Faculty of Science, University of South Bohemia, České Budějovice



# INSECT CHRONOBIOLOGY

**Ph.D. Thesis**

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## Annotation

The Ph.D. thesis consists of three original research articles dealing with relevant subjects of the field of insect chronobiology.

In the first paper, we present the functional molecular analysis of the *timeless* promoter from the drosophilid fly, *Chymomyza costata*. We have found and analysed four relevant *cis*-acting elements in the *tim* promoter (the canonical and incomplete E-boxes, TER-box and PERR-box) and assessed their respective impacts on *tim* transcription levels. In addition, daily expression profiles of the circadian clock genes *tim, per, vri* and *dbt* in adult heads of wild type and non-photoperiodic-diapause (*npd*) strains were described.

The second paper is focused on the regulation of cell division cycle during the entrance into the diapause in larvae of the drosophilid fly, *Chymomyza costata*. Seven regulators that likely participate in the diapause-linked cell cycle arrest, *cyclin E*, *cyclin D*, *wee1*, *myt1*, *cdc25*, *dacapo* and *pcna*, were partially cloned and their expressions were quantified in the central nervous system and imaginal discs of diapausing and nondiapauasing larvae.

In the third paper, we established the Mediterranean flour moth, *Ephestia kuehniella*, a primitive lepidopteran species, as a new non-drosophilid species for circadian clock studies. Two circadian clock genes *period* and *timeless* were cloned and characterized in the flour moth. Furthemore, the analysis of circadian controlled egg hatching behavior and locomotor activity of adult moths is presented.

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**Prohlášení**

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Alena Kobelková

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**List of original papers**

The dissertation is based on the following papers:

I. **Kobelková A**, Bajgar A, Doležel D (2010) Functional molecular analysis of a circadian clock gene *timeless* promoter from the drosophilid fly *Chymomyza costata.* J Biol Rhythms (*accepted*)

II. Košťál V, Šimůnková P, **Kobelková A**, Shimada K (2009) Cell cycle arrest as a hallmark of insect diapause: Changes in gene transcription during diapause induction in the drosophilid fly, *Chymomyza costata*. Insect Biochem and Mol Biol 39: 875-883

III. **Kobelková A**, Závodská R, Doležel D, Šauman I. The introduction of Mediterranean flour moth, *Ephestia kuehniella*, as a novel non-drosophilid species for circadian clock studies. (*manuscript*)

**CONTENTS**

## INTRODUCTION

### Chronobiology

Chronobiology is a word derived from three Greek stems: **chronos** for time, **bios** for life and **logos** for study. Chronobiology comprises the systematic scientific study of living timing processes in all organisms spaning from single-celled cyanobacteria through plants to animals, including the human species. The field deals with living internal clocks allowing internal estimation of time in the external world. The internal clocks play a vital role in the adaptation to life on the rotating Earth, not just drive passive responses of organisms to changes in the environment.

### Insect Chronobiology

The focus of early studies was recognizing behavioral rhythms in insects and documenting their features. Daily locomotor activity rhythms were reported in many insect species and have been studied particularly in the fruitfly, *Drosophila melanogaster* (Konopka and Benzer, 1971). Gated population rhythms as egg-hatching and pupal eclosion and circadian locomotor activity were found in insects with complete metamorphosis, including dipterans and lepidopterans (Šauman et al. 1996; Codd et al., 2007) Circadian rhythmicity acts as a gating process, allowing the emergence of new larvae or adults at the most appropriate time of day for maximal survival. Interestingly, calling during courtship has a nocturnal rhythmic character in crickets and katydids. Similarly, many silkmoth species release pheromones at specific time during night to attract males from long distances.

Molecular era in chronobiology began with cloning the first clock gene *period* from the fruitly, *D. melanogaster* (Diptera) (Bargiello and Young, 1994; Reddy et al., 1984). The following research on *Drosophila* clock genes has been quite comprehensive and led to understanding of molecular mechanism underlying the circadian clocks (reviewed by Dunlap, 1999; Stanewsky 2002). Recently, the clock genes from various insects orders have been sequenced and compared, for example from the housefly *Musca domestica* (Diptera), giant silkmoth *Antheraea pernyi* (Lepidoptera), cricket *Gryllus bimaculatus* (Orthoptera) and honeybee *Apis melifera* (Hymenoptera) (see the complete list of species in Sandrelli et al., 2008). Although homologous circadian genes have been found in all insect species mentioned above, their contribution to circadian timing system is different, indicating that the same genes have gained different roles during evolution. Detailed comparative studies are needed to solve whether the *Drosophila* model of circadian clocks is generally valid or rather species-specific circadian clock models have to be accepted.

Another phenomenon widely studied in insects is the photoperiodism. By analogy with circadian clock, the daily time measurement system, the photoperiodic calendar is a seasonal time measurement system (Nelson et al., 2010). Sensitivity to photoperiodic signal (photoperiodism) allows insects to recognize the season and adjust their life style and developmental destiny accordingly. Compared to our

knowledge on the molecular mechanism of circadian clocks, photoperiodic calendar mechanisms are just little understood. In 1936, Edwin Bünning proposed that the photoperiodic sensitivity is based on circadian functions. Over time, the connection between circadian and photoperiodic timing systems still remains controversal. Several hypotheses have been introduced (Bünning, 1960; Pittendrigh, 1972; Lees, 1973; Lewis and Saunders, 1987; Saunders, 2005) and supported by experimental work, however the concensus has not been reached yet.

**Circadian Clock – General Introduction**

Many processes in organisms show a rhythmic course as an adaptation to cyclic changes in light, temperature, and other environmental factors during day and night. But not all observable rhythms can be considered as circadian (**circa** for about and **dies** for day). The true circadian rhythms are defined by three major, well-established criteria:

1. The circadian rhythms persist **(free-run) in constant conditions** (light and temperature) with a **period** approximately **24 hours**.
2. The circadian clocks are **temperature compensated** i.e. temperature insensitive within a certain range of physiologically plausible temperatures.
3. The circadian rhythms **can be entrained** by certain environmental cues such as light-dark cycles, temperature cycles and other stimuli.

Circadian clocks are endogenous, genetically determined mechanisms. Circadian timing system consists of three main parts:

1. the **input** pathway provides the connection of external environment with the inner clock and it is necessary for entrainment
2. the **core oscillator** represents the actual molecular time-keeping device, the endogenous clock
3. the **output** pathway transfers time information from the core oscillator to the rest of the organism

All known circadian oscillators use molecular loops (Fig. 1) that close within cells and rely on positive and negative elements in oscillators in which transcription of clock genes yields clock proteins (negative elements), which act in some way to block the action of positive element(s) whose role is to activate the clock gene(s) and clock-controlled genes (Dunlap, 1999). Biological rhythms driven by circadian clocks can be observed and studied on molecular, physiological and behavioral levels.

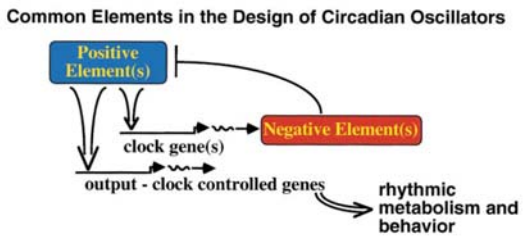


Fig. 1 The schematic representation of a core oscillator model (From Dunlap, 1999)

**Circadian Clock in *D. melanogaster***

*Drosophila melanogaster* is a premier species for circadian rhythm studies and serves as a model the others are compared to. Pioneering study of clock mechanism in *D. melanogaster* came from Konopka and Benzer (1971). They used chemical

mutagenesis and screened for genetic variants with unusual circadian rhythms. As result, they found aperiodic and phase-altering mutants and this three mutations mapped to the same locus on X chromosome, named *period* (*per*) gene. Molecular studies started by cloning *per* as the first circadian clock gene (Reddy et al., 1984). Since this discovery, the research of mechanism underlying circadian clock in *D. melanogaster* has continued in leaps and bounds. Another core circadian clock genes were cloned and characterized, *timeless* (*tim*, Seghal et al, 1994), *Clock* (*Clk*, Allada et al., 1998), *cycle* (*cyc*, Rutila et al, 1998), *vrille* (*vri,* Blau and Young, 1999), *PAR domain protein 1ε* (*Pdp1ε*) and *clockwork orange* (*cwo*, Kadener et al, 1997). These genes and their proteins participate in mechanism called transcription/translation negative feedback loops (Fig. 2).

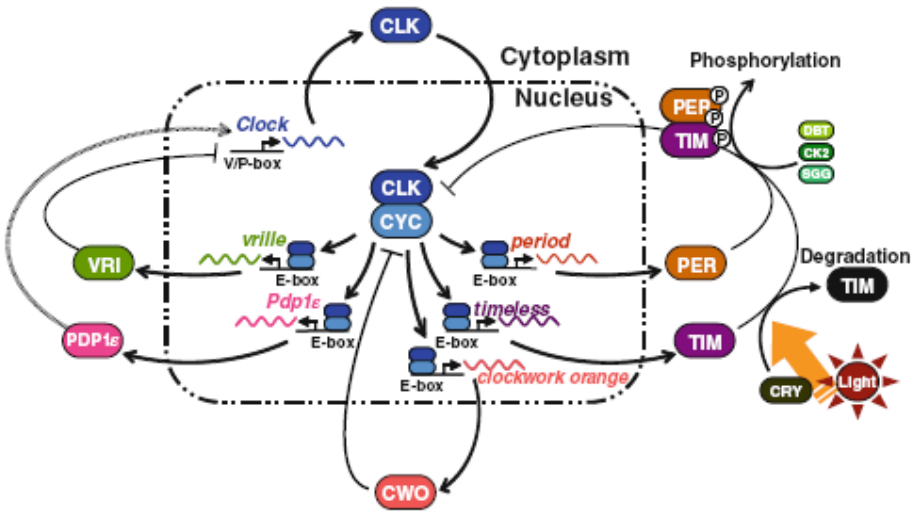


Fig. 2 A scheme illustrating current knowledge about molecular machinery of the *Drosophila* circadian clock (Tomioka and Matsumoto, 2010)

*per* and *tim* genes are activated by binding of CLK/CYC protein complex to their promoter sequences. *per* and *tim* mRNAs accumulate in cytoplasm during the light phase of the day until their abundance reaches maxima in the early evening (Hardin et al., 1990; Seghal et al., 1995; So a Rosbash, 1997). Both transcripts are translated in cytoplasm, while newly synthesized monomeric PER is destabilized by the kinase DOUBLETIME (Kloss et al., 1998; Price et al., 1998) and its accumulation is thus delayed. TIM protein is also degraded in cytoplasm during the light phase of day. This light-dependend degradation is mediated by CRYPTOCHROME. PER phosphorylation and TIM degradation cause the 6 hours *per/tim* transcription-to-PER/TIM translation delay, which is one of the most compelling feature of the feedback model. After the sunset TIM is no longer degraded and its level increases in cytoplasm and stabilizes PER protein. Subsequently, PER and TIM dimerize and enter a nucleus where they bind CLK/CYC and this protein complex represses transcription of *per* and *tim* as well as another clock controlled genes. The feedback loop is closed and the one turnover takes ~ 24hours.

Additionally to the major feedback loop described above, two more interacting feedback loops are involved in the circadian core oscillator. VRI and PDP1ε are transcriptional regulators that cycle in their abundance with different phases.

CLK/CYC dimer activates transcription of *vri* and *Pdp1ε*. The *vri* mRNA is soon translated to VRI protein, which enters the nucleus and inhibits transcription of CLK. The translation of Pdp1ε occurs in a rather delayed manner and PDP1ε binds to *Clk* promoter competitively with VRI and activates the *Clk* transcription. This mechanism ensure cycling of *Clk* mRNA and CLK protein, reaching peak levels around subjective dawn. The third loop consists of CLK/CYC and transcriptional repressor *cwo*. CLK/CYC complex promotes *cwo* transcription and CWO then inhibits its own as well as *per* and *tim* transcription (Fig. 2).

Described transcription/translation negative feedback loops model in *Drosophila* is greatly simplified and serves as a point of departure for the discussion in further text.

**Circadian Clock in Non-drosophilid Species**

As it was implied in the second chapter, the overt sequence homologies of the circadian clock genes in various insect species do not necessarily guarantee the same function in their circadian clocks.

*Drosophila* model was compared to other flies, the most detailed study so far is that in housefly*, Musca domestica* (Diptera; Codd et al., 2007). Unlike *Dm*PER protein, PER did not cycle in abundance during day in housefly heads. Furthemore, the spatial organization of clock cells in central nervous system differed in housefly as well as the expression patterns of PER and TIM in these cells. But in the same time, *Md*PER strongly rescued circadian locomotor rhythms in *Drosophila per* null mutant (Piccin et al., 2000).

Besides the dipteran species, the most of the comparative circadian studies were conducted on lepidopteran species, particularly on the giant silkmoth, *Antheraea pernyi*, the silkmoth, *Bombyx mori*, the monarch butterfly *Danaus plexippus* and the hawkmoth *Manduca sexta* (Šauman and Hashimi, 1999; Iwai et al, 2006; Šauman et al., 2005; Wise et al, 2002)

The striking results came from studies on the giant silkmoth that are in clear contradiction with the molecular mechanism of the circadian clock in the fruitfly (Tab. 1). *per* mRNA and PER protein levels oscillate in the silkmoth central brain and eyes (Reppert et al., 1994; Šauman and Reppert, 1996). In the eyes, *per* mRNA cycling precedes the protein in a similar manner as found in *Drosophila*. PER protein is also located in the photoreceptor nuclei during the subjective night (Šauman and Reppert, 1996). However, a dramatically different situation was found in the silkmoth central brain, where only 8 large neurosecretoty cells (four in each hemisphere) express PER and TIM proteins. The striking feature of the PER and TIM protein expression is that they are both restricted to the cytoplasm of the 8 neurons and do not enter the nucleus, which is a critical feature of the *Drosophila* negative feedback oscillator model (Šauman and Reppert, 1996). An unprecedented finding was that besides *per* mRNA the eight brain neurons also express an antisense *per* RNA. This antisense *per* transcript also oscillates in circadian manner, but with an opposite phase with respect to the *per* (sense)

mRNA cycling (Table 1, Šauman and Hashimi, 1999)

Table 1. Expression Patterns of Circadian Clock Genes in Adult Central Brain

| | | FRUITFLY | SILKMOTH |
|---|---|---|---|
| PER | | | |
| | Cell No. | >100 | 8 |
| | Cycling | Yes | Yes |
| | Nuclear | Yes | No |
| | Axons | No | Yes |
| | *per* mRNA cycling | Yes | Yes |
| | *per*/PER phase | Phase delay | Synchronous |
| | *per* antisense RNA | No | Yes |
| TIM | | | |
| | Co-localization | Yes | Yes |
| | Cycling | Yes | Yes |
| | Nuclear | Yes | No |
| | Axons | No | Yes |

The table contrasts the major differences in the circadian timing system between the fruitfly, *Drosophila melanogaster,* and the giant silkmoth, *Antheraea pernyi*. The data included in this table can be found in the following references: Siwicki et al., 1988; Hardin et al., 1990; Reppert et al., 1994; Reppert and Sauman, 1995; Hall, 1996; Sauman & Reppert, 1996; Dunlap, 1998a, 1990.

for survival and breeding and enter dormancy to overcome harsh periods. The photoperiod (i.e. ratio of the day length : night length) serves as major token stimulus for seasonal changes in life cycle at higher latitudes. Photoperiodic clock system allows insects to predict and prepare in advance for the coming season.

Regardless all the differences, the silkmoth *per* can rescue circadian behavior in arrythmic *Drosophila per* null mutant (Levine et al., 1995), indicating that *Ap per* is not only structural homolog, but also a functional homolog of the *Drosophila per* gene.

We would like to extend the research on lepidoptera in order to gain a better understanding of circadian clock evolution. We chose the Mediterranean flour moth, *Ephestia kuehniella*, which represents phylogenetically less diverse lepidopteran species, compared to the other lepidopteran species which are under molecular investigation of circadian clock (Chapter II – Paper III).

**Photoperiodic Calendar – General Introduction**

Insects are able to anticipate seasonal changes in their environment. Thus they are active under conditions most suitable

The system sensitive to photoperiod theoretically consists of four functional units:

1. The **input** pathway(s) perceive light and temperature conditions
2. The **photoperiodic clock** measures the actual night length
3. The **photoperiodic counter** records the number of experienced inductive photoperiods during sensitive period in insect life cycle
4. The **output** pathways transmit photoperiodic information to the rest of the body

Because the exact mechanisms underlying photoperiodic clock and counter have not been described yet, most studies rely on systematic variation of the input into the system and observing the outputs.

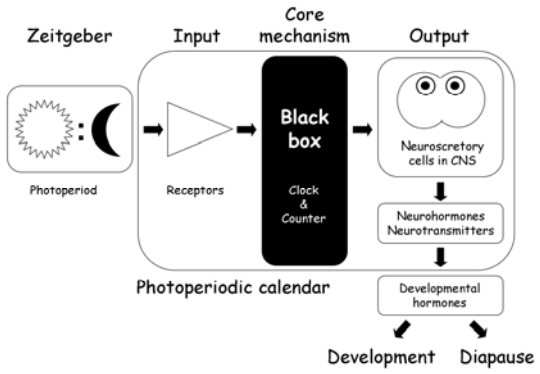


Fig. 3 A diagram illustrating current concept of photoperiodic calendar and its input and output pathways (Košťál, unpublished review)

Entering into diapause is by far the most common insect strategy for survival of cold temperate winter. Diapause is defined as a neurohormonally mediated developmental arrest. Phenomena associated with diapause are: low metabolic activity; increased resistance to environmental extremes; and altered or reduced behavioural activity. Diapause occurs during genetically determined stage of life cycle that varies among species. Photoperiodically controlled diapause can occur in adult, pupal, larval and embryonic stages depending on insect species (Saunders, 2002). Diapause is usually initiated in response to a number of environmental stimuli that precede unfavourable conditions, most prominent stimulus being photoperiod followed by temperature. Once diapause has begun, development stops and metabolic activity is suppressed even if conditions remain favourable for continuation in direct development (Tauber et al., 1986, p.21).

**Diapause in *Drosophila melanogaster***

*D. melanogaster* serves as a premier insect model species for circadian research due to its suitability for genetics and ease of observation of daily and circadian rhythms. Unfortunately, it is difficult to assay photoperiodic diapause in this species. When the adult female experiences short day and low temperature from 10°C to 14°C shortly after emergence, it enters into a weak and short reproductive diapause, during which the ovaries remain small, with no accumulation of yolk in oocytes. The incidence of diapause, however, varies widely in tested samples (Saunders et al., 1989). Therefore, *D. melanogaster* is not very suitable for research on photoperiodic induction of diapause.

**Diapause in Non-drosophilid Species (with respect to cell cycle arrest)**

The studies on diapause focused on other dipteran species (flesh fly *Sarcophaga crassipalpis,* mosquito *Culex pipiens*, and drosophilid fly *Chymomyza costata*) and several lepidopteran species (silkworm *Bombyx mori*, hornworm *Manduca sexta*, giant silkmoth *Antheraea pernyi,* giant silkworm *Hyalophora cecropia,* cabbage armyworm *Mamestra brassicae* etc.). I will provide more details for two species from the above list, silkworm *B. mori* and flesh fly *S. crassipalpis*. For these two species, data are available concerning cell cycle arrest during diapause, which is one of most remarkable outputs of photoperiodic timing system.

The silkworm, *Bombyx mori*, exhibits embryonic diapause which differs from that of most other model species. Diapause induction is under strict maternal control and is mediated by diapause hormone (DH) (Yamashita, 1996). DH is a neuropeptide released by the female adult during the period of egg maturation. DH acts upon the ovarioles, leading to the

production of diapause-destined eggs. After being oviposited, the silkworm embryos enter a diapause characterized by a G2 cell cycle arrest (Nakagaki et al., 1991). Expression patterns of several genes associated with the G2 cell cycle arrest have been monitored. The data suggested that *cyclin dependent kinase 2* (*cdc2*) and *Bombyx cdc 2 related kinase* (*Bcdrk*) are involved in the G2 cell cycle arrest (Takahashi et al., 1998).

During pupal diapause in the flesh fly, *Sarcophaga crassipalpis*, the cells of the brain are arrested in the G0/G1 phase of the cell cycle (Tammariello and Denlinger, 1998). Four G1 and S phase regulatory genes, proliferation enhancers *cyclin E* and *proliferating cell nuclear antigen* (*pcna*) and cell cycle inhibitors *p21* and *p53,* were examined by Northern blot analysis to evaluate their expression patterns in relation to the cell cycle arrest. C*yclin E*, *p21* and *p53* were expressed equally in diapausing and nondiapuasing flies. A significant difference between diapausing and nondiapausing individuals was shown only for *pcna*, which was highly expressed after diapause termination but not during diapause. Differential display screen in *S. crassipalpis* identified 4 diapause-up-regulated clones and 7 diapause-down-regulated clones. Again, *pcna* was revealed as one of the diapause-down-regulated clones (Flannagan et al., 1998). PCNA (synonymus *Mutagen sensitive 209* - *Mus209*), is an essential accessory factor of δ-polymerase, an enzyme necessary for leading strand DNA replication and DNA repair machinery (Prelich et al., 1987a,b; Shivji et al., 1992). PCNA is localized in the nucleus at sites of active DNA replication.

It is intriguing how the cell cycle synchronously halts during diapause initiation and later restarts after diapause termination. We chose a drosophilid fly, *Chymomyza costata,* as the model organism to extend research of cell cycle arrest during the diapause (Chapter II – Original Paper II).

**Diapause in Drosophilid Fly,**
***Chymomyza costata***

Drosophilid fly*, Chymomyza costata* (Diptera, Drosophilidae), is distributed over a cool-temperate, Holarctic area (Hackman et al., 1970; Toda, 1985), and enters winter diapause as 3$^{rd}$ instar larva in response to long night length and/or low temperature (Enomoto, 1981; Riihimaa and Kimura, 1988; Yoshida and Kimura, 1995). *C. costata* becomes sensitive to photoperiodic stimuli during an unspecified stage of its early development (embryo, 1st larval instar), the sensitivity gradually increases during the 2$^{nd}$ and early 3$^{rd}$ larval instars, and reaches its maximum just before the moment when it abruptly ceases at the age of 15–19 days after oviposition (Košťál et al., 2000). *C. costata* is a promising model for biochemical and molecular studies on diapause regulation due to: (i) its close systematic relationship to *Drosophila melanogaster* (Hackman et al., 1970); (ii) clear and robust photoperiodic response (Riihimaa and Kimura, 1989); and (iii) availability of non-photoperiodic-diapause (*npd*) mutant strain.

Riihimaa and Kimura (1988) selected a *npd*-mutant strain that was not responding to a photoperiodic signal and, instead of entering diapause under short days, the *npd* larvae continued development and

pupariated. Genetic linkage analysis confirmed that the larval non-photoperiodism and adult eclosion arrhythmicity in the *npd* strain were caused by a mutation in a single autosomal gene locus *npd* (Riihimaa and Kimura, 1989; Riihimaa, 1996). Homolog of *D. melanogaster per* and *tim* cDNA were sequenced, and the amount of respective mRNAs showed typical daily oscillation in the wild type (*wt*) *C. costata* (Shimada, 1999; Košťál and Shimada, 2001; Pavelka et al., 2003).

Sequence of *per* in *npd*-mutant carries a 6-bp deletion, and few others point mutations, but such mutated *per* is not responsible for the losses of the eclosion rhythms and photoperiodic response in *npd* strain (Shimada, 1999; Košťál and Shimada, 2001). Daily and circadian oscillations of *per* mRNA levels, however, were disrupted in the *npd* flies (Košťál and Shimada, 2001).

Polymorphism associated with the *tim* gene segregated with the *npd* phenotype (Pavelka et al., 2003). Disrupting *tim* transcription by RNA-interference in *wt* strain caused that certain proportion of *wt* individuals showed the *npd*-mutant phenotype (Pavelka et al., 2003). Such results indicated that the locus *npd* could code for the *tim* gene. Genomic DNA of *tim* locus was sequenced and compared in *wt* and *npd* strains (Stehlík et al., 2008). Several amino acid substitutions were revealed in coding sequence together with remarkable,1855-bp long, deletion in *tim* promoter of *npd* strain. Detailed quantitative PCR confirmed previous Northern blot results by Pavelka et al. (2003) that the *npd*-mutant flies show low levels of *tim* transcripts. We continued in this line of research with the aim to find out whether the 1855-bp long deletion in *tim* promoter is responsible for nearly null *tim* mutation in *npd* strain (Chapter II – Paper I).

Studies on *C. costata* revealed that the *npd*-mutants show disrupted both the circadian (loss of eclosion rhythms) and the photoperiodic (diapause) outputs. The pieces of evidence gathered so far point toward that the *timeless* gene codes for *npd* locus and may provide a link between circadian and photoperiodic timing systems. We decided to explore the impact of *tim* mutation on the circadian clock mechanism of *C. costata* by quantifying the expressions in several core clock genes, *tim, per, vri* and *dbt,* in the adult heads of *wt* and *npd*-strains (Chapter II – Paper I).

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## RESEARCH OBJECTIVES

The Ph.D. thesis consists of three papers dealing with relevant objectives in chronobiology of insect but they do not follow one common theme.

## PAPER I

Previous studies showed that *timeless* gene in drosophilid fly, *Chymomyza costat*, might represent a molecular connection between circadian and photoperiodic clock system. 1855 bp long deletion was found in *tim* promoter of non-photoperiodic-diapause (*npd*) mutant of *C. costata* and it was proposed that this deletion is responsible for *tim* null mutation. The aims of our work were to

(i) elucidate whether the 1855 bp deletion is truly responsible for the null phenotype in the *npd*-mutant of *C. costata* using a transfection assay in S2 cells

(ii) show how does the individual *cis*-acting elements in the *tim* promoter (the E-box, TER-box and PERR-box) contribute to the transcription of *tim* in *C. costata*

(iii) quantify the expression of the circadian clock genes *tim*, *per*, *vri* and *dbt* in adult heads of both *wt* and *npd*-strains to show the impact of *tim* null mutation on the circadian clock of *C. costata*

## PAPER II

Cell cycle arrest is one of the hallmarks of insect diapause. Surprisingly, there are not many studies elucidating a regulation of this process. Larvae of *C. costata* show a rapid growth cessation of central nervous system (CNS) and imaginal discs during their entrance to diapause. In this study we aim to

(i) detect the cell cycle phases at which the cells of CNS are arrested during larval diapause in *C. costata*

(ii) clone (at least partially) seven potential regulators participating in the diapause cell cycle arrest (*cyclin E*, *cyclin D*, *wee1*, *myt1*, *cdc25*, *dacapo* and *pcna*)

(iii) quantify the expression of genes listed above in the larval CNS and imaginal discs during entrance to diapause

## PAPER III

*D. melanogaster* (Diptera) represents paradigmatic model in circadian clock studies. Comparative analyses with this model were most intensively studied in the giant silkmoth, *Antheraea pernyi* (Lepidoptera) and the housefly, *Musca domestica* (Diptera). There were found some relevant and sometimes even striking differences among their circadian clock mechanisms. We would like to extend the research on lepidoptera in order to gain better understanding of circadian clock evolution. We chose the Mediterranean

flour moth, *Ephestia kuehniella*, relatively primitive lepidopteran species to

(i) document circadian rhythms in egg-hatching behavior and locomotor activity in adults

(ii) characterize two circadian clock genes *period* and *timeless*

(iii) quantify the expression of *per* and *tim* genes in adult heads in three different time regimes

(iv) localize *per*/PER and *tim* expressing neurons in central nervous system

## CHAPTER II – PAPER I

Kobelková A, Bajgar A and Doležel D. **Functional molecular analysis of a circadian clock gene *timeless* promoter from the drosophilid fly *Chymomyza costata***

Accepted in Journal of Biological Rhythms

Alena Kobelková has done 80% of the work


ABSTRACT

The circadian transcription of the *tim* gene is tightly regulated by the protein complex dCLK/CYC, which directly interacts with a series of closely spaced E-box and E-box-like elements in the *Drosophila timeless* promoter. The *tim* promoter from *D. melanogaster* has been studied in detail both in tissue cultures and in living flies, yet has never been investigated in other species.

Here we present a detailed functional analysis of the *tim* promoter from the drosophilid fly, C*hymomyza costata* in *Drosophila* tissue cultures. A comparison of *tim* promoters from *wt* and *npd*-mutants confirmed that the 1855 bp deletion in the latter removes crucial regulatory *cis*-elements as well as the minimal promoter, being subsequently responsible for the lack of *tim* mRNA expression. Deletion and substitution mutations of the *wt tim* promoter showed that the region containing the canonical E-box, TER-box and two incomplete E-box sequences is essential for CLK/CYC mediated expression while the PERR element appears to be a repressor in S2 cells.

Furthermore, the expression of the circadian genes *timeless, period, vrille* and *doubletime* was quantified in *C. costata* adults. We have found striking differences in expressions profiles for *tim*, *per* and *vri* between *wild type* and *npd*-mutant individuals.



ABSTRAKT

Proteinový komplex dCLK/CYC řídí cirkadiánní průběh transkripce genu *timeless* u octomilky *Drosophila melanogaster* tak, že se váže na tandemové sekvence E – boxů a E – boxu podobných sekvencí v promotoru genu *tim*. *tim* promotor byl detailně studován jak v tkáňových kulturách *Drosophily* tak u živých octomilek, ale dosud nebyl popsán u jiného druhu hmyzu.

Předkládaná publikace popisuje detailní fukční analýzu promotoru genu *tim* mušky *Chymomyza costata,* která byla provedena v buněčných kulturách *D. melanogaster*. Srovnání promotorů genu *tim* u divokého kmene (*wt*) a mutantního kmene (*npd*) potvrdilo, že 1855 bp dlouhá delece v *npd* promotoru je zodpovědná za minimální expresi *tim* mRNA, protože obsahuje důležité *cis*-regulační sekvence a minimální promotor. Delece a substituční mutace

ve *wt tim* promotoru ukázaly, že oblast obsahující kanonický E – box, TER – box a dva neúplné E – boxy je nezbytná pro expresi zprostředkovanou komplexem CLK/CYC, zatímco PERR – box funguje v S2 buňkách jako represor transkripce.

Současně byla stanovena exprese cirkadiánních genů *timeless, period, vrille* a *doubletime* v hlavách dospělců *C. costata*. Nalezli jsme významné rozdíly v expresních profilech genů *tim*, *per* a *vri* mezi divokým a mutantním *npd* kmenem.

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## CHAPTER II - UNPUBLISHED DATA

Následující pasáž o rozsahu 5 stran (str. 39 – 43) obsahuje utajované skutečnosti a je obsažena pouze v archivovaném originále diplomové práce uloženém na Přírodovědecké fakultě JU.

**CHAPTER III – PAPER II**

Košťál V, Šimůnková P, Kobelková A, Shimada K (2009) **Cell cycle arrest as a hallmark of insect diapause: Changes in gene transcription during diapause induction in the drosophilid fly, *Chymomyza costata*.** Insect Biochem and Mol Biol 39: 875-883

Alena Kobelková has done 10% of the work


ABSTRACT

The division cycle of CNS cells was arrested in G0/G1 (86.6%) and G2 (12.8%) phases in diapausing larvae of *Chymomyza costata*. A two-step response was observed when the diapause was induced by transferring the 3rd instar larvae from long-day to short-day conditions: first, the proportion of G2-arrested cells increased rapidly within a single day after transfer; and second, the increase of G0/G1-arrested cells started with a delay of 5 days after transfer. The changes of relative mRNA levels of seven different genes, which code for important cell cycle regulatory factors [Cyclins D and E, kinases Wee1 and Myt1, phophates Cdc25 (String), Dacapo (p27), and PCNA] were followed using qRT-PCR technique. Two reference genes (*Rp49* and *ß-tubulin*) served as a backround. Significant transcriptional responses to photoperiodic transfer were observed for two genes: while the relative levels of *dacapo* mRNA increased during the rapid entry into the G2 arrest, the *pcna* expression was significantly downregulated during the delayed onset of G0/G1 arrest. In addition, moderate transcriptional upregulations of the genes coding for two inhibitory kinases, *wee1* and *myt1* accompanied the entry into diapause. The other genes were expressed equally in all photoperiodic conditions.

ABSTRAKT

Buněčný cyklus v mozkových buňkách diapauzních larev mušky *Chymomyza costata* byl zastaven v G0/G1 (86.6%) a G2 (12.8%) fázi. Diapauza byla indukována přenosem larev třetího instaru z dlouhého do krátkého dne. Během takto indukovaného vstupu do diapauzy proběhlo zastavení buněčného cyklu ve dvou krocích: (1) podíl buněk zastavených v G2 fázi buněčného cyklu se významně zvýšil během jediného dne po přenosu larev do krátkého dne, zatímco (2) zastavení buněk v G0/G1 fázi proběhlo pomaleji, a to až pátý den po přenosu. Pomocí kvantitativní real-time PCR byly stanoveny změny v relativním množství transcriptů sedmi genů, které kódují důležité regulátory buněčného cyklu [Cykliny D a E, kinázy Wee1 a Myt1, fosfatázy Cdc25 (String), Dacapo (p27), a PCNA]. *Rp49* a *ß-tubulin* byly použity jako referenční geny. Významná změna v transkripci během fotoperiodicky indukovaného vstupu do diapauzy byla zjištěna u dvou genů: relativní množství transkriptu *dacapo* se zvýšilo během zastavení buněčného cyklu v G2 fázi a naopak exprese genu *pcna* se snížila během

zastavení buněk v G0/G1 fázi. Vstup do diapauzy byl navíc doprovázen mírným zvýšením transkripce dvou kináz *wee1* a *myt1*, které inhibují průběh buněčného cyklu. Exprese zbývajících genů zůstala neměnná při všech testovaných fotoperiodických podmínkách.

## CHAPTER IV – PAPER III

Kobelková A, Závodská R, Doležel D, Šauman I. **The introduction of Mediterranean flour moth, *Ephestia kuehniella,* as a novel non-drosophilid species for circadian clock studies.**

Manuscript

Alena Kobelková has done 70% of the work


ABSTRACT

Although homologous circadian genes are found in insect clocks, their contribution to circadian timing systems may differ. Striking differences in the clock gene regulation were found between master clocks of the fruitly, *Drosophila melanogaster* (Diptera) and the silkmoth, *Antheraea pernyi* (Lepidoptera).

Therefore we have extended our research to a relatively primitive lepidopteran species, Mediterranean flour moth, *Ephestia kuehniella,* in order to explore how well the silkmoth model is conserved within Lepidoptera. The Mediterranean flour moth has been used as a novel model organism for circadian rhythm studies. At first, characterization of circadian controlled egg hatching behavior and locomotor activity of the flour moth adults is presented. Futhermore, full-length cDNA sequences encoding the circadian clock genes *period* and *timeless* in *E. kuehniella* were cloned. Southern analyses confirmed one copy of *per* and *tim* gene, respectively, within both sexes. PER-like immunoreactivity was observed in nuclei and cytoplasm of most neurons in the central brain, in many photoreceptor nuclei, in the optic lobes and in the ventral part of suboesophageal complex. Similar expression pattern were revealed for *per* and *tim* mRNAs using *in situ* hybridization.

*Ephestia kuehniella* was established as a convenient non-drosophilid species for the circadian rhythm studies.



ABSTRAKT

Ačkoliv cirkadiánní geny vykazují u různých druhů hmyzu sekvenční homologie, některé z nich získaly během evoluce odlišné role v řízení cirkadiánních rytmů. Významné rozdíly v regulaci hodinových genů v centrálním oscilátoru byly nalezeny mezi octomilkou *Drosophila melanogaster* (Diptera) a martináčem dubovým *Antheraea pernyi* (Lepidoptera).

Našim cílem bylo prozkoumat nakolik je model cirkadiánních hodin martináče dubového konzervovaný v rámci řádu Lepidoptera. Pro tento účel jsme jako modelový organismus zvolili relativně primitivní druh motýla, zavíječe moučného (*Ephestia kuehniella*). Tento druh byl pro studium cirkadiánních hodin použit vůbec poprvé. U zavíječe moučného jsme nejdříve charakterizovali cirkadiánně řízené líhnutí larev a pohybovou aktivitu dospělců. Dále se podařilo zaklonovat úplné sekvence dvou cirkadiánních genů *period* a *timeless*. Southern

analýza potvrdila, že jak v samčím tak v samičím genomu zavíječe je pouze jedna kopie genů *per* a *tim*. PER imunoreaktivita byla pozorována v jádrech a cytoplasmě velkého množství neuronů v centrálním mozku, v jádrech fotoreceptorů, v optických lalocích a ve ventrální části subesophageálního komplexu. *in situ* hybridizace potvrdila podobný expresní profil pro *per* a *tim* mRNA.

Závěrem můžeme konstatovat, že zavíječ moučný byl zaveden jako vhodný hmyzí model pro studium cirkadiánních hodin.

Následující pasáž o rozsahu 31 stran (str. 54 – 84) obsahuje utajované skutečnosti a je obsažena pouze v archivovaném originále diplomové práce uloženém na Přírodovědecké fakultě JU.

**SUMMARY**

**PAPER I**

(i) We proved that the 1855 bp deletion in the *tim* promoter is responsble for *tim* null mutation in the *npd*-mutant of *C. costata*, as it removes functional elements necessary for *tim* transcription.

(ii) Detailed deletion and substitutional mutagenesis within *tim* promoter showed that the tandem arrangement of the canonical E-box (CACGTG) and TER-box (GCAGCACGTG) seems to be crucial for high levels of expression, while the incomplete E-boxI and E-boxII (ACGTG) assist in maintaining transcriptional activity. Aditionally, we recognized a repressive role for PERR-box (GTACGCACGA).

(iii) Using quantitative PCR we verified that *npd*-mutant does not express *tim* gene. As a consequence, this *tim* null mutation affects the expression profile of circadian genes *per* and *vri*, reducing their high amplitude cycling to flat, intermediate expression levels. The expression pattern of *dbt* is neither cyclic nor affected by the *npd* mutation.

Moreover, we showed that photoperiod influences the phase of *per*, *tim* and *vri* cycling in wild type *C. costata.*

**PAPER II**

(i) Using flow cytometry analysis, we showed that the cell cycle of central nervous system is arrested in G0/G1 (86.6%) and G2 (12.8%) phases in diapausing larvae of *C. costata*

(ii) We obtained partial sequences of *cyclin E*, *cyclin D*, *wee1*, *myt1*, *cdc25*, *dacapo* and *pcna* genes which enabled us to design suitable primers for quantitative PCR

(iii) We found significant *pcna* transciptional downregulation and *dacapo* transcriptional upregulation during larval diapause entrance in both CNS and imaginal discs.

Moderate transcriptional upregulations (up to 1.5 fold) of *wee1* and *myt1* accompanied the entry into diapause in CNS. These data suggest that inhibitory kinases *wee1* and *myt1* may play some role in the G2 arrest.

The other genes, *cyclin E*, *cyclin D* and *cdc25* were expressed equally during the diapause entry.

**PAPER III**

(i) The egg-hatching was diurnal and gated to 6 hours time window after dawn. The locomotor activity in adults of *E. kuehnilella* has clear circadian character with free running period ~24h.

(ii) The full-lenght sequences of *per* and *tim* genes were cloned. Just one copy of each gene was revealed in *E. kuehniella* genom.

(iii) *per* and *tim* transcripts appeared to cycle during day and those cycling were maintained in constant darkness. Circadian cycling was disrupted in constant light conditions.

(iv) Expressions of *per* and *tim* mRNAs were detected in the same regions in the brain-subesophageal complex of *E. kuehniella*. Expression of *per* mRNA agreed with the localization of PER-like positive cells

The prominent daily oscillations of PER protein, *per* and *tim* mRNAs were observed in nuclei of photoreceptors.

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**Kobelková A**, Bajgar A, Doležel D (2010) Functional molecular analysis of a circadian clock gene *timeless* promoter from the drosophilid fly *Chymomyza costata.* J Biol Rhythms (*accepted*)

Košťál, V., Šimůnková, P**., Kobelková, A**., Shimada, K., 2009. Cell cycle arrest as a hallmark of insect diapause: Changes in gene transcription during diapause induction in the drosophilid fly *Chymomyza costata.* Insect Biochem. Mol. Biol., 39: 875-883

**Presentations**

2008 XXIII International Congress of Entomology, Durban, South Africa
Mediterranean flour moth, *Ephestia kuehniella*, a new non-drosophilid species for circadian clock studies, Kobelková A., Doležel D., Závodská R. and Šauman I. – poster

2010 International Conference of Invertebrate Reproduction and Development, Prague, Czech Republic
Mediterranean flour moth, *Ephestia kuehniella*, a new non-drosophilid species for circadian clock studies – part II, Kobelková A., Závodská R., Doležel D. and Šauman I. – poster

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