INSTITUTE OF PHYSIOLOGY CAS

Review – PhD thesis

Author: Lenka Chodáková, MSc.
Title: ***Circadian clock genes in the circadian clock and photoperiodic timer in Phyrrocoris apterus***
Reviewer: Assoc. Prof. Alena Sumová, CSc., DSc.

Formal issues

The PhD thesis is based on 4 studies divided in 2 published papers and 2 manuscripts. The extent of the PhD thesis is 122 pages, involving 96 pages of the text and the references. The structure of the thesis is well designed allowing easy orientation in the text and understanding the major contribution of the results to the research field.

The "Introduction" first provides a brief informative overview of the current knowledge on the circadian and photoperiodic mechanisms in insect mostly based on D. melanogaster data. Special attention is paid to the hypotheses on the mutual interaction between the circadian clock and photoperiodic timer with the emphasis on the need for examination of other model species. Then, the advantage of using P. apterus as a suitable model for studies aimed at elucidating mechanisms of the circadian and photoperiodic interactions at molecular levels is clearly declared.
I appreciate involvement of the "Thesis overview" subchapter which takes the reader briefly through the thesis, explaining and justifying logical relationship among experiments described in the individual parts of the thesis. This subchapter explains the context of the results and makes the thesis more compact.

The "Results" chapter is divided into 4 parts, each representing one of the papers or manuscripts involved in the thesis.

The description of methods used in the individual studies is included in each of the part (in the papers and manuscripts). Nevertheless, a separate "Methods" chapter including more detailed information on the techniques and methods used to analyze the data would be useful especially for readers who are not familiar with the circadian field.

The "Discussion and conclusions" chapter is 4 pages long and includes a scheme drawn by the author summarizing the proposed model of the circadian clock in P. apterus and incorporating the author's data obtained in the PhD thesis. The graphic is informative and stresses importance of the results.

Topic, major findings and contribution to the current knowledge

The aim of the thesis is to contribute to comparative analyses of molecular mechanisms underlying the circadian clock and photoperiodism in P. apterus. The thesis provides significant progress in understanding these mechanisms and the results represent a real breakthrough in the field. The major findings are summarized below:

I) The results demonstrated variation in free-running periods among populations of the P. apterus collected at various geographic regions. This finding raises a question about underlying mechanisms and provides rationale for speculations about the possibility that adjustment of this basic property of the circadian clock represents an adaptation to environmental cues involving the geography-related photoperiodic variation.

II) The results provided comparative analysis of the functional role of individual clock genes in P. apterus, further extending the previously identified variability of the clockwork mechanisms among individual insect species. Moreover, a possible new role for one of the genes, *taiman*, has been suggested.

III) Using silencing of specific clock gene by RNA interference revealed their possible role in diapause induction.

IV) Behavioral analyses and unique CRISPR/Cas9 gene engineering technique revealed the role of *PaCry2* in the circadian clock and photoperiodism of P. apterus. The results confirmed diversification of molecular clockwork mechanism in the insect species.

Questions

I find the results obtained in the thesis extremely interesting. They inspired me to ask rather speculative questions related to the topic:

Q1: In mammals, the information about external photoperiod seems to be processed to the organism via modulation of mutual phases among individual oscillators coupled in the master clock driving systemic rhythms rather than via modulation of the TTFL mechanism. Is there any data suggesting similar mechanism in insect? Could the absence of the clock gene expression rhythm found in the brain of P. apterus under the long photoperiod be possibly related to a larger dispersion of phases among cellular oscillators in the brain?

Q2: The finding of the correlation between free-running period and geographic location in P. apterus raises many questions that have also been mentioned in the Discussion. The major question remains to be answered whether this phenomenon represents an adaptation of the circadian clock to the external environment depending on the geographic location. If this were the case, one would expect that there would be the same trend in modulation of the free-running periods in other species living in the extreme photoperiods. However, the finding of opposite correlation between the photoperiod and free-running period in D. melanogaster and P. apterus does not support the speculation. Also, could the author explain the adaptive advantage for selecting the period length depending on the habitat's location? Why longer period would be advantageous for organisms living in the north (more extreme photoperiodic changes) and the shorter period for those living in the south (milder photoperiodic changes)?

Q3: The finding that RNAi of the Clk and Cyc genes leads to diapause induction even in a reproduction-stimulating environment provides a basis for testing the hypothesis about position of the photoperiodic timer relative to the circadian clock on the pathway mediating the diapause induction and termination. The data suggest that dampening the circadian clock releases the photoperiodic timer to induce this adaptation response. Could this be tested in an opposite way (that is, to show that rescuing the clock will stop the diapause)? And more generally, could the author speculate whether this finding is an evidence for casual and specific involvement of Clk and Cyc genes in the mechanism mediating the diapause induction? I mean, would manipulation with the circadian clock of P. apterus via other mechanism lead to the same effect?

Minor comments

The thesis is well written and there is only a limited number of typos, e.g., "circadian ge<u>r</u>nes" (p. 2), "recent studies indicate<u>s</u>" (p. 17), c<u>l</u>ues (p. 42, p. 72), etc.

Final conclusion

The PhD thesis is of a high quality and summarizes results which significantly advanced understanding of insect circadian physiology. The author confirmed her expertise in performing the experiments, reviewing and citing the relevant literature, and analyzing and interpreting the data. To my opinion, the PhD thesis fulfils the criteria to be successfully defended.

In Prague, 7.1.2019

Sumová

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Review of Lenka Chodáková's dissertation, "Circadian clock genes in the circadian clock and photoperiodic timer in *Pyrrhocoris apterus*"

By Jeffrey L. Price

This dissertation documents an excellent foray into the circadian and photoperiodic systems of *Pyrrhocoris apterus.* It establishes the basic clock gene network in this insect with a combination of in silico, RNAi and CRISPR/Cas9 approaches and shows that several of the circadian genes are involved in the photoperiodic response as well. *Pyrrhocoris apterus* is an ideal model system for the analysis of photoperiodism because it has a diapause response that is strongly regulated by photoperiod, unlike the *Drosophila melanogaster* circadian model. As a circadian model, this insect is of interest because it is the first insect model to have a *timeless* (*tim*) ortholog without a cryptochrome 1 (CRY1) photoreceptor that triggers TIM degradation in other insects, so the mechanism of response to light is clearly different. The work is well performed and analyzed and has already led to two publications for the student – one as a first author. My only criticism is that the student did not offer much discussion of how follow-up work should proceed; this can be addressed during the defense. I will cover the chapters in sequential order.

1) Unexpected Geographic Variability of the Free Running Period in the Linden Bug *Pyrrhocoris apterus*

The student analyzed 59 lines of linden bugs from various latitudes of Europe and Israel and found that the lines had a remarkable range of average periods and arrhythmicities. Period length correlated with latitude and possibly altitude, becoming longer at more northern latitudes and higher altitudes, with the exception of the two southernmost lines from Israel and one from Spain, which had longer periods. The arrhythmicity correlated with the range of periods found in each line (higher arrhythmicity correlates with more variance in period). There was no correlation between the arrhythmicity and the average period length or the arrhythmicity and the latitude. Sequencing of the *period (per)* intron revealed three different clades, but the broad period range was found in two of the clades (The Israeli clade was the third and consisted only of these two lines). The period differences did not produce obvious phase differences in the LD activity profiles.

a) The shorter period lines appear to have narrower peaks of activity in light:dark cycles. Could this be a real correlation?
b) It is stated, "If that is the case, then P. apterus might "sacrifice" the precision of the circadian clock and use circadian genes to adjust the functioning of the photoperiodic timer." How would lack of precision in circadian genes adjust the functioning of the photoperiodic time?

2) Circadian clock genes of the linden bug *Pyrrhocoris apterus*

From an in silico analysis of the transcriptome, numerous orthologs of the mammalian and Drosophila circadian genes were found. The *period* (PER), *Clock* (CLK), *Cycle* (CYC) and CRY orthologs appear more similar to the mammalian orthologs than to the Drosophila ones, as PaPER does not have homologies with the CLK- interacting domain in DmPER but does have a mammalian CRY-interacting domain, PaCLK is missing the poly Q activation domain of DmCLK, and PaCYC has a mammalian BMAL-like activation domain. In addition, *Pyrrhocoris apterus* is missing the Drosophila CRY1 ortholog but has the mammalian CRY2 ortholog. Surprisingly it has an ortholog of Drosophila TIM, which is targeted by CRY1 in other insects that have CRY1. None of these clock genes appear to oscillate in whole brain mRNA, and this lack of oscillation and the lack of a CRY1 interactor for TIM pose questions about the nature of the circadian clock in *Pyrrhocoris apterus* adults (oscillations are shown in nymphs in a later chapter). RNAi treatment for all of these genes led to decreased levels and effects on the circadian rhythms of locomotor activity. In addition, RNAi for Taiman (Tai), a heterodimeric partner of the juvenile hormone receptor MET and also involved in ecdysone signaling, lengthened period, but no alterations of juvenile hormone or ecdysone had similar effects. Knock-down of the ecdysone receptor and E75 had mild effects on arrhythmicity.

a) What is the source of the *Pyrrhocoris apterus* transcriptome data base that was searched to identify the circadian genes?
b) Is it possible that germ-cell expressed (GCE), another receptor for juvenile hormone, has effects on the clock? Could this interact with TAI to explain the effects of TAI?

3) The role of circadian clock genes in the photoperiodic timer of the linden bug *Pyrrhocoris apterus* during the nymphal stage

This paper investigates the effect of photoperiod on the daily activity rhythms of the linden bug adults and nymphs and the effects on circadian gene expression, as well as the effects of knock-down of circadian gene expression on diapause. In both short day (SD) and long day (LD) photoperiods, activity rhythms are observed, and *per* and *cyc* gene expression is rhythmic. The paper demonstrates effective knock-down of *Clk* and *cyc* with RNAi, and this produces a diapause state in long day photoperiods, which otherwise would not cause diapause. The diapause state is indicated by low levels of vitellogenin, high levels of a diapause storage protein, and nonreproductive ovaries. The results suggest that the circadian system regulates diapause.

a) The levels of *Clk* and *cyc* mRNAs do not differ much between long day and short day photoperiod regimes, so it would seem that altered photoperiod does not regulate diapause by altering these mRNA levels, even though the RNAi experiment induce diapause in this manner. So how do you think photoperiod regulates diapause via the clock genes?
b) In the discussion, it is argued that circadian processes might regulate reproduction downstream of the photoperiodic timer. How could one distinguish direct and downstream effects?

4) Circadian clock and photoperiodic timer of the CRISPR/Cas9 mutants in Pacry2 gene

Since RNAi phenotypes of linden bug *cry2* were not particularly strong, the student analyzed the circadian phenotypes of two *cry2* CRISPR/Cas9 mutants. One was a 27 bp insertion that generated an in-frame insertion at the N terminus and the other was a 13 bp deletion that produced a stop codon at the C terminus. Both caused high levels of arrhythmicity and short periods. In addition, both led to reproductive females under short day conditions that would typically induce diapause – the opposite phenotype from *cry* and *Clk* RNAi.

a. Why does the 27bp in-frame insertion have a stronger phenotype than the 13 bp deletion/truncation? Most of the protein is still intact in the former.
b. As noted in the introduction to this review, where do you go from here?

In conclusion, this is an excellent dissertation. It presents a comprehensive analysis of the circadian and photoperiodic systems in this insect and establishes an interplay between them. The student has demonstrated both methodological and analytical proficiency, and the dissertation is very well written. I recommend it for the award of the PhD degree.

In Kansas City, January 9, 2019

Jeffrey L. Price, PhD