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Circadian regulation of caterpillar feeding and growth

Agnieszka Suszczynska[a], Magdalena Maria Kaniewska[a], Piotr Bebas[a], Jadwiga Maria Giebultowicz[b], Joanna Kotwica-Rolinska[a,*]

[a] Department of Animal Physiology, Institute of Zoology, Faculty of Biology, University of Warsaw, Warsaw, Poland
[b] Department of Integrative Biology, Oregon State University, 3029 Cordley Hall, Corvallis, OR 97331, USA

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ABSTRACT

Circadian clocks orchestrate many physiological processes in adult organisms. For example, rhythmic feeding behavior is regulated by the central clock in the nervous system in coordination with metabolic rhythms, which in turn depend mostly on peripheral clocks localized in many tissues. Disruption of the circadian clock leads to metabolic dysregulation both in mammals and in the model insect *Drosophila melanogaster*. Circadian coordination of feeding and metabolism has been studied mainly in adult insects and not in larval stages, which are dramatically different from adults in species with complete full metamorphosis. The goal of this study was to determine whether feeding and metabolism in lepidopteran larvae are subject to circadian regulation. We show that cotton leafworm caterpillars, *Spodoptera littoralis*, display rhythmic feeding behavior and that circadian clock genes are expressed in two peripheral tissues, the midgut and fat body. Even though both tissues display rhythmic circadian clock gene expression, the main component of the clock, *per*, is arrhythmic in the gut and rhythmic in the fat body. In both tissues, the presence of rhythmic physiological processes was observed, which suggested that metabolism is already driven by the circadian clock in the insect's juvenile stages.

1. Introduction

Circadian clocks are endogenous cellular mechanisms that synchronize behavior and physiology with environmental changes and allow for anticipation of the upcoming day or night. The molecular clock mechanism is based on transcriptional/translational feedback loops involving clock genes (Bell-Pedersen et al., 2005). Among insects, the clock mechanism is best understood in *Drosophila melanogaster*, in which several clock genes and proteins were shown to act in two feedback loops. The first loop consists of positive elements, including two transcription factors: CLOCK (CLK) and CYCLE (CYC). CLK/CYC heterodimers regulate expression of *period* (*per*) and *timeless* (*tim*), which are two negative elements of the circadian clock. PER and TIM proteins form heterodimers and translocate to the nucleus to bind to CLK/CYC and repress transcriptional activity, including their own transcription (Bell-Pedersen et al., 2005; Darlington et al., 1998; Hardin et al., 1990). A second feedback loop of the *Drosophila* circadian clock participates in the regulation of *Clk* gene expression. The CLK/CYC heterodimer activates expression of the zinc finger transcription factors *PAR domain protein 1* (*Pdp1*) and *Vrille* (*vri*), which play a role in activating and repressing *Clk* transcription, respectively (Bell-Pedersen et al., 2005; Cyran et al., 2003; Kim et al., 2012; Tataroglu and Emery, 2015). Circadian clocks are able to synchronize (entrain) to a new light:dark regime. In *Drosophila*, this process is achieved using the blue-light photoreceptor protein CRYPTOCHROME (referred to later as CRY1), which promotes TIM degradation after light-activation. While most molecular components of the circadian clock are well conserved between insects and mammals, there is diversity in the number and role of *cry* genes. Above mentioned CRY1 of *Drosophila* is phylogenetically distant from CRY proteins in mammals (referred to as CRY2-type). Some insects, such as the butterfly *Danaus plexipus*, the mosquitos *Anopheles gambiae* and *Aedes aegypti,* and the cockroach *Blattella germanica* possess two *cry* genes encoding photosensitive CRY1 and mammalian-type CRY2 (Bazalova et al., 2016; Merlin et al., 2007; Yuan et al., 2007). *Spodoptera littoralis,* which we investigated in this study, expresses *cry1* and *cry2* genes in addition to all core clock genes (Jacquin-Joly et al., 2012; Merlin et al., 2007).

Clock genes are expressed in the central nervous system and in many other tissues (peripheral clocks) where they govern local physiological processes. The circadian clock restricts an animal's activity to a particular part of the day. Cyclical locomotor activity is associated with rhythmic foraging, which imposes daily regulation of metabolism (Eckel-Mahan and Sassone-Corsi, 2013). Circadian foraging has been reported in the adult stage of several insect species, including *D. melanogaster* (Xu et al., 2008), *Bombus terrestris* (Stelzer et al., 2010), *Rhodnius prolixus*, and *Aedes aegyptii* (Meireles and Kyriacou, 2013).

* Corresponding author.
E-mail address: jaszmije@gmail.com (J. Kotwica-Rolinska).

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Rhythmic digestive processes have also been observed in the guts of insects (Mohammadi et al., 2015). In insects, peripheral clocks are photosensitive and can function independently of the clock localized to the brain (Giebultowicz, 2001a). Although the importance of central clocks in controlling behavior is well-documented, functions of an insect's peripheral clocks are poorly understood (Giebultowicz, 2000, 2001b; Tomioka et al., 2012). Components of a circadian clock are expressed in the alimentary tract of several insect species. For example, expression of *per* was detected in *D. melanogaster* (Karpowicz et al., 2013; Liu et al., 1988), *per* and *tim* were detected in *Gryllus bimaculatus* (Uryu and Tomioka, 2010) and all core clock genes are expressed in the gut of *Pyrrhocoris apterus* (Bajgar et al., 2013). Insect metabolism is mainly governed by a multi-functional fat body tissue (Arrese and Soulages, 2010) and only a few studies reported circadian regulation of metabolism in adults (Erion et al., 2016; Katewa et al., 2016; Xu et al., 2008). The studies discussed above were performed on adult insects, and the question of whether similar rhythms may occur in larval stages remains unexplored. Larvae of insects undergoing full metamorphosis have very different anatomy and feeding habits compared to their adult forms. To explore circadian organization in larvae, we investigated daily feeding profiles, measured circadian clock gene expression and studied metabolic processes in the gut and fat body tissues of larvae of the noctuid moth *S. littoralis*, which is a species that has a widely studied peripheral clock function (Kotwica et al., 2006, 2011; Merlin et al., 2009). Our results demonstrate that there are rhythmic fluctuations in feeding behavior and growth in the larval stage as well as expression of clock genes in the gut and fat body. Our data show that the rhythmicity of the circadian clock gene expression is strongly tissue dependent. Nevertheless, in both tissues, we found processes that undergo circadian regulation, which suggests that metabolism is already affected by the circadian clock during larval development.

2. Material and methods

2.1. Insect rearing

Spodoptera littoralis larvae were reared in the photoperiod LD 16:8 (16 h of light and 8 h of darkness) at 26 °C. The larvae were maintained on an artificial diet adapted for Noctuidae moths (Bebas and Cymborowski, 1999). Last (6th) instar larvae that were 1–3 days-old were used in all of the experiments. To obtain synchronous cohorts of 6th instar larvae, 5th instar individuals nearing ecdysis according to head capsule slippage were selected and used on the following day. Larvae used in most experiments were collected every 4 h around the clock starting in the morning after ecdysis. Measurements and sample collections during the dark phase were performed under a dim red light.

2.2. Feeding behavior and larval growth

On the first day of the 6th instar, groups of ten *S. littoralis* larvae were placed on a 5-g chunk of artificial diet in LD and DD (constant darkness). Every 4 h, the diet and the larvae were weighed and a new diet was substituted. Measurements of the amount of diet consumed and larval weight were performed at 4-h intervals during days 1–3 of the 6th instar.

2.3. Gene expression analysis

Daily expression profiles of circadian clock genes were measured in midguts and fat bodies collected from larvae on the second and third day of the last larval instar in LD and DD. Genes involved in metabolic processes were analyzed only on the second day of the last larval instar in LD. Tissues were collected at 4-h intervals. Total RNA was extracted from tissues using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions and then treated with recombinant DNAse I (Takara). The samples were quantified on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and analyzed through agarose electrophoresis. First strand cDNA synthesis was performed using the iScript cDNA synthesis Kit (Biorad) on 1 µg of total RNA. Gene expression analysis was performed with a qRT-PCR method using the KAPA SYBR FAST qPCR KIT (Kapa Biosystems) with the Step One Plus detection system (Applied Biosystems). Primer sequences were designed based on sequences deposited in the BioInformatics Platform for Agroecosystem Arthropods (http://bipaa.genouest.org/is/lepidodb/spodoptera_littoralis) and are shown in Supplementary Table 1. Primer binding specificity was tested through gel electrophoresis and sequencing of the PCR product. Primer efficiency was evaluated by an RT-qPCR method on a six-point standard curve prepared separately from purified PCR products for every gene tested. Gene transcript levels were normalized to the expression level of the housekeeping gene *28S rRNA*. RT-qPCR expression analysis followed MIQE guidelines (Bustin et al., 2009).

2.4. Immunohistochemistry and Western analysis

Immunolocalization of PER protein was performed on guts collected every 4 h from 6th instar larvae kept in LD and fat bodies in LD or DD. PER localization in fat bodies was performed on whole mount tissues. Fat bodies were fixed in 4% PFA in PBS, washed 3 × 30 min in PBST (supplemented with 0.5% Triton X -100) and blocked for 1 h in 5% Normal Goat Serum (NGS) in PBST. For PER localization, we used polyclonal anti – *Drosophila* PER (PER-Ab; a generous gift from Dr. R. Stanewsky) diluted 1:1000 in 5% NGS in PBST. Fat bodies were incubated 48 h in antibody solution at 4 °C followed by several washes in PBST and a 1 h incubation (at room temperature) with anti-rabbit Alexa Fluor 488 (Molecular Probes) secondary antibody diluted 1:1000 in PBST. Nuclei were stained with Hoechst 33258. After additional PBST washes, tissues were mounted in Vectashield mounting medium (Vector Laboratories). The guts, after they were fixed in 4% PFA in PBS, they were incubated in 10%, 20% and 30% of sucrose solution in PBS followed by freezing in OCT medium (Tissue – Tek). The guts were then sectioned on a Leica cryostat into 15 µm sections and processed with ICC staining as described for the fat bodies. Densitometric analysis of the intensity of PER staining was performed with ImageJ software (Schneider et al., 2012) on at least 30 cells from each of 10 individuals. Control of the specificity of the antibody binding was performed by Western blot analysis (Fig. S1) following a previous protocol with *Drosophila* heads used as a control (Kotwica et al., 2009). An additional control was performed for *S. littoralis* tissues in which the primary antibody was omitted (data not shown).

2.5. α – Amylase and protease activity

Digestive enzyme activity was measured in fluid midgut content collected every 4 h during the second day of the last instar. Briefly, larval midguts were carefully opened and the whole peritrophic membrane along with ingested food was snap frozen in liquid nitrogen and stored at −80 °C until further use. Thawed samples were centrifuged for 10 min at 14 000 rpm, and the supernatant was collected and used to measure amylase and proteases activity. For α - amylase activity, we used a 3,5-Dinitrosalicylic acid (DNS) method in which 10 µl of supernatant were incubated for 1 h at 37 °C with 500 µl of activity buffer (50 mM Tris-HCl pH 10.0, 30 mM NaCl and 30 mM $CaCl_2$) with the addition of 40 µl of 1% starch as a substrate. After incubation, the reaction was stopped by adding 100 µl of DNS reagent and heating the samples for 10 min in a 100 °C water bath. Afterwards, the samples were put on ice for 5 min and then briefly centrifuged. Amylase activity was quantified spectrophotometrically in supernatants at 440 nm. α - Amylase activity was calculated as the amount of reducing sugars created in the sample after starch digestion. Serial dilutions of maltose were used as a standard curve (Darvishzadeh et al., 2014).

To measure total protease activity, 10 µl of gut content and