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Temperature cycle amplitude alters the adult eclosion time and expression pattern of the circadian clock gene *period* in the onion fly

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ABSTRACT

Soil temperature cycles are considered to play an important role in the entrainment of circadian clocks of underground insects. However, because of the low conductivity of soil, temperature cycles are gradually dampened and the phase of the temperature cycle is delayed with increasing soil depth. The onion fly, *Delia antiqua*, pupates at various soil depths, and its eclosion is timed by a circadian clock. This fly is able to compensate for the depth-dependent phase delay of temperature change by advancing the eclosion time with decreasing amplitude of the temperature cycle. Therefore, pupae can eclose at the appropriate time irrespective of their location at any depth. However, the mechanism that regulates eclosion time in response to temperature amplitude is still unknown. To understand whether this mechanism involves the circadian clock or further downstream physiological processes, we examined the expression patterns of *period* (*per*), a circadian clock gene, of *D. antiqua* under temperature cycles that were square wave cycles of 12-h warm phase (W) and 12-h cool phase (C) with the temperature difference of 8 °C (WC 29:21 °C) and 1 °C (WC 25.5:24.5 °C). The phase of oscillation in *per* expression was found to commence 3.5 h earlier under WC 25.5:24.5 °C as compared to WC 29:21 °C. This difference was in close agreement with the eclosion time difference between the two temperature cycles, suggesting that the mechanism that responds to the temperature amplitude involves the circadian clock.

1. Introduction

To know the time of day and show daily behavioral and physiological rhythms, most organisms including insects refer not only to exogenous factors, but also to an endogenous circadian clock with an approximate 24 h period. Light and temperature act as time cues from the environment, i.e., Zeitgebers, to establish entrainment of the circadian clock to 24-h environmental cycles (Rensing and Ruoff, 2002; Saunders, 2002). Various circadian clock genes consisting of molecular feedback loops underlying a circadian clock have been isolated in the fruit fly *Drosophila melanogaster*. Of these, *period* (*per*) and *timeless* (*tim*) are important components of a major loop. The expression of *per* and *tim* is activated during the day and in the early evening, and their mRNA levels achieve a peak early at night. The protein products of *per* and *tim*, PER and TIM, respectively, accumulate in the cytoplasm and form a PER-TIM heterodimer late at night. This heterodimer translocates into the nucleus and suppresses the transcription of *per* and *tim*, resulting in reduced PER and TIM during the daytime, and thereby the transcription of *per* and *tim* is activated again. Thus, the expression levels of *per* and *tim* and the abundance of their products oscillate with a periodicity of approximately 24 h (Tomioka and Matsumoto, 2010; Zheng and Sehgal, 2008). It has been demonstrated that the oscillation of the expression level of these genes can be entrained to light–dark cycles and temperature cycles with a stable phase relationship (Boothroyd et al., 2007; Glaser and Stanewsky, 2007; Majercak et al., 1999; Qiu and Hardin, 1996).

In the lifetime of an insect, timing of adult eclosion is one of the important events related to survival and reproductive success. In many insect species, eclosion occurs at a specific time of a day using a circadian clock (Myers, 2003; Saunders, 2002).

Abbreviations: C, 12-h cool phase; LL, continuous light; mesor, midline estimating statistic of the rhythm; PAC, PAS associated C terminal; PAS, PER-ARNT-SIM; *per*, *period*; φ_E, phase of the eclosion peak; *RpL32*, *ribosomal protein L32*; *tim*, *timeless*; W, 12-h warm phase; ZT, Zeitgeber time.

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In *D. melanogaster*, several studies have shown that *per* and *tim* affect eclosion time. Mutation at the *per* and *tim* loci can change the period length of the circadian clock that controls eclosion time or can induce arrhythmic eclosion (Konopka and Benzer, 1971; Konopka et al., 1994; Rothenfluh et al., 2000; Rutila et al., 1996; Sehgal et al., 1994). In addition, the increase and decrease of *per* gene dosage shorten and lengthen the period of this circadian clock, respectively (Smith and Konopka, 1982). Therefore, analysis of the expression pattern of circadian clock genes such as *per* and *tim* is considered important to investigate the relationship between eclosion time and the circadian clock in insects.

The onion fly, *Delia antiqua*, pupates at a depth of 2–20 cm in the soil where no light penetrates. Eclosion occurs in the early morning (Akita Agricultural Experiment Station, 1950; Inoue, 1967; Tanaka and Watari, 2003; Watari et al., 2006). Tanaka and Watari (2009) demonstrated that high temperatures significantly disturb expansion of the wing in newly emerging adults of *D. antiqua* although desiccation has no or little effect on wing expansion. Therefore, it is likely that the eclosion time is restricted to the early morning to avoid the high temperatures on the soil surface in the middle of the day. As in many other insects, a circadian clock controls the timing of *D. antiqua* eclosion (Watari, 2002a,b). The sensitivity of this clock to Zeitgebers is found only in the late pupal stage (Watari, 2005). Therefore, it is considered that the circadian clock of *D. antiqua* is entrained to 24 h by daily changes in soil temperature and results in emergence of new adults from the soil at the optimal time of the day (Tanaka and Watari, 2003; Watari, 2002b, 2005). This fly is able to detect a temperature difference as small as 1 °C to establish the timing of eclosion (Tanaka and Watari, 2003; Watari and Tanaka, 2014).

D. antiqua advances the eclosion time as the amplitude of diel temperature cycles (thermoperiods) decreases (Tanaka and Watari, 2003, 2011; Watari and Tanaka, 2010, 2014). This response has probably evolved to regulate timing of eclosion at different soil depths. With increasing soil depth, the daily change in soil temperature is gradually dampened and the phase of the temperature cycle is delayed because of low heat conductivity of soil. *D. antiqua* compensates for the depth-dependent phase delay of temperature change by advancing the eclosion time as the amplitude of the temperature cycle decreases with an increase in depth. Therefore, pupae located at any depth in the soil can eclose at the appropriate time (Tanaka and Watari, 2003). Similar responses have been observed in the flesh fly *Sarcophaga crassipalpis* and in the cabbage moth, *Mamestra brassicae*, which pupate underground (Miyazaki et al., 2011; Tanaka et al., 2013), and in the Indian meal moth, *Plodia interpunctella*, which pupates within the food substrate such as grains (Kikukawa et al., 2013).

The mechanism that regulates eclosion time in response to temperature amplitude is still unknown. Is this mechanism involved in the circadian clock or is it involved in more downstream physiological processes? If the former is the case, the expression pattern of circadian clock genes would be affected by temperature amplitudes and a decrease in temperature amplitude would advance the oscillation phase. In the latter case, the expression pattern would be similar regardless of temperature amplitude. In the present study, we isolated the fragment of *per* of *D. antiqua* and examined its expression pattern under temperature cycles with the same mean temperature and different amplitudes.

2. Materials and methods

2.1. Insects

A stock culture of *D. antiqua* was originally supplied by the Hokkaido Prefectural Central Agricultural Experiment Station, at Naganuma, near Sapporo, Hokkaido (43°03′N), Japan in 1981, and was maintained by mass rearing on fresh onion slices. This species enters diapause at the pupal stage in response to a short-day photoperiod or low temperature during the larval stage (Ishikawa et al., 1987; Watari, 2003). In the present study, only those diapause pupae that had been reared as larvae in 12-h light: 12-h darkness at 15 °C were used and these were then maintained in continuous darkness at 7.5 °C for more than 3 months to terminate diapause. The post-diapause pupae kept at 25 °C eclose as adults within 8–15 days (Watari, 2002a; Watari and Tanaka, 2014).

2.2. Recording the eclosion time

The apparatus used to record the eclosion time was similar to that described by Watari (2002a). It consists of a plastic box equipped with an infrared light emitter and a detector (GT2; Takenaka Electronic Industrial Co., Kyoto, Japan) and is based on the "falling ball" principle (e.g., Saunders, 1976; Truman, 1972). When a stainless-steel ball pushed out by an eclosing fly's head crosses the infrared beam, a signal is sent to a computer and the number of eclosions is counted. The recording apparatus filled with post-diapause pupae was placed under continuous light (LL) in an incubator (Nippon Medical and Chemical Instruments Co., Osaka, Japan), in which thermoperiods consisting of square wave cycles of 12-h warm phase (W) and 12-h cool phase (C) could be programmed. The time of temperature increase was defined as Zeitgeber time 0 h (ZT 0). The temperature difference between W and C was 8 °C (WC 29:21 °C) and 1 °C (WC 25.5:24.5 °C) with the same average temperature (25 °C). The programmed constant temperature was kept within a range of ±1.0 °C. It was confirmed by using a portable data logger that experimental temperatures correspond to the set values of the incubator (Ondotori, TR-74Ui Illuminance UV Recorder; T&D Co., Matsumoto, Japan). A 10 W fluorescent lamp (FL10WB; Hitachi Lighting Ltd., Tokyo, Japan) in the incubator gave about 1.9 W m^{-2} of light. As flies emerged over several days, the daily data obtained during the whole emergence period were pooled, and the phase of the eclosion peak (φ_E) was represented by the median time of eclosion.

2.3. Cloning

Fragments of *per* and *ribosomal protein L32* (*RpL32*) of *D. antiqua* were obtained by reverse transcription polymerase chain reaction (RT-PCR). Generally, *per* is highly expressed in the head and *RpL32* is ubiquitously expressed. Therefore, we used RNAs from the head of 20 adult flies for *per* cloning and those from the whole bodies of 10 adult flies for *RpL32* cloning. Total RNAs were extracted with Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, dissolved in nuclease-free water, and stored at −20 °C until use. cDNAs were synthesized from total RNAs using M-MLV reverse transcriptase and oligo$(dT)_{12-18}$ Primer (Invitrogen). PCR was performed with cDNA and 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen) in a final concentration of 1× PCR buffer formulated by Invitrogen, containing 0.5 μM of each primer (PtPer-F1 and PtPer-R1 for *per* and RPL-49L-F1 and RPL49L-R1 for *RpL32*), 0.2 mM dNTP, and 1.5 mM $MgCl_2$ in a total volume of 20 μL. Primers were PtPer-F1 (5′-ATG AAR AAR GGB CAR ACG GC-3′), PtPer-R1 (5′-CTB GTR TTW GTV ACA CTG C-3′), RPL-49L-F1 (5′-CAC CAG TCG GAT CGN TAT GCC-3′), and RPL49L-R1 (5′-GAC AGC TGC TTG GCN CGN TC-3′). Amplification for *per* was achieved by initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 45 °C for 15 s, and elongation at 72 °C for 40 s, followed by a final elongation for 7 min at 72 °C. For *RpL32*, the following PCR conditions were used: 94 °C for 2 min; followed by 32 cycles of 94 °C for 15 s, 45.8–48.9 °C for 15 s, and 72 °C for 30 s, and post-heating