.8–2.9 W/m^2 depending on the proximity to the light source and crickets were maintained at a constant temperature of 25.0 ± 0.5°C. They were fed with laboratory chow (CA-1, Clea Japan, Tokyo, Japan) and water. From this laboratory colony, adult males were collected to use for further experiments.

Recording of locomotor activity

Locomotor activity of adult male crickets was recorded in transparent plastic activity chambers (18 × 9 × 4.5 cm) with a rocking substratum as described previously in Moriyama et al. (2008). The rocking substratum seesaws with movements of a cricket and a magnetic reed switch placed at the bottom of the activity chamber sensed the rocking movement. The number of rocking movements per 6 min was recorded using a computerized system. Laboratory chow (CA-1, Clea Japan) was provided as food and water was provided through the wet absorbent cotton attached to the plastic tube of a water bottle. The activity chambers were placed in an incubator (MIR-153, Sanyo Biomedica, Osaka, Japan), in which lighting conditions were provided by a cool white fluorescent lamp (FL15N, Panasonic) connected to an electronic timer and the light intensity was 1.8–2.9 W/m^2 varying with the proximity to the lamp. Two-day-old adult male crickets were isolated from the laboratory colony of crickets maintained under standard laboratory conditions of LD cycles at 25°C. For the locomotor activity experiments, after sexual maturation the male crickets were initially entrained to LD cycles at 25°C for three days and subsequently transferred to temperature cycles (TC) of 12 h 30°C: 12 h 25°C combined with LD in which L and D corresponded to the warm (30°C) and cold (25°C) phases, respectively, or TC under constant darkness (DD/TC) for 5 days. The temperature transition from 25°C to 30°C occurred in 23 ± 2 min and transition from 30°C to 25°C occurred in 12 ± 1 min inside the incubator. In a subset of animals, three days of LD cycles were followed by seven days of DD/TC. After 7 days of DD/TC, the onset of cold phase of the temperature cycle was phase advanced by 6 h. The raw data obtained as the number of seesaw movements per 6 min were used to generate double-plotted actograms or daily average histograms to assess the activity patterns, and evening activity onset was used as the phase marker to estimate the number of transients required to attain a stable phase relationship to the shifted TC. The phase of evening activity onset was determined, for each animal, with the bin size of 6 min as the Zeitgeber time when the activity level exceeded twofold of the daily average. This method effectively detected the evening activity onset which exceeded basal activity levels.

Optic lobe sampling protocol

For optic lobe sampling, adult male crickets were entrained initially to LD cycles for three days and subsequently placed in LD, LD/TC or DD/TC as described above. After seven days in each condition, the optic lobe, the clock tissue of crickets, was sampled at 4 h intervals for 24 h to examine whether TC can entrain transcription rhythm of clock genes. For phase shift experiments, after 7 days of entrainment under TC, crickets were exposed to a 6 h phase advance (PA) of TC by changing the onset of cold phase. The optic lobes were sampled at 4 h intervals on day 1 (PA1), day 3 (PA3) and day 7 (PA7) after the TC was shifted by 6 h.

Measurement of mRNA levels

To assess the mRNA transcription profile of clock genes, three replicates were used for each time point under LD and for phase advance experiments. Each replicate consisted of 3 pairs of optic lobes obtained from three individuals. Four replicates were used for LD/TC and DD/TC experiments. Total RNA was extracted and purified from six optic lobes with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). To avoid any genomic DNA contamination in the sample, total RNA obtained was treated with DNase I. About 500 ng total RNA of each sample was used for reverse transcription. cDNA synthesis was carried out with random 6-mers using PrimeScript RT reagent kit (Takara, Shiga, Japan) and quantitative real time RT PCR (qPCR) was run by using the Mx3000P™ Real-Time PCR Sys-

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tem (Stratagene, La Jolla, CA) to measure mRNA levels. Temporal expression profiles of core clock genes, *Gb'per* (GenBank/EMBL/DDBJ accession No.BAG48878), *Gb'tim* (BAJ16356), *Gb'cry2* (LC202053), *Gb'cyc* (AB762416) and *Gb'Clk* (AB738083), were measured under temperature cycles and also under phase advanced temperature cycles. Primers used for qPCR are listed in Table 1. In all cases, a single expected amplicon was confirmed by melting analysis. The quantification was made based on a standard curve obtained with known amounts of templates. *Gb'rpl18a* mRNA with stable expression across time was used as the reference gene. Clock gene mRNA values shown are relative to *Gb'rpl18a* mRNA. For temperature entrainment experiments, mRNA abundance was normalized by using the highest value as 1. For phase advance experiments, PA1, PA3 and PA7 mRNA abundance values were normalized with the highest value of Day 0.

Statistical analysis

To analyse the phase of activity onset, we determined the phase of evening activity peak which occurred at light to dark and warm to cold transition. These values were subjected to circular vector analysis (Batschelet, 1981) to obtain the magnitude (r, on a scale 0 to 1), and direction (a^0, on a scale of 1–360° or 1–24 h) of the phase-coherence vectors. A magnitude of 1 would mean that all individuals in a given set have exactly the same phase, whereas a magnitude of 0 would mean that individuals in the group are completely phase desynchronized. We subjected the phase values to the Watson-Williams test following ANOVA to determine if the phase was significantly different between LD, LD/TC and DD/TC. The statistical analyses were implemented on Statistica for Windows Release 5.0 B (StatSoft 1995, Tulsa, OK, USA).

Cosinor analysis as outlined in Nelson et al. (1979) was implemented in MATLAB-R2016a to test for rhythmicity, and estimate the peak phase and the amplitude (peak/trough ratio) of relative mRNA expression. For analysis of amplitude, the best fit curve was used even when the rhythm was not significant. Error bars in all the graphs represent standard error of the mean (SEM).

RESULTS

Temperature cycles can entrain behavioural rhythm of crickets

To assess whether temperature cycle (TC) can entrain circadian locomotor activity rhythms of crickets, we recorded locomotor activity of adult male crickets under LD (n = 11), LD combined with TC (LD/TC) (N = 21), and TC under constant darkness (DD/TC) (n = 19). Under LD and LD/TC conditions, crickets showed a nocturnal rhythm with the evening activity onset occurring at the light-off (Fig. 1A, B). Under DD/TC, the evening activity onset occurred earlier, preceding the warm/cool transition (Fig. 1C, F). To assess detailed daily activity pattern we calculated daily average activity profiles. As shown in Fig. 1D, under LD, the cricket showed two peaks at lights-on (ZT 0.1) and lights-off (ZT 12.1). The lights-on or morning peak was of short duration and has been shown to be a masking effect caused by light, while lights-off or nocturnal peak persisted longer and has been shown to be an endogenous rhythmic component (Tomioka and Chiba, 1987). Under LD/TC, the lights-off peak was similar to that under LD, but the lights-on peak was greatly reduced (Fig. 1E). Under DD/TC, the morning peak disappeared and the nocturnal peak occurred earlier (Fig. 1F). We further assessed the phase of activity onset and found that it occurred at the lights-off under LD and at the warm to cold phase transition under TC. The activity onset occurred at ZT 12.06 ± 0.01 h, ZT 11.91 ± 0.05 h, and ZT 10.89 ± 0.13 h, under LD, LD/TC, and DD/TC, respectively. Activity onset occurred at the lights-on is at ZT 23.54 ± 0.1 h under LD condition. Watson Williams test on phase of onset of evening activity occurring at lights-off/warm to cold phase transition showed that DD/TC (r = 0.98) is significantly different from LD (r = 0.99) (P < 0.0001) and LD/TC (r = 0.99) (P < 0.0001) (Fig. 1G). This suggests that temperature cycles alone phase-advance the evening locomotor activity onset in crickets.

We then tested re-entrainment of the nocturnal activity peak to DD/TC advanced by 6 h in 12 crickets. The mean phase value of evening activity onset during DD/TC prior to phase advance is ZT 10.70 ± 0.19 h. After confirming entrainment to TC, the onset of cold phase was advanced by 6 h. The activity onset advanced gradually and established a stable phase relationship with the shifted TC after ~16 transient cycles (Fig. 2A, B). On the 17th day (PA17), the mean phase of activity onset was ZT 10.81 ± 0.56 h. From 17th day (PA17) onwards, the phase of activity onset occurred ~1 h prior to cold phase and the mean phase value of activity onset from the 17th to the 23rd day (PA17-23) is ZT 10.69 ± 0.3 h. ANOVA revealed that the mean phase values obtained for DD/TC (ZT 10.70 ± 0.19 h) and PA17-23 are not significantly different (Fig. 2C). The re-entrained rhythm was phase-locked as the evening activity onset occurred about 1 h prior to the onset of the cold phase. These results suggest that TC can entrain the circadian activity rhythm of crickets.

Temperature cycle can entrain the molecular oscillation of the circadian clock

To assess whether temperature cycle can entrain the circadian clock and affect its phase, we estimated transcription profile of core clock genes such as *period* (*Gb'per*), *timeless* (*Gb'tim*), *cryptochrome2* (*Gb'cry2*), *cycle* (*Gb'cyc*) and *Clock* (*Gb'Clk*) in the optic lobe of adult male crickets under LD, LD/TC, and DD/TC (Fig. 3). Under LD, *Gb'per*, *Gb'tim* and *Gb'cry2* showed a clear circadian expression rhythm (cosinor analysis, p < 0.001, P < 0.003, and P < 0.0001, respectively) (Fig. 3A): their mRNA levels were low during the light phase and started to increase at the early day (*Gb'tim*), midday (*Gb'per*) or late day (*Gb'cry2*) to peak at ZT 13.84 ± 0.44 h (*Gb'tim*), ZT 15.78 ± 0.55 h (*Gb'per*) and at ZT18.90 ± 0.38 h (*Gb'cry2*), respectively. *Gb'cyc* also showed a rhythmic pattern (cosinor analysis, P < 0.04), but

Table 1. PCR primers used for quantitative RT-PCR.

Primers	Forward	Reverse
Gb'per	5'-AAGCAAGCAAGCATCCTCAT-3'	5'-CTGAGAAAGGAGGCCACAAG-3'
Gb'tim	5'-GATTATGAAGTCTGTGATGATTGG-3'	5'-AGCATTGGAGAGAACTGAAGAGGT-3'
Gb'cry2	5'-AGCACCATCACACACTTCACA-3'	5'-ACACTCAGCGCAATCCACAC-3'
Gb'Clk	5'-AGCACCATCACACACTTCACA-3'	5'-ACACTCAGCGCAATCCACAC-3'
Gb'cyc	5'-GGCCGAAGCTCATAAAGTGG-3'	5'-AACCGCACAAAGGAACCATC-3'
Gb'rpl18a	5'-GCTCCGGATTACATCGTTGC-3'	5'-GCCAAATGCCGAAGTTCTTG-3'

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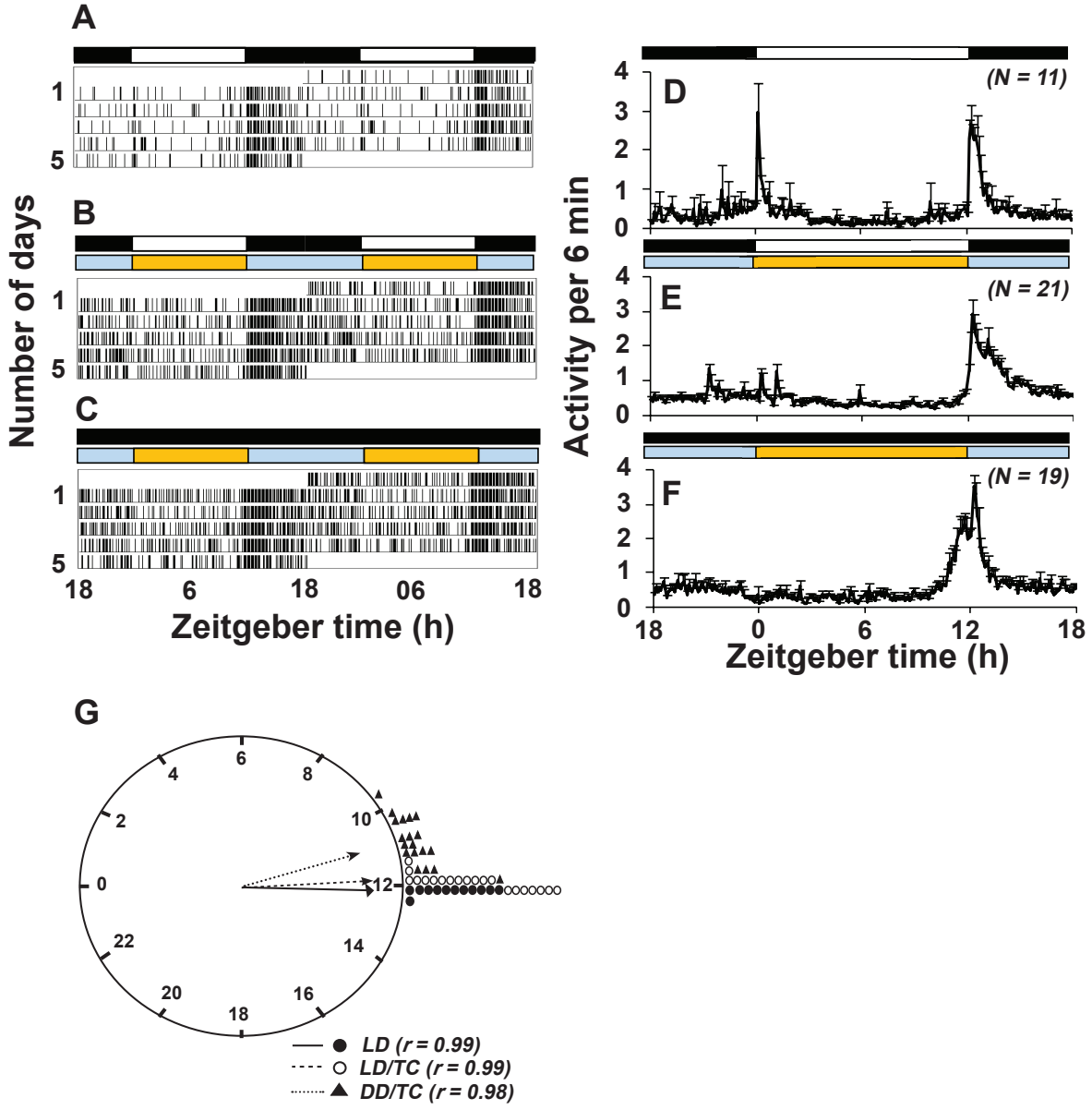


Fig. 1. Locomotor activity-rest rhythms under light/dark and temperature cycle. A–F: Representative locomotor activity rhythms **(A–C)** and daily activity profiles averaged over five days **(D–F)** of the cricket *Gryllus bimaculatus* under light/dark (LD) cycle **(A, D)**, LD combined with temperature cycle (TC) of 30°C 12 h: 25°C 12 h **(B, E)**, and TC in constant darkness (DD) **(C, F)**. Black and white bars above panels indicate dark (black) and light (white) phase, respectively. Light blue and orange bars indicate cold phase (light blue) and warm phase (orange), respectively. Y axis in D–F represents mean activity counts per 6 min. Numbers in the parenthesis show the number of animals used. G: Phase of onset of evening activity under LD, LD/TC and DD/TC. Numerals indicate Zeitgeber time. The symbols in the circular plot depict the phase determined from the onset of evening activity. Black circles, open circles and black triangles are the phases under LD, LD/TC and DD/TC, respectively. Phase coherence vectors are depicted as arrows, the length indicates the magnitude and the direction of the mean phase (black solid arrow, LD; black dashed arrow, LD/TC; black dotted arrow, DD/TC). Watson Williams test on phase of onset of activity occurring at lights-off/warm to cold phase transition showed that DD/TC (r = 0.98) is significantly different from LD (r = 0.99) ($P < 0.0001$) and LD/TC (r = 0.99) ($P < 0.0001$).

its mRNA levels were low at night, and peaked at early day (ZT 10.88 ± 1.43 h).

Under LD/TC, *Gb'per*, *Gb'tim* and *Gb'cry2* showed a clear daily rhythmic profile (cosinor analysis, $p < 0.0001$, $p < 0.002$ and $p < 0.0001$, respectively) (Fig. 3B), and their peak maintained a similar phase to those under LD, occurring at ZT 16.32 ± 1.11 h, ZT 12.35 ± 0.38 h, and ZT 17.89 ± 0.66 h, respectively (Fig. 3D). *Gb'cyc* mRNA showed a rhythmic profile (cosinor analysis, $P < 0.04$) (Fig. 3B) with no significant reduction in amplitude, while its phase advanced compared to that under LD, peaking at ZT 6.43 ± 0.66 h (Fig. 3B, D).

Under DD with TC, *Gb'per*, *Gb'tim*, and *Gb'cry2* mRNA levels again showed a clear daily cycling (cosinor analysis,

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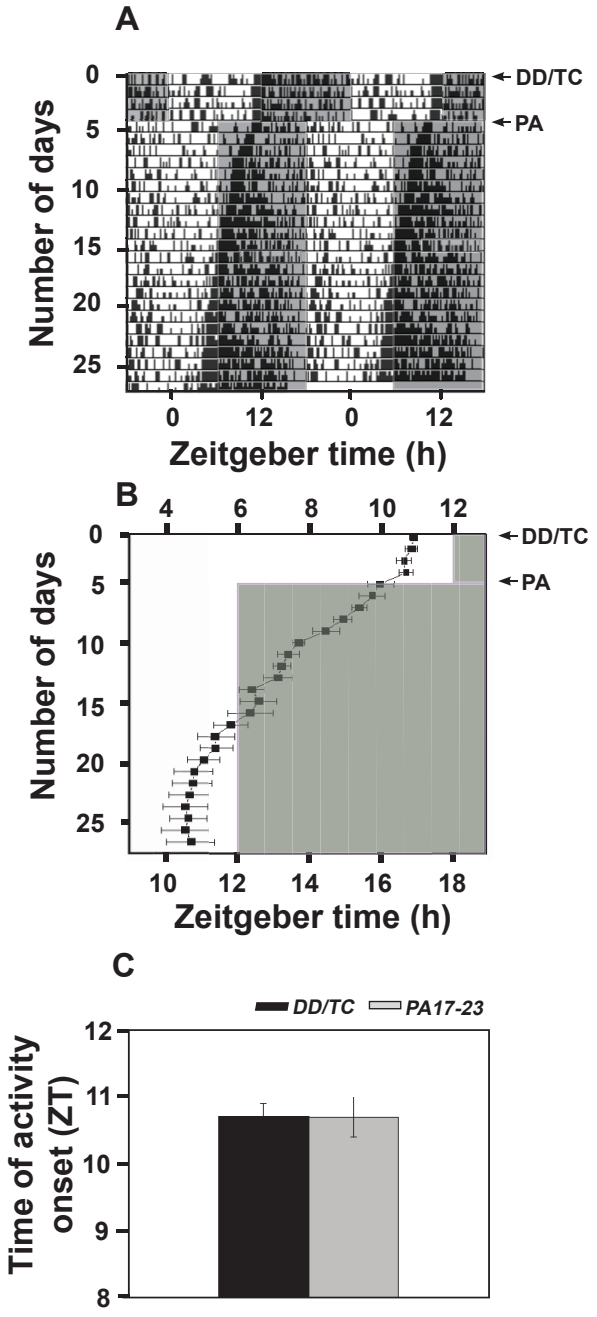


Fig. 2. Locomotor activity-rest rhythm of crickets under phase advanced temperature cycle. **(A)** Representative double plotted actogram of adult male crickets. Crickets were subjected to 12:12 h of warm phase/cold phase cycles (TC) under constant darkness (DD). After four days of temperature cycle entrainment, the onset of the cold phase of the temperature cycle was phase advanced by 6 h (PA). Gray bars represent the duration of the cold phase of temperature cycle. **(B)** The trajectories of the phase of evening activity onset before and after the phase advance of temperature cycle are plotted as means with SEM (n = 12). Values given on the top X axis is the ZT before the phase shift and the ZT values after the phase shift is given on the bottom X axis. Note that the activity rhythm required ~16 transient cycles to reach a stable phase relationship with the phase advanced temperature cycles. **(C)** Mean phase of evening activity onset under DD/TC and during days 17–23 under phase advanced temperature cycle. ANOVA on mean phase values exhibit no significant difference under the two conditions ($P > 0.05$).

$p < 0.0001$, $p < 0.002$, and $p < 0.04$), but their peak occurred earlier at ZT 11.36 ± 0.58 (*Gb'per*), ZT 11.09 ± 0.41 (*Gb'tim*) and ZT 13.96 ± 0.41 (*Gb'cry2*) compared to those under LD and LD/TC (Fig. 3C, D). The amplitude of *Gb'per* expression was higher than that under LD but not different from that under LD/TC (Fig. 3). The amplitude of *Gb'tim* expression was higher than that under both LD and LD/TC (Fig. 3). The amplitude of *Gb'cry2* mRNA expression was intermediate between those under LD and LD/TC (Fig. 3). *Gb'cyc* also showed a significant daily oscillation (cosinor analysis, $p < 0.01$) with an advanced peak (ZT 4.98 ± 0.90 h) under DD/TC compared to that under LD (ZT 10.88 ± 1.43 h), while no change was observed in amplitude (Fig. 3).

Gb'Clk exhibited a slight increase during the day phase or warm phase under these three light and temperature conditions, but the significant rhythm was detected by cosinor analysis only under LD/TC conditions ($p < 0.05$, Fig. 3).

Resetting of the circadian clock to the changes in the timing of temperature cycles

To elucidate how circadian clock resets with the changes in temperature, we assessed the readiness of molecular rhythm of circadian oscillator components to re-entrain to the phase advanced temperature cycles. When TC was phase advanced, both *Gb'per* and *Gb'tim* mRNA amplitudes were reduced and exhibited no rhythmic changes on the first day (cosinor analysis $p > 0.05$) (Fig. 4A, B, E). With increasing number of days, mRNA levels of both genes slowly regained a rhythmic expression. On the third day of TC phase advance, *Gb'per* and *Gb'tim* mRNA showed a gradual increase during warm phase and a reduction through cold phase, resulting in a significant daily cycling (cosinor analysis, $p < 0.02$ for *Gb'per* and $p < 0.003$ for *Gb'tim*) with an amplitude nearly the same as (*Gb'per*) or smaller than that before the phase shift (*Gb'tim)* (Fig. 4A, B, E). On day 7 of phase advanced TC, they gained a stable rhythmic expression with a peak near warm/cool transition (cosinor analysis, $p < 0.002$ for *Gb'per* and $p < 0.0001$ for *Gb'tim*) (Fig. 4A, B). The amplitude of *Gb'per* expression regained nearly the original level. On day 7, the peak of *Gb' tim* and *Gb' per* expression was delayed by 2 to 3 h compared to that before phase shift occurring near warm/cool transition (Fig. 4A, B, D). Although in this study the mRNA levels were measured at 4 h intervals and could not make exact phase comparison between before and after the phase shift of TC, it seemed likely that they at least nearly re-entrained to the shifted TC on day 7.

In contrast to *Gb'per* and *Gb'tim*, *Gb'cry2* maintained a significant expression rhythm after TC was phase advanced (cosinor, $p < 0.02$). The 6 h phase advance in TC did not affect the amplitude of *Gb'cry2* mRNA rhythm on day 1 (Fig. 4C, E), while the amplitude of *Gb'cry2* expression was greater than the original level on day 3 and day 7 (Fig. 4C, E). The peak of *Gb'cry2* mRNA occurred at ZT 22.53± 0.29 h on day 1, delayed by 2.57 h compared to that of the original phase angle, whereas on day 3, the peak phase advanced to ZT 21.41 ± 0.91 h. The peak subsequently advanced to peak at ZT 16.2 ± 0.27 h on day 7, which was 2.24 h later than that before the shift (Fig. 4C, D).

In contrast to the quick re-entrainment of *Gb'per* and *Gb'tim*, *Gb'cyc* mRNA rhythm exhibited a feeble response

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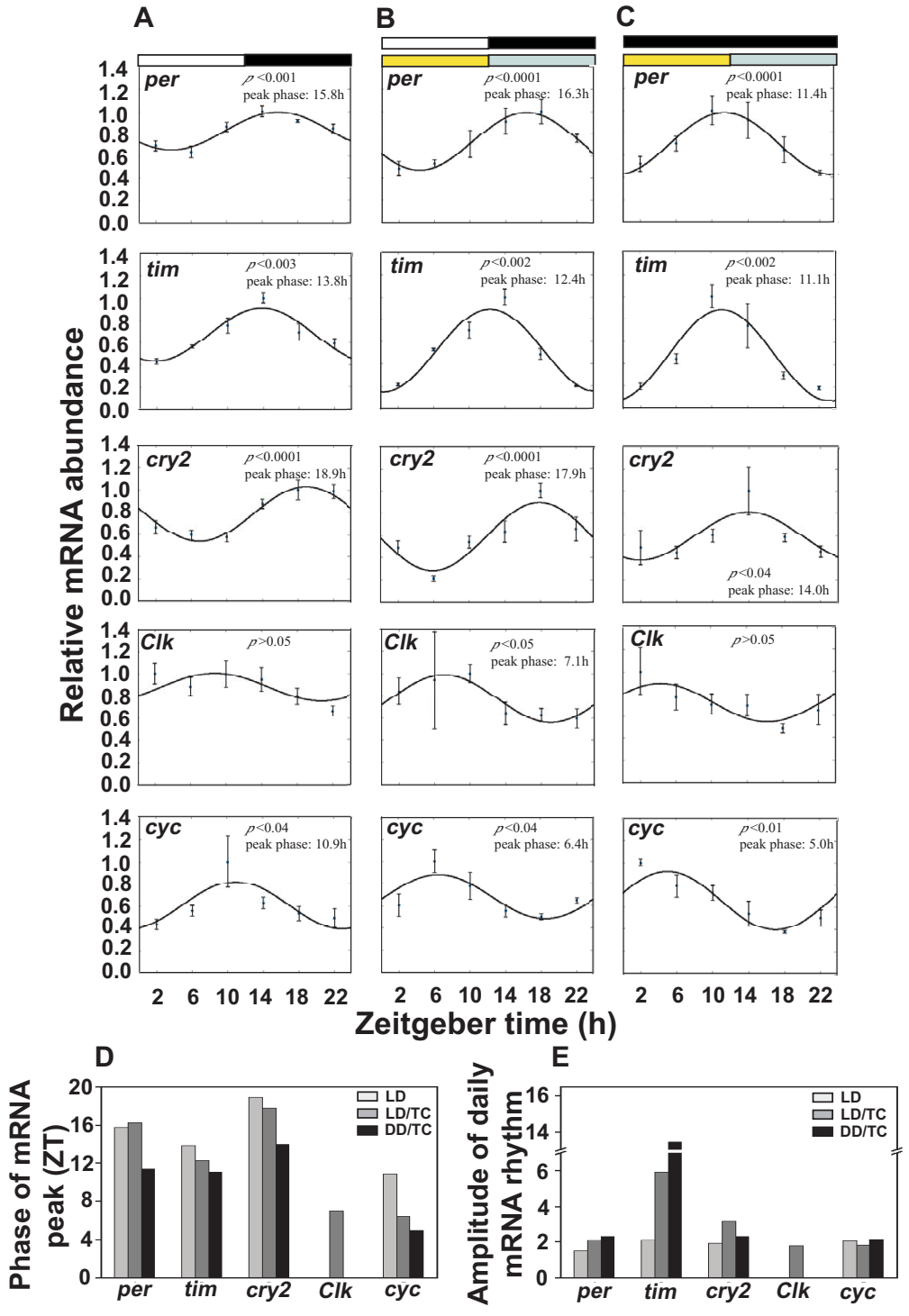


Fig. 3. Expression profiles of clock genes *Gb'per*, *Gb'tim*, *Gb'cry2*, *Gb'cyc* and *Gb'Clk* in the optic lobe of *Gryllus bimaculatus* under LD, LD/TC, and DD/TC. A–C: Daily changes in clock gene mRNA levels under LD **(A)**, LD/TC **(B)**, and DD/TC **(C)**. The abundance of mRNA was measured by quantitative real-time RT-PCR. The abundance of *rpl18a* mRNA was used as an internal reference. Black and white bars above panels indicate dark (black) and light (white) phase, respectively. Light blue and orange bars indicate cold phase (light blue) and warm phase (orange), respectively. Three replicates were used for LD and four replicates for LD/TC and DD/TC for each time point and the mean value was plotted with SEM. *Gb'per*, *Gb'tim*, *Gb'cry2*, and *Gb'cyc* mRNA level exhibit daily rhythm under the three conditions and the peak was phase advanced under DD/TC. *Gb'per* and *Gb'tim* peaked almost at the same phase under LD and LD/TC, while *Gb'cyc* showed a phase advance even under LD/TC. *Gb'Clk* showed a tendency to be high in light phase or warm phase, but statistically significant rhythm was detected only under LD/TC. The solid line in the figure is the cosine fitted curve for the mean value of mRNA expression of clock genes at 4 h intervals over 24 h. *p* values were obtained from cosinor analysis. **(D)** Peak phase of mRNA rhythm of *Gb'per*, *Gb'tim*, *Gb'cry2*, *Gb'cyc* and *Gb'Clk* under LD, LD/TC and DD/TC. The peak of *Gb'per*, *Gb'tim* and *Gb'cry2* expression rhythms was phase advanced under DD/TC compared to those under LD and LD/TC. *Gb'cyc* was phase advanced under both LD/TC and DD/TC compared to LD. **(E)** Amplitude (peak/trough ratio) of clock gene mRNA rhythm under LD, LD/TC and DD/TC. Both *Gb'per* and *Gb'tim* mRNA exhibited an increase in amplitude under DD/TC compared to LD, whereas amplitude of *Gb'cry2* mRNA was increased only under LD/TC. *Gb'cyc* mRNA exhibited no significant difference in amplitude between these three different conditions. *Gb'Clk* mRNA exhibited rhythm only under LD/TC conditions. For further details, see text.

to the advance in temperature phase. Under DD/TC, *Gb'cyc* mRNA oscillated with a peak after the onset of warm phase (ZT 4.98 ± 0.9 h). After the phase advance of the temperature cycle, *Gb'cyc* maintained a rhythmic profile (cosinor analysis, $P < 0.001$) (Fig. 5). The peak of mRNA expression remained nearly at the original phase on the first and the third day (Fig. 5A, C). Although phase advance of *Gb'cyc* expression rhythm was observed on the seventh day, it did not regain the original phase relationship (Fig. 5A, C, D). *Gb'Clk* exhibited no significant rhythm under phase advanced TC (cosinor analysis, $P > 0.05$; Fig. 5B).

DISCUSSION

Although temperature is considered as one of the pre-

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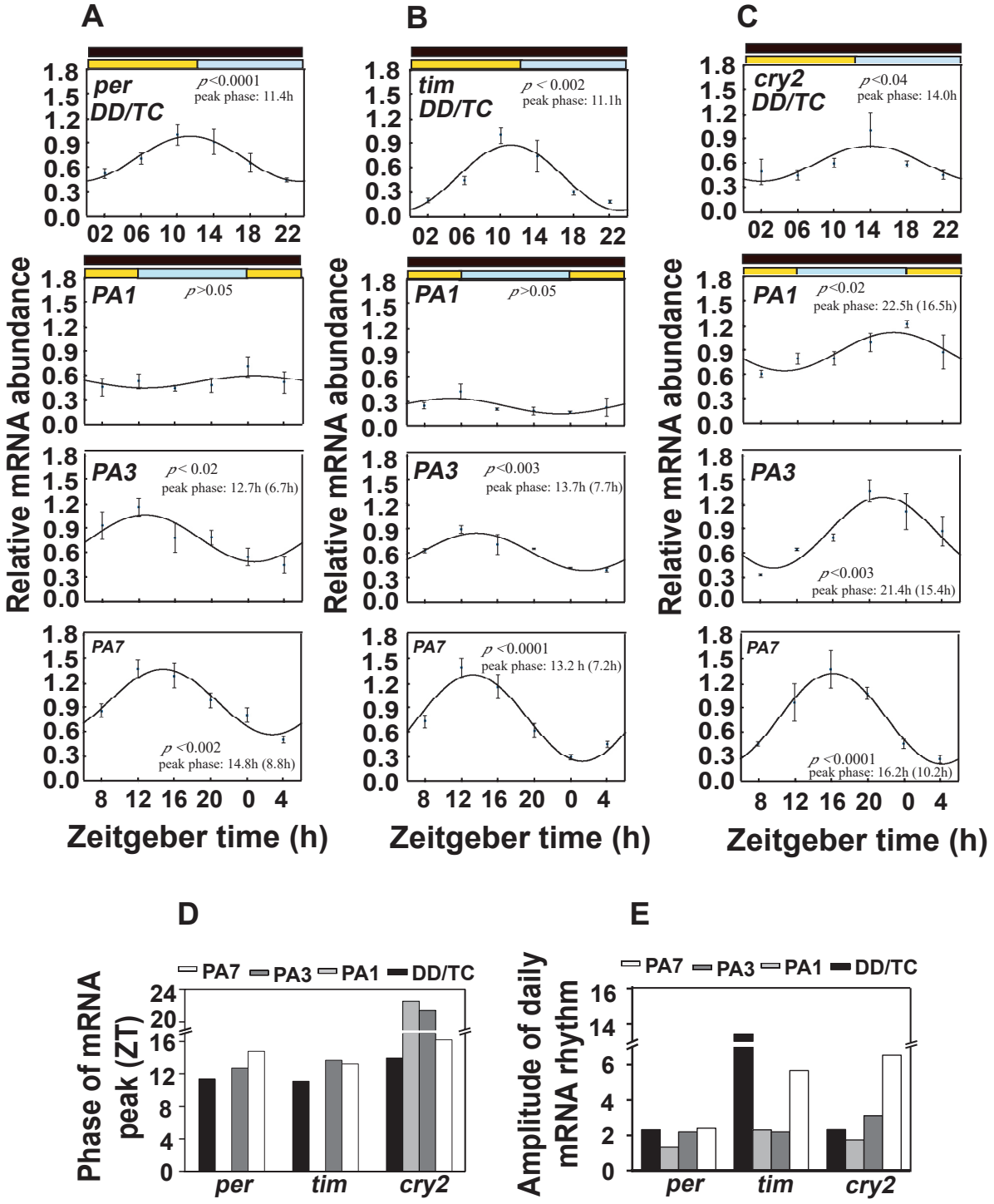


Fig. 4. Expression profiles of *Gb'per*, *Gb'tim*, and *Gb'cry2* mRNA in the optic lobe under 6 h phase advanced temperature cycles. **(A–C)** Daily changes in mRNA levels of *Gb'per* (A), *Gb'tim* **(B)**, and *Gb'cry2* **(C)** before and after the shift of TC under DD. After 7 days of entrainment under TC, crickets were exposed to a 6 h phase advance of temperature cycle. The optic lobe, the clock tissue of crickets, were sampled at every 4 h intervals on day 1 (PA1), day 3 (PA3) and day 7 (PA7) after the shift. *Gb'per* and *Gb'tim* mRNA rhythms re-entrained to near original phase on day 3 whereas *Gb'cry2* re-entrained gradually. The solid line in the figure is the cosine fitted curve for the mean value of mRNA expression of clock genes at 4 h intervals over 24 h. *p* values were obtained from the cosinor analysis. Peak phase values given are calculated with respect to the ZT after the phase shift and those before the phase shift are shown in parenthesis. **(D, E)** Peak phase and amplitude (peak/trough ratio) of clock gene *per, tim* and *cry2* mRNA before the shift (DD/TC) and on day 1 (PA1), day 3 (PA3) and day 7 (PA7) after the shift. (D) The mRNA of *Gb'per* and *Gb'tim* peaked close to the original phase on day 3, while peak of *Gb'cry2* oscillation gradually advanced to occur close to the original phase on day 7. However, their peaks occurred about 2 h later than their original phase. (E) Both *Gb'per* and *Gb'tim* mRNA levels were reduced on the first day (PA1) of phase shift of TC, while the amplitude of *Gb'cry2* mRNA rhythm showed no changes on day 1, but increased on day 7. For further details, see text.

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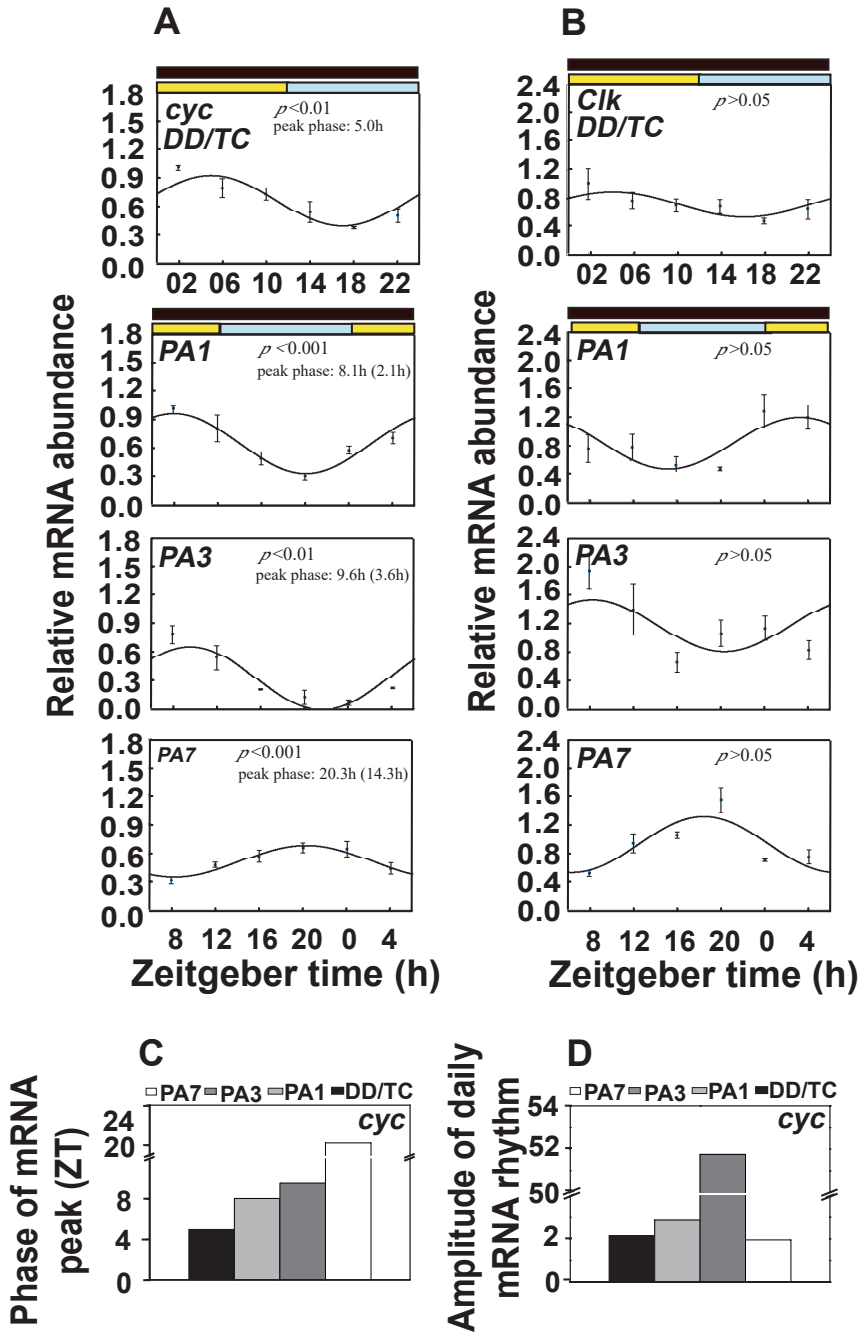


Fig. 5. Expression profiles of *Gb'cyc* and *Gb'Clk* mRNA in the optic lobe under 6 h phase advanced temperature cycles. **(A)** After the phase advance of temperature cycle, the peak of *Gb'cyc* mRNA level remained nearly at the same phase on the first and third day of phase advanced temperature cycle. On day 7 it shifted several hours but did not attain the original phase relationship. **(B)** *Gb'Clk* mRNA showed no rhythmic expression under the phase advanced temperature cycle. The solid line in the figure is the cosine fitted curve for the mean value of mRNA expression of clock genes at 4 h intervals over 24 h. Peak phase values given are calculated with respect to the ZT after the phase shift and those before the phase shift are shown in parenthesis. C, D: Peak phase **(C)** and amplitude (peak/trough ratio) **(D)** of *cyc* gene mRNA rhythm under DD/TC, PA1, PA3 and PA7. *Gb'cyc* showed a significant shift on day 7, but the resultant phase is far apart from the original phase. For further details, see text.

vailing time cue among the so called "zeitgebers" (Rensing and Ruoff, 2002), the molecular mechanism of thermal entrainment remains largely unresolved. Our present study showed that temperature cycles can drive circadian rhythmicity in locomotor activity and the expression of core clock genes in the optic lobe, the circadian clock tissue, of the cricket, *Gryllus bimaculatus*. The phase of nocturnal activity onset was almost the same under LD and LD/TC but advanced under DD/TC. Likewise, phases of mRNA expression rhythms of *Gb'per*, *Gb'tim*, *Gb'cry2*, and *Gb'cyc* were advanced relative to those under LD cycles. Crickets are predominantly nocturnally active at higher ambient temperature (25°C), and become diurnal at lower ambient temperature (20°C) under light/dark cycles (Ikeda and Tomioka, 1993). The results of our present study showed that phase of nocturnal activity onset was advanced under temperature cycles alone, corroborating the crucial temperature dependency of the phase of activity rhythm in crickets.

Phase advance of temperature cycle can trigger changes in the phase of circadian oscillatory components in a gene specific manner and it required ~16 transients for the activity-rest rhythm to re-entrain to the temperature cycle advanced by 6 h. We also assessed the re-entrainment of activity-rest rhythm to TC (25°C/30°C) delayed by 6 h. Our preliminary results showed that 6 h delayed TC did not effectively entrain the locomotor rhythm and most crickets showed a rhythm running with a period close to 24 h. These are in contrast to light entrainment, in which activity rhythms are successfully re-entrained to 6 h advanced or delayed light/dark cycles by approximately five transients (Komada et al., 2015). Taken together, these results indicate that temperature is a weaker zeitgeber than light for the cricket's circadian timing system.

Our findings also revealed that temperature cycle can affect both the phase and amplitude of *Gb'per* and *Gb'tim* mRNA oscillations. In addition, change in the phase of external thermal stimuli can elicit transient changes in the phase of both *Gb'per* and *Gb'tim* mRNA oscillations. These phase changes occur in a manner that is consistent with the direction of the phase shifts in behavioral rhythms, and the molecular rhythms of *Gb'per* and *Gb'tim* attain the phase relationship similar to that of locomotor rhythm seven days after the shift of TC cycle while the peak of both rhythms occurred about 2 h later than their original phase (Figs. 2 and 4). This fact suggests that *Gb'per* and

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Gb'tim are the initial clock-oscillatory components that respond to changes in the external temperature cycle. Another feature of *Gb'per* and *Gb'tim* mRNA rhythms observed during re-entrainment is that they peaked at the warm-cold temperature transition. Moreover, advance in the onset of the cold phase of temperature cycle reduced the transcription level of *Gb'per* and *Gb'tim* mRNA during the cold phase on the first day of the shift. Although further studies are needed, these results suggest that cold temperature hampers transcription of core clock oscillatory components *Gb'per* and *Gb'tim* in the cricket. This finding is reminiscent of a previous report that cold temperature suppresses the protein levels of clock gene *frequency* in *Neurospora* (Liu et al., 1998).

So far studies suggest that *cryptochrome* is needed for light entrainment and in addition it seems to be important for the maintenance of central clock function at extreme temperature (Dolezelova et al., 2007) whereas studies also suggest that it hampers temperature entrainment in fruit flies (Gentile et al., 2013; Yoshii et al., 2010). In non-drosophilid insect species, including crickets, *cryptochrome*s can probably act as transcriptional repressor within the clock network alone or along with *per* (Rubin et al., 2006; Tokuoka et al., 2017). In the cricket, *Gb'cry2* mRNA is expressed rhythmically under temperature cycles and it showed a gradual re-entrainment under phase advanced temperature cycles (Figs. 3 and 4), suggesting its close relation to the behavioral rhythm.

In addition to *Gb'per*, *Gb'tim* and *Gb'cry2*, *Gb'cyc* transcript is expressed rhythmically in crickets (Uryu et al., 2013), showing the features similar to the mammalian orthologue *Bmal1*, while it does not oscillate in fruit flies (Rutila et al., 1998). Our present study showed that *Gb'cyc* transcript oscillation is phase advanced under temperature cycle than under light (Fig. 3) (Uryu et al., 2013). However, it was re-entrained much more slowly than phase-advanced TC in comparison with *Gb'per* and *Gb'tim* and did not regain the original phase even seven days after the shift of TC (Figs. 4, 5). Further studies are required to test whether *Gb'cry2* and *Gb'cyc* are apparently linked to resetting the circadian clock with the changes in temperature and to reveal their specific roles in temperature entrainment. In contrast to other clock genes, *Gb'Clk* transcript exhibited significant rhythmic changes only under LD/TC (Fig. 3). It has been suggested that *Gb'Clk* could be rhythmically expressed in some condition (Uryu et al., 2013), and our results suggest that its expression may be intricately regulated by light and temperature.

Our results showed that temperature cycle can entrain the behavioral rhythm and transcriptional rhythms of core clock genes such as *Gb'per*, *Gb'tim*, *Gb'cyc* and *Gb'cry2*. *Gb'per* and *Gb'tim* were re-entrained to the shifted temperature cycles with a lesser number of transient cycles compared to *Gb'cry2* and *Gb'cyc*, and *Gb'Clk* exhibited no rhythmicity. It seems likely that *Gb'cyc* requires more transient cycles to attain a stable phase; the transient cycles may correspond to the time that the locomotor activity rhythms require to be re-entrained to the 6 h advanced TC cycle. These results suggest that the clock constituents have different sensitivity to temperature and play differential roles in resetting the clock caused by changes in temperature like in photic entrainment (Kutaragi et al., 2016; Lee et al., 1996; Shigeyoshi et al., 1997). Since temperature exerted influence on the transcript levels of *Gb'per* and *Gb'tim* (Figs. 3 and 4), it may have direct effects on the clock machinery. However, how temperature entrains the core molecular clock still remains to be answered. Detailed examination of the expression of clock constituent genes during the transition period combined with their RNAi may provide a clearer picture of this process. There is a possibility that the temperature receptor located outside the clock neurons like the thoracic chordotonal organ and antennal thermoreceptor may be involved in temperature resetting of the circadian clock as in the case of *Drosophila* (Sehadova et al., 2009; Yadlapalli et al., 2018). It is possible that in the cricket, antennal thermoreceptors (Nishikawa et al., 1985) may perceive, integrate, and relay temperature information to neural circuits to modulate the molecular clock and behavioral outputs with the changes in environmental temperature. This should be examined in future studies.

We also have to consider the clock neuronal network underlying the temperature entrainment. In *Drosophila*, studies have shown that there is functional differentiation among circadian clock neurons: some are entrainable to temperature while some to light (Miyasako et al., 2007; Yoshii et al., 2009). Although in the cricket, the clock neurons have not yet been identified within the optic lobe, there might be a similar situation in which temperature entrainment is achieved through the temperature-entrainable neurons. As the neuropeptides are the key molecules mediating the entrainment of the circadian timing system (Taghert and Nitabach, 2012), it will be interesting to further explore the novel neuropeptides and receptors associated with thermosensory neurons, and to characterize the neural mechanisms underlying temperature entrainment of cricket circadian timing system.

ACKNOWLEDGEMENTS

We thank Dr. Sheeba Vasu for suggesting improvements on the manuscript, Shahnaz Rahman Lone and Nikhil K L for helping with the statistical analysis. This study was supported in part by a grant from Takeda Science Foundation in the form of research fellowship to NK.

COMPETING INTERESTS

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: NNK KT. Performed the experiments: NNK YT MN AT. Analyzed the data: NNK KT. Contributed reagents/materials: KT. Wrote the paper: NNK KT.

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(Received September 17, 2018 / Accepted November 21, 2018)

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