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CIRCADIAN GENES AND REGULATION OF DIAPAUSE IN INSECT

Ph.D. Thesis

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Annotation

This thesis considers various roles of circadian clock genes in insect physiology. Application of molecular-biology methods in *Pyrrhocoris apterus*, non-model insect species, enable us to investigate involvement of circadian clock genes in photoperiod induced physiological responses. We discover involvement of neuroendocrine cells, and a role of Juvenile hormone (JH) signalization in transduction of photoperiodic signalization to peripheral tissues. We found new principles of JH signal diversification in tissue specific manner, and in addition described molecular mechanism of photoperiod induced changes in gut physiology. Comparison of gut and fat body tissue reveals that mechanism observed in the gut is tissue specific, and that circadian clock genes exhibit tissue specific functional pleiotropic effect.

Declaration [in Czech]

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Adam Bajgar

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

Two of the presented papers are already accepted in respected journals, the other three are in form of manuscript (prepared for final correction and submission in impact journals)

Kobelkova A., Bajgar A., Dolezel D. 2010. Functional molecular analysis of a circadian clock gene *timeless* promoter from the drosophilid fly *Chymomyza costata*. The Journal of Biological Rhythms, 25, 6, 399-409, (Dec 2010).

Adam Bajgar helped with preparation of constructs with modified promotor sequences and with luciferase reporter assays.

Bajgar A., Jindra M., Dolezel D. 2013. Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling. PNAS. 110, 11, 4416-21, D OI: 10.1073/pnas.1217060110, (Feb2013).

Adam Bajgar performed most of the experiments presented in this articles. Participated on research design, writing, and revision of the article.

Bajgar A., Dolezel D., Hodkova M. 2013. Endocrine system regulation of circadian clock genes *cry2* and $Pdp1_{iso1}$ expression in the insect gut. (in preparation)

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Bajgar A., Smykal V., Fexova S., Buricova M., Provaznik J., Takaki K., Jindra M., Dolezel D., Hodkova M. 2013. Insect reproduction and metamorphosis depend on distinct branches of juvenile hormone signaling. (in preparation)

Adam Bajgar and Vlastimil Smýkal contributed equally to this work. Adam performed most of the experiments analyzing JH influence of vitellogenesis. Adam also participated on writing and revision of the manuscript.

Bajgar A., Fexova S., Dolezel D. 2013. Expression patterns variability of *cry2* and $Pdp1_{iso1}$ genes in response to reproductive or diapause photoperiodic conditions suggests their pleiotropic tissue specific effect in *P. apterus*. (in preparation)

Adam Bajgar performed most of the experiments presented in this manuscript. Participated on research design, writing, and revision of the manuscript.

Contents

Introduction

Many environmental conditions are changing with certain periodicity. Differences in exposure of the Earth surface to light, cause periodical fluctuations of temperature, moisture, seasonal climatic changes, and many subsequent features. The periodic changes of environmental factors are associated with regular changes of light conditions, which could be used like a specific signal (Saunders 2002). It is obvious, that light conditions promotes cyclic changes during a day and during a year, which enable individuals to predict upcoming conditions in advance. But individuals must be able to measure time for predicting changes of environmental conditions and sufficiently prepare on it (Danks 2005). This ability serves to synchronization of many physiological and behavioral functions which brings to individual considerable benefits.

The evolutionary emergence of the circadian clock has turned organisms from merely responders to predictors, and increased their fitness so that the clocks become widespread (Pegoraro and Tauber 2011). That's why we can find circadian clock behavior and time measuring mechanisms in majority of living organisms. This wide spread occurrence enable us to partially generalize results from research made on model organisms (fly, mouse) to the humans (Kostal 2011, (Mohawk et al., 2012).

Investigation of circadian clock mechanism in the last decade indicated, that the circadian clock mechanism has common components which could play various roles in various species. This fascinating diversity complicate investigation of circadian clock mechanism itself, but on the other hand enable us to observe how genes with highly conserved amino-acid sequence can manifest functional polymorphism. It was described several times, that the circadian clock oscillator mechanism is composed from many transcription factors, which reciprocally regulate their own expression patterns (Cyran et al. 2003,(Peschel and Helfrich-Forster, 2011; Tomioka and Matsumoto, 2010)).

The cyclic expression of transcription factors further directs several peripheral clock mechanisms in the whole organism (Ito et al. 2008). Circadian clock mechanism synchronizes many behavioral and physiological processes with external light conditions. Typical behavioral circadian phenotypes are wake/sleep cycles and daily activity changes. From physiological functions we can for example mention daily temperature and metabolic changes.

After identification of main components of the circadian clock mechanism (circadian clock genes), it was surprising to find them in a wide range of another physiological functions. Circadian clock genes were found in connection to photoperiodic time measurement (Schiesari et al. 2011), regulation of photoperiodic tissue specific phenotypes (Dolezel et al. 2008), and in regulation of several other biological processes (sleep, metabolism, immunity, cell cycle (overview in Rosenwasser at al. 2010)).

Contrary to circadian clock, photoperiodic clock mechanism measures ratio between light and dark phase of a day, and can thus be used for prediction of upcoming season. The typical photoperiod responding phenotypes are migration and diapause (Wilde 1962). These are two possibilities how to avoid upcoming inconvenient environmental conditions. Diapause can be triggered simply by external

light conditions in insect and is usually accompanied with obvious morphological markers. It makes from the insect diapause induction an ideal experimental object for studying and dissecting photoperiodic clock mechanism.

Despite intense study of photoperiodic clock mechanism, the core mechanism still remains elusive. It is mainly because of missing photoperiodic phenotype in commonly used model organisms of molecular biology such as *Drosophila, Coenorhabditis,* and *Mus*. Newly introduced insect model organism performing both circadian and photoperiodic phenotypes enable us to study molecular components necessary for function of both time measuring mechanism.

In this work, I want to present data which brings some new insight into mechanism of photoperiodic regulation of metabolism and reproduction in *Pyrrhocorisapterus*. In addition, I want to show which neural structures are responsible for photoperiodic clock signal transduction and how this kind of signal can influences photoperiodic phenotype and expression of circadian clock genes in periphery by tissue dependent manner.

1. Circadian clock

1.1. Central circadian clock mechanism

It has been shown many times, that substantial part of transcriptome exhibits circadian fluctuations in expression level. These are genes involved in several physiological mechanisms typical for respective time of a day. The expression of these genes is directed by output from central circadian pacemaker (Pegoraro and Tauber 2011). Small subset of the genes with circadian cyclic expression, are evolutionary conserved components of central clock oscillator, which keep the clock mechanism still working.

According to generally accepted theory, the circadian clock mechanism has evolved in relation to necessity of DNA replication machinery protection from UV light exposure causing DNA damage (Gehring and Roshbash 2003). Evolutionary important advantage, orchestrating DNA replication in the dark part of a day, has been soon interconnected with several other processes. Further advantage of synchronization of physiological processes reveals with emergence of multicellular organisms. Thus we can summarize, that circadian clock mechanisms works on single cell level (cell autonomous clock system) and share the same conserved components over wide range of organisms.

In animals the circadian clock mechanism resides in central nervous system (Saunders and Bertosa 2011). Complex system of interconnected negative feedback loops promotes natural circadian oscillations and orchestrates expression of many downstream genes.

Interesting findings on circadian clock mechanism was brought by Fritz et al. (1996), who showed that circadian clock mechanism works on unicellular level and do not need intercellular crosstalk. The result of this internal mechanism is spontaneously cycling expression pattern of these genes with approximately 24 hour lasting period. This time lap is called free-running period and is specie

characteristic, although free running period may vary between individual strains, including those originating from different latitudes (Lankinen 1979; Lankinen 1986). The period is not lasting exactly 24-hours and it is necessary to synchronize the clock mechanism by external signal to get precise time measurement (Zeng et al. 1996, Emery et al. 1998). The strongest synchronizing clue is light, but it was documented that synchronization can be made by temperature(Glaser and Stanewsky, 2005; Sehadova et al., 2009), or social interactions (Levine et al. 2002). The core mechanism of the central circadian clock is well studied in a typical molecular biological model organism (fly, mouse). Research based on selection of natural mutants and mutations mapping led to detection of the first circadian clock genes (Bargielo et al. 1984, Sehgal et al. 1994).

Nowadays we have identified several genes necessary for properly working circadian clock machinery and we know a lot about their reciprocal interactions. Although we can found conserved homologues of circadian clock genes in almost all animals, recent observations on non-model organisms (mainly insect) reveal unexpected variability in the central clock mechanism (Yuan et al. 2007). One of the most conserved part of the circadian clock, common for both *Drosophila* and mammals, involves negative feedback loops of clock proteins accumulated in the cell cytoplasm, that enter the nucleus to repress their own expression. Afterwards, when the amount of protein is significantly decreased the expression of the genes is restored again. *Drosophila* central clock mechanism is schematically described on figure 1. In*Drosophila*, the two transcription factors (CYC, CLK(Allada et al., 1998; Rutila et al., 1998)) dimerize through their PAS domains and initiate expression of several transcription factors by binding their E-box regulatory sequences (Curtin et al. 1995, Darlington et al. 1998). Active transcription of *Period* (*Per*) and *Timeless* (*Tim*) causes accumulation of PER-TIM heterodimer in cytoplasm during light part of a day, and after reaching of certain abundance in cytoplasm, enters the nucleus and repress *cyc* and *Clk* gene expression. Stability of PER –TIM heterodimer is in light dependent manner regulated by other circadian clock genes CRY (*cryptochrome*) and JET(*Jetlag*)(Peschel et al., 2009). Beside this main negative feedback loop, the circadian positive elements (CLK-CYC) activate expression of *vrille*(*vri*) and *Par domain protein1* (*Pdp1*), members of second interlinked feedback loop. VRI and PDP1 influence expression of *Clk*oppositely (VRI – repress, PDP1 – activate), while they are competing for the same promoter region (Cyran et al 2003). The last part of the mechanism is CLK-CYC activated *Clockwork-orange*, which down-regulates expression of *Clk*as well (Richier et al. 2008, Kadener et al. 2007, Matsumoto et al. 2007) (Fig. 1). This machinery described in *Drosophila* works with some differences also in mammals.

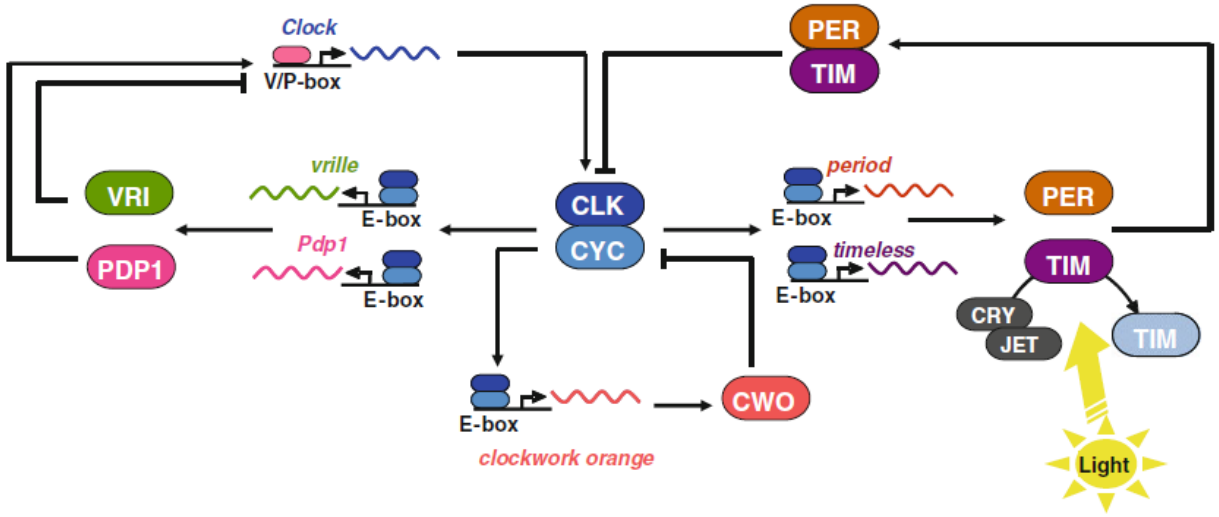

Fig. 1.Molecular oscillatory mechanism of *Drosophila* circadian clock. The clock consists of three main interlocked transcriptional translational loops. Light induced degradation of TIM through CRY and JETLAG (JET) resets the clock in a time of day dependent manner. Involved circadian clock genes: *Clock* (*Clk*), *cycle* (*cyc*), *period* (*per*), *timeless* (*tim*), *cryptochrome* (*cry*), *Jetlag* (*Jet*), *vrille* (*vri*), *Par domain protein1* (*Pdp1*), *clockwork orange* (*cwo*) (adopted from Tomioka et al. 2012).

The mammalian central clock mechanism is more complicated. Circadian clock genes have more paralogs and the genes promote functional redundancy (overview in Takahashi et al. 2008). The largest functional difference is in function of *cry* gene. In *Drosophila* central clock mechanism *d-cry* acts just like synchronizing external element, while in mammals CRY binds to PER (instead of *Drosophila* TIM)(Kume et al., 1999) and act as a relevant component of the main negative feedback loop (Yuan et al. 2007).

This understandable mechanism can explain diurnal cycling of circadian clock genes expression patterns (Fig. 2), however the recent studies show that the model is more complicated. It has been proved, that expression of several circadian clock genes (in Drosophila *Clk*, *cwo*, *vri*) is regulated post-transcriptionally by micro-RNA machinery (miRNA). miRNAs regulate expression by binding regulatory sequences or by degradation of messenger-RNA. For example the well-studied miRNA called *bantam* occurs in circadian clock neurons and binds regulatory sequence of *Clk* gene. Overexpression of this miRNA prolong natural circadian period (Yang et al. 2008). Another important component of central circadian clock mechanism is post-translational modifications. Specific phophorylations and dephosphorylation guarantee appropriate delay between gene expression and protein action(Kim and Edery 2006(Chiu et al., 2011; Sathyanarayanan et al., 2004; Weber et al., 2011)).

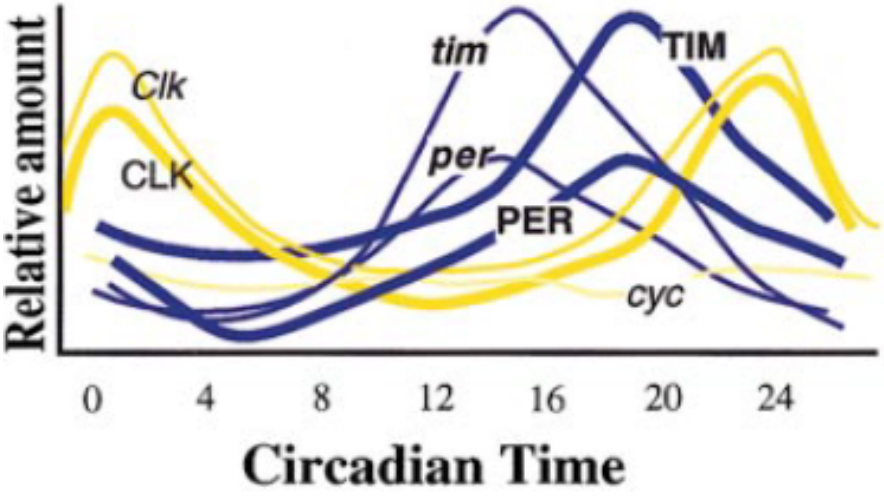

Fig. 2 Temporal expression pattern of genes *period* (*per*), *timeless* (*tim*) and *Clock* (*Clk*) in central clock mechanism of *D. melanogaster* (adopted from Dunlap et al. 1999).

Our knowledge of central circadian clock mechanism shows high complexity and suggests that similar relationships can be expected also in peripheral and seasonal clocks.

Despite well characterized central clock mechanism there are still some unanswered questions. We still don't know how the central clock mechanism orchestrates peripheral clock mechanism. What are the mechanisms of the peripheral clock oscillators? How are circadian clock genes involved in seasonal timing? How distinct functions can circadian clock genes have? Data from other experimental insect species than *Drosophila* and mouse reveal, that there is a wide variability in clock mechanisms between species, and that the circadian clock genes can adopt in this mechanism distinct functions

1.2. Pluripotency of circadian clock mechanisms

Studies of central circadian clock mechanism in non-model insect species show that there is a wide range of deviances from *D. melanogaster* (Saunders and Bertosa 2011). Same circadian clock genes changed their function in the clock mechanism and some genes are even missing, substituted by another gene with redundant function. In several insect species is beside (or instead of) *cry1* its homolog *cry2* gene. The *cry2* was detected in *Dannausplexipus*, *Apismelifera*, *Pyrrhocorisapterus*, *Triboliucastaneum*, some Lepidoptera species, and in mosquitoes. The *cry2* is according to its aminoacid composition more similar to mammalian type of *cry* and play different role in central clock mechanism. In contrast to CRY1, CRY2 participate in negative feedback loop as a transcription repressor of CLK-CYC heterodimer formation and repress through this mechanism other circadian clock genes(Yuan 2007). Its function is light independent and resembles function of *cry1* in *Drosophila* peripheral oscillators (discussed later in the text).

Another well documented gene with dual or changed function is *Timeless* (*tim1*) (Barnes et al. 2003). In majority of species was observed another form of this gene called *timeout* (*tim2*) (Benna et al. 2010). Well understood function of *tim1* can be substituted by *cry2*, while *tim2* can act as a light entraining component of this loop (Benna et al. 2010). In species with missing *tim1* is most probably its role replaced by *tim2*, as was recently shown in flies (Schurko et al. 2010).

The diversity in the circadian clock mechanism imply, that there is a space for divergence of clock gene function and thus these genes can act in different relationships and mechanisms. Study of peripheral clock mechanisms can reveal novel principles how circadian oscillators can works.

1.3. Peripheral circadian clock mechanism

Central circadian pacemaker (described in previous chapter), residing in CNS, regulates daily oscillations of many behavioral and physiological functions (Saunders 2002). Although the master clock is necessary for circadian physiology regulation and entrainment with external conditions, there are many of tissue specific peripheral circadian clocks in the organism (Tomioka et al. 2012). These peripheral oscillators are responsible for tissue specific circadian organization of organ function in more or less autonomous manner.

The autonomous rhythms can be found for example in sensory organs, digestive and reproductive system (Kostal 2001). In *D. melanogaster* it has been shown that the peripheral clock is self-sustained oscillator with molecular machinery slightly different from that of the central clock (Stanewsky et al. 1998). It is obvious, that all peripheral oscillators have to be synchronized to work in harmony and have to be adjusted to dial period (Fig3). Recently, molecular studies on insect reveal circadian tissue specific changes in expression level of several circadian clock genes suggesting presence of tissue specific oscillators (Hege et al. 1997; Plautz et al.1997; Merlin et al. 2006; Uryu and Tomioka 2010).

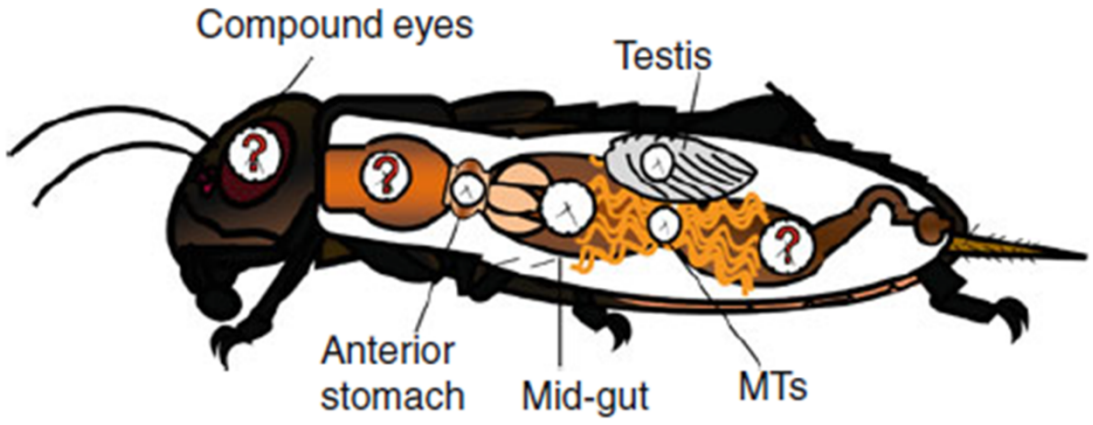

Fig. 3 Central and peripheral clocks in crickets. The cricket's central clocks are located in the optic lobes in the brain. The central clocks control overall rhythms such as activity, feeding and mating. In contrast, there are clocks in many body parts, so-called peripheral clocks, which assign circadian rhythmicity with their specific functions (adoptedfrom Tomioka et al. 2012).

It suggests that the mechanism of peripheral clock share common components with central clock machinery. Since the peripheral clocks were found in wide range of animals (Dibner et al. 2010) this evolutionary conserved system should has a great importance in organisms. The studies on non-model insect species enable us to compare the mechanisms of peripheral clock system and we can see the variability in peripheral clock mechanism on both intra- and inter-specie level.

Study of peripheral clock mechanism is pretty complicated, because it is crucial to distinguish between autonomous tissue specific regulatory system and impact of the central clock mechanism. Therefore the experiments are often held on isolated organs or tissue cultures,this fact makes the whole process more complicated (Tomioka et al. 2011). Results from experiments carried on eyes, gustatory system, malpighian tubules, and epidermis proved involvement of many circadian clock genes, such as *per*, *tim*, *cyc*, *Clk*, *cry* in peripheral clock mechanism(Kamae et al. 2010, Ikeno et al. 2011). It is well documented that the peripheral and central machineries contain the same components but work differently. For example the role of *cry* in *Drosophila* central clock oscillator is to synchronize PER-TIM loop with the external conditions, while in peripheral clock CRY binds PER and act like a transcription repressor(Collins et al., 2006). In addition *cry* mutant flies have abolished the peripheral cyclic phenotypes (Stanewsky et al. 1998; Ivanchenko et al. 2001) (Fig. 4). Another example of difference between central and peripheral clock mechanism was observed in moths. In moth there is a deviance from *Drosophila* in central clock mechanism in PER-TIM loop. PER was not detected in nuclei of central clock neurons, which obstructs its function in *Clk* repression. Contrary the situation in the central clock oscillator, PER shows clear nuclear expression in periphery, where in addition promotes circadian fluctuations (Gvakharia et al. 2000; Schuckel et al. 2007; Kotwica et al. 2009).

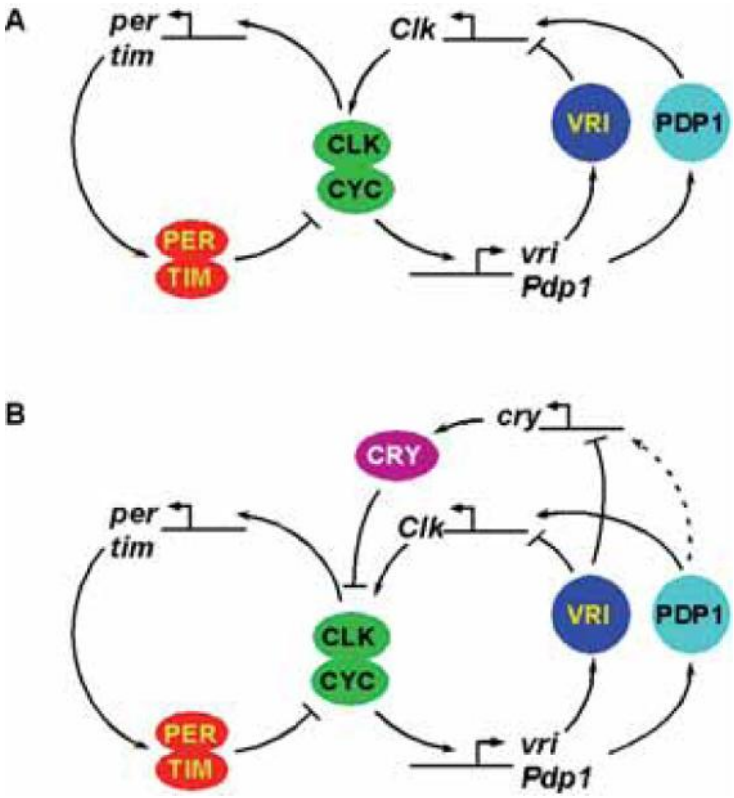

Fig. 4 Model of the molecular clock feedback loops in *Drosophila*. The clock is composed of two interconnected transcriptional-translational feedback loops. (*A*) In s-LN_Vs, *cry* is not required for rhythms and is omitted from the model, although it acts as a cell-autonomous photoreceptor in these cells. (*B*) In peripheral clocks, CRY has an important clock role and acts as a transcriptional repressor of CLK/CYC activity in conjunction with PER. Because *cry* expression is regulated by VRI and because VRI- and PDP1-binding sites are very similar, *cry* is probably also regulated by PDP1 (*dashed line*) (adopted from Blau et al. 2007).

The relationship between central and peripheral clock oscillators is pretty complicated issue. Some tissue specific peripheral oscillators are absolutely subordinated to the central clock, while the others show partial autonomy. The autonomous tissues show cyclic phenotype in vitro and have the ability of self-synchronization by light or temperature stimuli (Levine et al. 2002; Glaser and Stanewsky 2005). Thus, must contain a complete set of circadian clock machinery including genes necessary for entrainment. Also after transplantation of autonomous tissues into the individual with opposite circadian phase these organs maintain their original settings (Giebultowicz et al. 2000).

It is now generally accepted, that peripheral oscillators in *Drosophila* are entrained even after central clock neurons ablation (with exception of prothoracic gland), which has been described in several other insect species (Merlin et al. 2007, 2009; Saifullah and Page 2009; Uryu and Tomioka 2010). But the mechanism of light entrainment of the peripheral clock is still unknown. It is possible that the mechanism is the same like in central clock and entraining element is CRY, affecting PER-TIM complex. For function of this model is necessary direct access of light to the synchronized cells (Ivanchenko et al. 2001). *cry* expression has been observed in peripheral tissues, but its mutation disrupt cycling of clock mechanism suggesting that *cry* plays an essential role in peripheral

Drosophila oscillator (Stanewsky et al., 1998; Krishnan et al., 2001). This mechanism could work in some small insect species, with light colored cuticle, but not in large and dark colored species, such as some bugs and beetles. Some insect species like crickets, bugs and cockroaches cannot entrain peripheral oscillators to the external conditions after interruption of optic lobe innervation (Tomioka and Chiba 1984). That suggests that the only source of light information is processed by retinal photoreceptors and transmitted to periphery by still unknown pathway.

The wide variability in central and peripheral clock mechanism is result of different evolutionary necessity in dependence to individual life-style. For example diversity in feeding times has to evoke different adjustment of peripheral clock system (Tomioka et al. 2012).

In some cases, the peripheral clock function is dependent on central clock signalization. The signalization to the peripheral tissues and regulation of tissue specific clocks will be described in the next part. Recent application of molecular biological techniques in non-model insect species can reveal molecular mechanism of peripheral clock and enable us to understand principle of orchestration of the multi-clock system.

1.4. Regulation of peripheral clock mechanisms by central clock oscillator

The synchronization of multiple clock system has a crucial effect on proper function of organism. Cooperation of various physiological processes in organs with self-oscillating clock mechanism is adaptive and beneficial (Sharma 2003). Many physiological processes, such as metabolic settings, reproduction, and homeostasis maintenance require interconnection and proper timing of physiological mechanisms of different tissues and behavioral phenotypes (Xu et al. 2008). This effect can be achieved by signalization from the central clock pacemaker residing in CNS. Relatively little is known about the central circadian clock output pathways directing the others clock systems in periphery (Kostal 2011).

Characterization of involved neuronal circuits in *Drosophila* and experiments with interruption of their descendent neuronal pathways suggested involvement of hormonal signalization. The only one well described neurotransmitter involved in circadian signalization is *Pigment-dispersing factor* (*Pdf*) (Homberg et al. 1991, (Renn et al., 1999; Yoshii et al., 2009)). PDF occurs in all central clock neurons and transfer their output to downstream neurons with neurosecretory function (Hyun et al. 2005; Shafer et al. 2008). Although most of these information has been obtained from *Drosophila*, PDF seems to play similar function also in other insect species (Petri and Stengl 2001; Schneider and Stengl 2005).

PDF act as a parathormone released from central clock neurons in periodic manner. Experiments with *Pdf* null mutant flies reveal that PDF is essential for maintaining of activity diurnal cycles(Renn et al., 1999). PDF responding neurons in dorsal protocerebrum are important regulatory units influencing release and synthesis of many neurohormones directly regulating behavior, or

synchronizing rhythmicity of peripheral clocks. Direct innervation of *prothoracic gland* and *corpora allata* (part of *ring gland* in flies) indicates engagement of juvenile hormone (JH) and ecdysteroids (Colombani et al. 2005).

It has been described in mammals that there exist another non-hormonal signal synchronizing peripheral clock systems. Oscillation of circadian clock genes in liver, heart and kidney of rat was influenced by short feeding stimulus (Wu et al. 2012), although liver clock system can be shifted by glucose and insulin signalization, respectively (Yamajuku et al. 2012). In addition Dibner et al. (2010) documented, that central master-clock system regulates peripheral clocks through body temperature in mammals.

Plurality of circadian signal transduction to peripheral organs implies that we can expect discovery of several regulatory systems in insect as well. Unfortunately, *Drosophila* peripheral clock systems seem to be synchronized directly by light. Therefore we have to use non-model insect species for study this phenomenon. In relation to above described organization of circadian clock hierarchy, we can suggest a similar system also in photoperiod perceiving mechanism. Whether photoperiodic clock system consist of central and peripheral oscillators remain still unknown, although novel results indicates some perspectives.

2. Photoperiodic clock

2.1. Central photoperiodic clock mechanism

Photoperiod is natural ratio between light (photophase) and dark (scotophase) phase of day. This photoperiodic ratio and prolonging or shortening of photophase is typical for certain season of the year. The ability of measuring of photoperiod is widely used for upcoming season forecast and preparation on changing environmental conditions in advance (Tauber et al. 1986). The mechanism of photoperiod measuring was for a long time absolutely unknown mainly due to weak or missing photoperiodic phenotype in commonly used model organisms. Introduction of novel non-model experimental species (mammalian and insect) and implementation of molecular methods has brought progress in understanding of photoperiodic clock mechanism.

One of the strongest photoperiodic phenotype is diapause incidence (Kostal 2011). This process of reproduction arrest, metabolic calming down and synthesis of reserves necessary for over-wintering can be induced simply by changes in light/dark ratio by prolonging dark phase of the day (Wilde 1962). Light/dark ratio in which half of the experimental individuals enter the diapause is called critical photoperiod (CPP) and is specie and population specific (changes with latitude (Lankinen and Lumme 1984)).

From observation of the general photoperiodic phenotype features, we can conclude that photoperiodic clock mechanism consist of four functional subunits: light receptor necessary for an external light signal transduction, photoperiodic clock distinguishing between long and short day

conditions, photoperiodic counter, assuming proceeding shortening or prolonging of days, and output pathways directing function of subordinated physiological processes (Saunders 1981). Investigation of photoperiodic clock system was mainly based on experiment modulation of the input information and characterization of output response, so that the mechanism of photoperiodic clock was a black box.

The important part of the photoperiodic clock mechanism is photoreceptive organ. From the surgical experiments on some insect species we can say, that the photoreceptive cells reside in eyes. Experiments with induction of diapause in different light conditions imply that most effective light is blue-green, while red far-red is not sufficient (Lees 1966, Saunders 2002). Also vitamin-A lacking diet reduced efficiency of diapause entry (Goto et al. 2010). It suggests, that primary photoreceptor of photoperiodic clock in insect is UV or short wavelength opsin, although it could be different in distinct species. Extirpations and operations of certain brain parts demonstrate that these light responding neurons are in *optic lobes* and *ventral neuropile* of *protocerebrum* (Steel and Lees 1977).

It is impossible to find one general photoreceptive organ, because the wide variability which occurs in insect. Light information can be reported through compound eyes, ocelli, andstemata (Shimizu 1982). Even more the light signal can be detected by more than just one type of cells and photoreceptive molecule and these input pathways can cooperate (Morita and Numata 1999). The input pathwaysseem to be multiple,redundant, and cooperative.

Molecular mechanism of photoperiodic clock system still remains elusive. There are several hypotheses about its function, but no one has been proved empirically. Several experiments held mainly on *D. melanogaster* and some non-model insect species determined genes playing important role in photoperiodic clock mechanism or influence its function (Saunders 2011). Based on mentioned theoretical background, researchers target the studies on candidate genes which participate in the circadian clock mechanism. The circadian clock genes *per*, *cry*, *tim*, (Saunders 1998, Chen et al. 2006, Collins et al. 2005) were found to be important for correct function of photoperiodic clock system.

Several genes promotes different expression pattern under long day and short day conditions. Cyclic phenotype of *per*, *Clk*, *cry*, *Pdp1* and *tim* is influenced by photoperiodic regime (Goto and Denlinger 2002, Stehlik et al. 2008, Kobelkova et al. 2010, Dolezel et al. 2008), but the results are specie specific and often give a contrary results. In these studies it is very important to measure and compare exactly the same tissues, because it has been shown, that in the head there are many peripheral circadian oscillators with partial or absolute autonomy (Page and Koelling 2003). Different expression settings of these oscillators can influence the results of such gene expression quantification.

To induce diapause state, there must be certain number of days with respective photoperiod. The number of these days is registered by photoperiodic counter (Kostal 2006). When the number of these days reaches specific threshold, the photoreceptive neurons translate the information by modulating output pathway signalization. The number of days is called *required day number* RDN and is specie specific (Saunders 1971). The induction of diapause is increased by length of *sensitive photoperiod* (SP), which is period in which the RDN is counted. The length of this SP can be changed by other

environmental factors, such as temperature, diet, social interactions, etc. (Saunders 2002). Molecular mechanism of the photoperiodic counter is completely unknown. It is obvious that the counter works in cooperation with photoperiodic clock system. The photoperiodic clock weight the situation and make a decision (yes/no), whereas the counter summarize these answers and in appearance to other conditions send the signal to downstream mechanisms (VazNunes 1990).

Moreover, experiments with mutant flies or photoperiodic modulation of expression cannot bring convincing evidence about involvement of these genes in photoperiodic clock mechanism. It is very hard (or almost impossible) to distinguish between effect of abolished circadian clock system on photoperiodic phenotype and direct involvement of circadian clock genes in photoperiodic mechanism. The only solution of this complicated relationship is determination of respective photoperiodic clock residing neurons and quantification of expression in single cell recording experiments.

To aim this issue, the microsurgical interventions were made and reveal *dorsolateral protocerebrum* to be essential for photoperiodic clock function in *Anthereapernyi* (Williams 1969). The later experiments identified these cells to be site of circadian clock system connected to *corpora allata* and PTTH (Sauman and Reppert 1996). These PER expressing neurons regulate hormonal release of the mentioned two neuro-secretory glands. Shiga et al. (2003) showed, that ablation of these PER expressing neurons prevent induction of diapause in *Manducasexta*. In flies, there were found five groups of *per* and *tim* expressing cells. Both the genes have the expression maxima during dusk, which suggest their involvement in photoperiodic clock mechanism (Muguruma et al. 2010). Even more all five groups co-express *Pigment dispersing factor* (PDF) which play an important role for downstream signalization in circadian clock mechanism (Shiga and Numata 2009). Ablation of lateral neurons abolish circadian behavioral phenotypes and ability to discriminate the photoperiod. All these results indicate that these five neuronal groups act as a multi-oscillatory cooperative system and all of them participate in diapause induction (Muguruma et al.2010).

Results of RNAi experiments show that *per* down-regulation in *Riptortuspedestris* disables diapause induction whereas *cycle*RNAi induced it, their effect on JH secretion was documented as well (Ikeno et al. 2010). In spite of divergent role of circadian clock genes in various peripheral organs and central, circadian clock mechanism it is hard to decide whether observed effect of systemic RNAi is due to disrupted photoperiodic clock mechanism, circadian clock mechanism, some tissue specific role of that gene, or signalization pathway.

Even though there are several examples documenting interconnection of circadian and photoperiodic clock, the direct involvement of circadian clock gees in photoperiodic mechanism is till discussed question which gave to rise of several theories.

2.2. Possible role of circadian clock genes in photoperiod measurement

It has been predicted that the photoperiodic clock and counter reside in the brain, but there are no experiments determining neurons responsible for photoperiod measuring (Numata et al. 1998). First theories and experiments have identified same general properties of photoperiodic clock mechanism and raise a possibility of direct circadian clock mechanism involvement. Crosstalk of both circadian clock and photoperiodic clock mechanism is intensely discussed question. Bunnig postulates the theory that the circadian clock genes underlie photoperiodic clock mechanism in 1936. The Bunnings hypothesis was then elaborated by Pittendrigh to the three main models of photoperiodic clock mechanism: external coincidence, internal coincidence and resonance model (Schiesari et al. 2011).

Bunnings hypothesis is based on occurrence of short photoperiodic sensitive period in circadian manner. This dial responsive window is in the time of sun-dusk and the difference between long (light is still on) and short (light is already off) day than can be recognized. This hypothesis works with dual role of light, which is important for entrainment and photo-induction. Application of Nanda-Hamner behavioral test shows that the hypothesis is relevant for some insect species, such as *Sarcophagaargyrostoma, Megouravbicie, Aphis phabae, and some Lepidoptera species* (Saunders 2011). Behavioral experiments with light pulse night phase interruption reveal that there are two sensitive time points during the night. In early night it is called the time point A, whereas in the late night it is called time point B. According to external coincidence model, through these two time points organism measures length of night more than length of day (Saunders 1975). Weak point of this hypothesis is that it cannot explain some experimental observations of diapause incidence in extremely long light phase of day or lacking diapause entry in constant dark.

Another theory of photoperiod measuring is internal coincidence model. In contrast to external coincidence model, it proposes necessity of two or more circadian oscillating cycles which influences each other. The light signal has a single role of entrainment in this model (Pitendrigh 1966). One oscillator is dawn (morning) and the other is dusk (evening). The changing external light conditions change the relation of these two oscillators and results in different regulation of downstream gene expression. Recent works on *D. melanogaster* identified two groups of neurons responding to morning or evening light conditions and regulates morning or evening activity cycles (Meigen by Grima*et al*. 2004, Stoleru*et al*. 2004). The last discussed model called circadian resonance model is based on multi-oscillatory system too. This model proposes that the circadian clock system itself is not involved in measurement of night length, but its presence in organism is necessary for proper function of photoperiodic clock. The performance of the circadian clock system is a function of its proximity to resonance (Vas Nunes and Veerman 1982).

It is obvious that the involvement of circadian clock genes in photoperiodic clock mechanism was (and still is) intensely discussed. Recent research held mainly on non-model insect species brings novel information in this research field (Saunders and Bertosa 2011, Kostal 2011). In experiments

with *per* mutant strains of *D. melanogaster*, it was shown that despite the flies were absolutely arrhythmic they were still able to distinguish between long and short day conditions but the mutants have shortened critical photoperiod (Saunders 1989). These results suggest that the *per* gene itself is not involved in photoperiodic mechanism, but circadian clock cycling is essential for correct photoperiod measurement (Saunders et al. 1998).

Another approach of circadian clock gene involvement study is comparison of sequence variability between populations occupying different habitats. Allelic variant of *per* and *Tim* has been identified to have different effect on diapause incidence (Lankinen and Forsman 2006, Han and Denlinger 2009, Sandrelli et al. 2007).

Several circadian clock genes have been shown to have changed expression level or expression pattern under different photoperiodic conditions. These experimental evidences from various insect species strongly suggest involvement of circadian clock genes in photoperiodic clock response and diapause induction (Dolezel et al. 2008, Muguruma et al. 2010, Koga et al. 2005). By the analogy with variability in circadian clock mechanism, we can assume that different species would have different photoperiodic mechanisms as well. It is possible that functional relationship of circadian clock expression and photoperiod is assessed species dependently (Kostal 2011). Results of these experiments suggest that circadian and photoperiodic clocks are two separated mechanisms that cooperate and can influence each other in insect (Saunders 2010, Kostal 2011). The selection of a suitable model organism is a key factor in further investigation and understanding of circadian and photoperiodic clock crosstalk.

Complicated relationship between both clock mechanisms makes the investigation of photoperiodic clock mechanism extremely hard. These complications and missing strong phenotype in model organism cause, that the true mechanism still remains to be solved. In contrast to the central mechanism, its output signalization and regulation of specific organ role in photoperiodic manner seems to be more promising.

2.3. Peripheral photoperiodic clock mechanisms

Different photoperiodic regimes influence wide range of physiological functions in organism. Photoperiodic clock system regulates function of many various tissues and organs by specific manner (Saunders 2002). In similar system of circadian clock system, there is a central pacemaker hormonally signaling to peripheral clock oscillators. These oscillators regulate function of tissue and organs in a specific way (Tomioka et al 2012(Dibner et al., 2010; Saini et al., 2011)).

Recent researches suggest that several circadian clock genes are expressed differently in peripheral tissues under various photoperiodic conditions. The circadian clock genes could serve like transducers of photoperiodic signal in periphery and regulate tissue specific expression in response to specific photoperiodic conditions (Dolezel et al. 2008, Ikeno et al. 2008). Experiments showing clear photoperiod induced phenotype are relatively reared. It is necessary to have experimental model which

enable us to clearly distinguish photoperiodic phenotype in peripheral tissues. The typical phenotypes are synthesis of storage proteins and metabolic adaptation and synthesis of protein necessary for activation of reproductive tissues.

In *Pyrrhocorisapterus*, there were described differences in peripheral tissue expressions under impact of long day and short day photoperiodic regimes. Measured expression of circadian clock genes in gut tissue reveal strong difference in *cry2* (mammalian type of *cryptochrome*) and $Pdp1_{iso1}$ (specific isoform of *Pdp1*). Expression of these genes was shown to be regulated by receptor of juvenile hormone (*Methoprene tolerant*) and by two circadian clock genes (*Clock* - *Clk*, *cycle* - *cyc*). All these three genes are essential for maintaining of photoperiod specific differences (Bajgaret al.2013). This new results indicate that there is a complicated mechanism of circadian clock gene regulation in the gut. Even more striking is observation, that *cry2* and $Pdp1_{iso1}$ genes are expressed in the same individuals differently in fat body tissue. Expression in the fat body shows circadian cyclic pattern and weak or no response on changing photoperiod in contrast to the gut. In addition circadian clock genes *cyc*and *Clk* are in the fat body necessary for maintaining of cyclic expression pattern of *cry2* and $Pdp1_{iso1}$ (Bajgar et al. unpublished data). The fact that circadian clock gene expression in periphery is influenced by photoperiod was documented several times before. For example Dolezel et al. (2008) show that *per* and *Pdp1* expression vary in the fat body in diapause and reproductive individuals. Another observation was obtained by another group studying photoperiodic phenotype in *heteroptera*specie *Riptortuspedestris*. According to their results, down-regulations of circadian clock genes *cyc* and *per* have neither impact on ovarian development nor effect on JH signalization (Ikeno et al. 2010).

It is obvious, that the circadian clock genes play an important role in peripheral tissue regulation and coordination under both circadian and photoperiodic light condition. Investigation of this phenomenon in species promoting both photoperiodic and circadian phenotype can help us to understand to real function of circadian clock genes in tissue specific manner.

2.4. Regulation of peripheral tissues by central photoperiodic clock mechanism

If we concede the existence of circadian clock based mechanism which regulates organ specific physiology in photoperiod responding way, there must be some mechanism of signal transduction. This signal should be able to spread through organism simply and direct different expressional changes in various tissues, because of their distinct functions (Saunders and Bertossa 2011). Although we still don’t know complete mechanism of signal transduction, we have already pretty strong data documenting photoperiodic regulation of several physiological functions.

The first step in photoperiodic clock mechanism investigation, was identification of neuronal clusters responsible for processing of photoperiodic output signal. Transplantation experiments in two insect species *Anthereapernyi* and *Pyrrhocorisapterus* determine cells of *pars intercerebralis* and *pars lateralis* to be essential in photoperiodic phenotype regulation (Shiga et al. 2003, Hodkova 1976). These two neuronal groups directly regulate neurosecretory glands *corpora cardiaca* and *corpus allatum*, which release important hormones. One of these hormones, juvenile hormone (JH), plays an important role in induction of adult diapause in insect (Hodkova 1976). Ablation of cells of *pars intercerebralis* and neurosecretory gland *corpus allatum* significantly influence expression level of circadian clock genes in the gut (unpublished data) as well as in the fat body (Dolezel et al. 2008). These experiments, for the first time showed the circadian clock gene response on hormonal signalization in insect and can thus elucidate a possible photoperiodic clock output signalization pathway.

Recent discovery of JH receptor in *P. apterus*enable us to perform series of knockdown experiments and applications of JH analog Methoprene (Miura et al. 2005). Receptor of JH (*Methoprene tolerant* gene - *Met*) is a member of bHLH-PAS (*basichelix-loop-helixPer-Arnt-Sim* protein) domain transcription factor family. Willingness of JH receptor to dimerize with another transcription factor is dependent on JH presence (Charles et al. 2011). Recently, the ability of *Met* to dimerize with circadian clock gene *cyc* has been documented in *Aedesaegipti* (Shin et al. 2012). Heterodimer of MET-CYC proteins is able (similarly to CYC-CLK heterodimer in circadian clock mechanism) to bind e-box promoter region and acts like a transcriptions regulator (Miura et al. 2005).

From the mentioned facts we can conclude, that the general mechanism of photoperiodic clock information to peripheral tissues is based on hormonal signalization. Hormones released from neurosecretory cells interact with receptors and transcription factors in tissue specific manner and regulate expression of circadian clock genes. The circadian clock genes are necessary for maintaining of photoperiod induced changes and regulation of downstream expression. The JH role in this signalization is not exclusive. It was observed several times, that photoperiodic phenotype in insect can be regulated through other hormones, such as ecdyson, prothoracicotropic hormone, and insulin (Denlinger et al. 2005, Shiga et al. 2003, Tatar et al. 2001).

3. Other circadian clock gene dependent processes

The circadian clock gene function in circadian clock mechanism and photoperiodic clock mechanism is well documented (see Saunders 2011 for overview). Beside these generally respected functions, there is wide range of recently discovered processes in which the circadian clock genes play an important role.

It has been already reliably proved that circadian clock gene *per* is overexpressed in mouse cerebral cortex after sleep deprivation. In addition, high levels of PER2 negatively regulate sleep deprivation recovery (Franken et al. 2007). PER2 is a possible connection between circadian clock mechanism, sleepiness, and mood disorders.

Circadian clock genes were recently associated with metabolism and its regulation. Cyclic expression pattern of many genes connected with metabolic processes (glucose transport, gluconeogenesis, lipolysis, etc.) imply that there is direct regulation by circadian clock genes (Kohsaka and Bass 2007). Well documented isconnection between circadian clock genes *Clock, Bmal1* and metabolic function through signalization of *REV-ERBa*. Also nutrient intestinal uptake is influenced by mutation in circadian clock genes and peripheral circadian clock disruption in the gut (Balakrishnan et al 2012). One another interconnection between circadian clock and metabolism is *Nocturnin*(*Noc*) gene. *Noc* gene responsible mainly for post-transcriptional modifications, shown robust rhythmic cycling and is regulated by circadian clock mechanism. Mice lacking *Noc*, have changed rate of main metabolic pathways and suffer from many metabolic diseases. Therefore, *Noc* is a potential key post-transcriptional mediator in the circadian control of many metabolic processes (Stubblefield 2012).

Disrupted circadian rhythmicity was connected with several systemic pathologies such as inflammation, metabolic diseases, cell cycle, and cancer (Yu and Weaver 2011). Increased number of activated cytokines has been observed as a result of missing *cry* gene and thus disrupted circadian clock mechanism. This reaction is caused by missing CRY protein, which leads to continuous activation of Nf-κB signalization and increased expression of pro-inflammatory cytokines in mammals (Narasimamaurthy et al. 2012).

Drosophila *cry* usually acting as a synchronizing element in circadian clock was recently described to be involved in light perceiving and vision. Ability to change its conformation after light exposure, and many conserved binding domains, determine CRY to be involved in many physiological processes (Mazzotta et al. 2013).

The best evidence of circadian-clock gene multi-functionality is involvement of *cry* in magnetoreception. Ability of perception of the Earth´ magnetic field and utilization of this information for navigation or simple passive lateralization was occurred in many animal species. CRY was proposed to be a primary magnetoreceptive molecule (Gegear et al. 2008) in light dependent kind of magnetoreception. This protein charged by photo-induction use the specific energetic state and

undergoes radical-pair reaction. The yields of this reaction are CRY molecules in singlet and triplet energetic states. It was proposed that the radical-pair reaction of CRY is moderated by magnetic field of the Earth and could serve as a signal for further transduction of magnetic field signal (Muller and Ahmad 2011).

The described diversity in circadian clock mechanisms and wide variety of other functions in which the circadian clock genes are involved can be explained by gene duplications and loses during evolution. It is generally accepted, that gene duplication is a way how genes can escape from selection pressure and developed in some new functions (Yuan et al. 2007). Since circadian clock genes are effective transcription factors, containing many binding domains, and willingly creating complexes and clusters, they are predetermined to involve in some new signalization or regulation.

Introduction summary

Circadian clock genes and their roles in insect were discussed in previous paragraphs. These genes occur in wide range of species from unicellular bacteria to human. The circadian clock genes has been determined to be crucial components of several physiological processes such as central clock mechanism, peripheral clock mechanism, photoperiodic signal transduction, photoperiod influenced peripheral tissue regulation, metabolism, and immunity modulation. Their highly conserved amino-acid sequence and similarity of the clock mechanism in phylogenetically distant species enable us to generalize result even from research of insect model organisms to mammals. It was mentioned many times, that the main insect model organism *D. melanogaster* is not fully satisfying. The*Drosophila* clock mechanisms differ significantly from other species and regulatory pathways directing peripheral oscillators are practically missing. Although the components of mechanisms are identical, the interconnection of genes is slightly different in both central and peripheral oscillators. For that reason raises requirement of novel insect model organism which facilitate the study of circadian clock genes and their diverse function. One example of such new organism is *P.apterus*. In this bug, we can study both circadian and photoperiodic clock mechanisms. In addition it is possible to make surgical interventions to identify neuronal connections and signalization to peripheral tissues. Since *P. apterus* physiology has been studied for decades, we can introduce modern molecular techniques into already known and well established physiological environment. Investigation of circadian clock involving processes can reveal us surprising discoveries applicable to mammals.

The aims of this study

The main aim of our work was to investigate involvement of circadian clock genes in photoperiodic response in non-model insect species *P. apterus*. We have focused on expression patterns of circadian clock genes in peripheral tissues which are responding to photoperiodic and circadian clock signals. By using qRT-PCR, we wanted to characterize expression patterns of circadian clock genes in tissue respecting manner (chapter 4). Further we wanted to examine pathway downstream from photoperiodic clock mechanism which regulates expression of circadian clock genes in the gut. We used wide range of techniques (RNA interference, ablations, hormone analogue application) to uncover the pathway on all its levels. We characterized effect of *pars intercerebralis* and *corpus allatum* in long and short day photoperiodic conditions on peripheral clock gene expression. To simulate activation of *corpus allatum,* we topically applied juvenile hormone analogue and detect changes involved by this intervention in the gut. For better understanding of regulatory mechanism of JH signalization and circadian clock gene role in peripheral tissues, we used RNAi to artificially down-regulate expression of involved genes (chapter 1, 2 and 4). In addition, we put together results from three cooperating research groups to characterize mechanisms of JH signalization pathway regulating various physiological processes in distinct tissues differently in *P. apterus* (chapter 3). At last, we characterized circadian clock gene interaction in central clock mechanism in a detail by using luciferase reporter assay. We compared and functionally characterize promoter sequences of circadian clock gene *timeless* (*tim*) in wild type (*wt*) and *non-photoperiodic diapause (npd)* mutant flies of *Chymomyzacostata*. We determined regulatory sequences necessary for correct *tim* regulation and compared expression profiles of circadian clock genes in *npd*and *wt* flies (chapter 5).

Following chapters are considering these issues:

1. Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling
2. Endocrine system regulation of circadian clock genes *cry2* and $Pdp1_{iso1}$expression in the insect gut
3. Insect reproduction and metamorphosis depend on distinct branches of juvenile hormone signaling
4. Expression patterns variability of *cry2* and $Pdp1_{iso1}$ genes in response to reproductive or diapause photoperiodic conditions suggests their pleiotropic tissue specific effect in *P. apterus*
5. Functional molecular analysis of a circadian clock gene *timeless* promoter from the *Drosophilid*fly *Chymomyzacostata*

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Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling

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In temperate regions, the shortening day length informs many insect species to prepare for winter by inducing diapause. The adult diapause of the linden bug, *Pyrrhocoris apterus*, involves a reproductive arrest accompanied by energy storage, reduction of metabolic needs, and preparation to withstand low temperatures. By contrast, nondiapause animals direct nutrient energy to muscle activity and reproduction. The photoperiod-dependent switch from diapause to reproduction is systemically transmitted throughout the organism by juvenile hormone (JH). Here, we show that, at the organ-autonomous level of the insect gut, the decision between reproduction and diapause relies on an interaction between JH signaling and circadian clock genes acting independently of the daily cycle. The JH receptor Methoprene-tolerant and the circadian proteins Clock and Cycle are all required in the gut to activate the *Par domain protein 1* gene during reproduction and to simultaneously suppress a mammalian-type *cryptochrome 2* gene that promotes the diapause program. A nonperiodic, organ-autonomous feedback between Par domain protein 1 and Cryptochrome 2 then orchestrates expression of downstream genes that mark the diapause vs. reproductive states of the gut. These results show that hormonal signaling through Methoprene-tolerant and circadian proteins controls gut-specific gene activity that is independent of circadian oscillations but differs between reproductive and diapausing animals.

reproductive diapause | photoperiodism | basic helix-loop-helix protein | oogenesis

To cope with adverse winter conditions, animals either migrate or minimize their metabolism and hibernate or diapause (1). Animals including insects anticipate these annual rhythms by measuring the changes in night or day length (i.e., photoperiod) through a seasonal clock whose mechanism has yet to be elucidated (2, 3). The hallmarks of diapause in insects such as the linden bug, *Pyrrhocoris apterus*, and the bean bug, *Riptortus pedestris*, include cessation of reproduction (4–6) and changes in the physiology of the digestive system (7) and the fat body (8). The arrest is induced by short days and results in small diapause ovaries. Conversely, long days promote ovarian maturation through the action of juvenile hormone (JH), produced by the corpora allata glands (9–11).

JH is an insect sesquiterpenoid that controls reproduction (12) and entry into metamorphosis (13). The connection between JH and reproductive diapause is well documented in various species (14). Application of the JH-mimicking analogue methoprene to diapausing *P. apterus* or *R. pedestris* bugs is sufficient to terminate diapause and induce ovarian growth (6, 15). Endogenous JH or added methoprene act through the Methoprene-tolerant (Met) protein to prevent premature metamorphosis in *P. apterus* juveniles (16). Met is a transcription factor of the basic helix–loop–helix Per-ARNT-Sim (bHLH-PAS) family (17), and it has been characterized as a JH receptor (18, 19). JH-dependent interaction between Met and another bHLH-PAS protein, FISC [synonymous to Taiman (Tai)_in *Drosophila melanogaster*; FlyBase], have been implicated in oogenesis of *Aedes aegypti* mosquitoes (20). Recently, *A. aegypti* Met and the bHLH-PAS circadian clock protein Cycle (Cyc) have been shown to dimerize and activate circadian rhythm-dependent gene expression in response to JH (21).

Whether photoperiodic regulation of seasonal diapause/reproduction timing involves the circadian clock is still debated (2, 22–24). Clinal polymorphism of the circadian gene *timeless* was observed in *D. melanogaster* (25, 26), and diapause in another drosophilid fly, *Chymomyza costata*, is altered by a *timeless* mutation (27, 28). However, whether these *timeless* mutations affect a central "photoperiodic clock" in the brain or compromise the execution of diapause in peripheral tissues remains unknown. A systemic RNAi-mediated knockdown of *cyc* in reproductive *R. pedestris* under long-day (LD) conditions switched the bugs into a diapause mode, whereas *period* (*per*) and *cryptochrome* (*cry*) RNAi terminated diapause and induced reproduction in adults experiencing short days (6, 29, 30). These data led the authors to propose that the three circadian genes, *cyc*, *per*, and *cry*, constituted the photoperiodic clock of *R. pedestris* (6, 29, 30). An alternative explanation is that the circadian clock genes have pleiotropic functions, one of which is to regulate the seasonal physiology by acting downstream of a presently undefined photoperiodic clock (22, 31).

To address the role of circadian genes in the regulation of diapause, we examined expression of circadian genes in the gut of reproductive and diapausing females of *P. apterus*, a species with robust and well characterized diapause biology (8, 32). We discovered an organ-autonomous regulatory feedback between Cryptochrome 2 (Cry2) and another circadian clock component, a basic leucine-zipper transcription factor, Par domain protein 1, isoform 1 ($Pdp1_{iso1}$) (33). We show that *cry2* represses $Pdp1_{iso1}$ and triggers a diapause-specific genetic program in the gut, whereas $Pdp1_{iso1}$ counteracts *cry2* and promotes the reproductive state of this organ independently of the daily cycle. Induction of $Pdp1_{iso1}$ and suppression of *cry2* transcription by JH mimic require the JH receptor Met and the circadian proteins Clock (Clk) and Cyc. Therefore, our data indicate organ-autonomous, yet noncircadian, involvement of clock genes and hormonal signaling in diapause regulation.

Author contributions: D.D. designed research; A.B. and D.D. performed research; M.J. contributed new reagents/analytic tools; A.B. and D.D. analyzed data; and M.J. and D.D. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. 1563012 ($Pdp1_{iso1}$), 1563013 ($Pdp1_{iso2}$), 1562940 (*cyc*), 1562939 (*Clk*), 1563010 (*cwo*), 1563014 (*tgo*), 1562945 (*tai*), 1563016 (*lip*), 1563019 (*def*), 1563020 (*est*), 1563024 (*sod*), and 1563021 (*tfl*)].

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Manuscript in preparation

Endocrine regulation of non-circadian behavior of circadian genes in insect gut

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Abstract

The linden bug *Pyrrhocoris apterus* exhibits a robust diapause response to photoperiod. Photoperiod strongly affected the basal levels of circadian gene transcripts in the gut, via the neuroendocrine system. *cryptochrome 2* (*cry2*) mRNA level was much higher in diapause promoting short days (SD) than in reproduction-promoting long days (LD), while *Par Domain Protein 1isoform1* ($Pdp1_{iso1}$) mRNA level was higher in LD than in SD. The effect of photoperiod on gene expression was mediated by the neurosecretory cells of the *pars intercerebralis* (PI) and the juvenile hormone (JH) producing *corpus allatum* (CA). In LD-females, CA ablation resulted in SD-like levels of gene transcripts, while PI ablation had only little effect. Conversely, in SD-females, CA ablation had only little effect, while PI ablation resulted in LD-like levels of gene transcripts. Thus, the CA is responsible for LD-like characteristics of gene expression in reproducing females and the PI is responsible for SD-like characteristics of gene expression in diapausing females. Simultaneous ablation of both PI and CA revealed two roles of PI in SD-females: (1) the inhibition of CA, and (2) a weak CA-independent stimulation of *cry2* mRNA. Overall, our results indicate that the peripheral circadian gene expression in the gut reflects rather the physiological state of females (with respect to diapause or reproduction) than the external light-dark cycle.

Manuscript in preparation

Insect reproduction and metamorphosis depend on distinct branches of juvenile hormone signaling

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Abstract

Juvenile hormone (JH) is one of the most important insect hormones regulating progress of many physiological functions. JH regulates larval development, metamorphosis, ovary maturation and reproduction (Wheeler and Nijhout 2003). It has been shown recently, that JH hormone and bHLH PAS (*basic helix–loop–helix Per-Arnt-Sim*) transcription factors *Methoprene tolerant* (*Met*), *cycle* (*cyc*) and *Clock* (*Clk*) are essential for photoperiodic regulation of gut physiology in *P. apterus* (Bajgar et al. 2013). In this article, we proved that JH signal, JH receptor (*Met*), and transcription factor *Krüppel homolog 1* (*Krh1*) are essential for maintenance of larval development, while JH signal, JH receptor (*Met*) and transcription factor *taiman* (*tai*) are necessary for ovary development. Effect of the other transcription factors from bHLH PAS family were experimentally excluded from these mechanisms. Based on these dates, we propose mechanism of JH signal diversification in *P. apterus* and suggest that the crucial component is specificity of *Met* binding partner.

Manuscript in preparation

Expression patterns variability of *cry2* and *Pdp1$_{iso1}$* genes in response to reproductive or diapause photoperiodic conditions suggests their pleiotropic tissue specific effect in *P. apterus*

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Abstract

Circadian clock genes function and their involvement in circadian clock mechanism, photoperiodic clock mechanism, and other physiological processes has recently become a frequently discussed question. In our previous research, we described the role of *cryptochrome2* (*cry2*, mammalian type) and *Par domain protein 1* (*Pdp1$_{iso1}$*) genes as transcription factors directing gut physiology into diapause or reproductive state. In addition, we showed involvements of the JH signaling (*Methoprene tolerant* - *Met*) and two bHLH-PAS circadian clock genes *cycle* (*cyc*) and *Clock* (*Clk*) in photoperiodic signal transduction in the gut (Bajgar et al. 2013). Here, we present diurnal expression profiles of *cry2* and *Pdp1$_{iso1}$* genes in gut and fat body in response to reproductive and diapause photoperiodic regimes, respectively. The data suggest different tissue-specific roles of both observed genes. In addition, results from knockdown experiments of transcription factors (*Met*, *cyc*, *Clk*) indicate that both *cry2* and *Pdp1$_{iso1}$* are involved in completely different tissue-specific molecular mechanisms, and their role in an individual organism is pleiotropic. Expression analysis of above-mentioned transcription factors was made, and their reciprocal effect was determined.

Functional Molecular Analysis of a Circadian Clock Gene *timeless* Promoter from the Drosophilid Fly *Chymomyza costata*

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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. This article presents a detailed functional analysis of the *tim* promoter from the drosophilid fly, *Chymomyza 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 2 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. Striking differences were found in expression profiles for *tim*, *per*, and *vri* between *wild-type* and *npd*-mutant individuals.

Key words *timeless* promoter, E-box, *Chymomyza costata*, circadian and photoperiodic clock, *Drosophila* S2 cells, *timeless* null mutation

According to the current model, the *Drosophila* circadian clock system relies on the existence of 3 interlocked feedback loops (reviewed by Tomioka and Matsumoto, 2010; Hardin, 2005; Hall, 2003). The central loop consists of the genes *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), and *cycle* (*cyc*). Transcription of *per* and *tim* genes is activated by the protein complex dCLK/CYC. PER and TIM proteins then repress transcription of their own genes by binding to the dCLK/CYC dimer. The temporal delay between the transcription peak of *per* and *tim* mRNA and the accumulation of PER and TIM proteins in the cytoplasm and their subsequent entry into the nucleus is ensured by the action of 2 kinases, DOUBLETIME and SHAGGY. The second loop comprises the *Clk*, *cyc* genes, and 2 other transcription factors, *vrille* (*vri*) and *Par Domain Protein 1* (*Pdp1*). The dCLK/CYC complex activates the expression of both *vri* and *Pdp1* genes, and the resulting VRI and PDP1 proteins in turn regulate the rhythmic transcription of *dClk*. The

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Final conclusion – contribution of particular chapters

This dissertation consists of five main chapters. Each of these chapters was designed like a self-sufficient article. Two of them were already accepted and are published in respected journals. The other three chapters are already prepared for publication or in a final adjustment. Most of the presented work is based on my own results obtained in the laboratory of molecular chronobiology, or raised from my collaboration with members from laboratories under leadership of prof. MarekJindraCSc. and Doc. RNDr. Magdalena HodkovaCSc..

One of the contributions of this work is implementation and establishment of molecular biological approaches in research of photoperiodic clock in non-model insect species*P. apterus.* This species is very useable for chronobiological research, mainly for its strong photoperiodic and circadian phenotype and very simple manipulation and raring. RNAi application has been shown to be working in this species and thus experimental manipulation of gene expression is possible. Nowadays the transcriptome and genome of this bug is in analysis, which would significantly facilitate the molecular methods and their applications. Even more, knowledge of *P. apterus* physiology (which is object of physiological research for more than fifty years) and ability to make surgical interventions makes this bug very potential for further research of clock genes and mechanisms they are involved in.

The presented data can be summarized in several new observations and findings. Here, I would like to mention the most interesting results of each chapter and display a cross-link between them. In **chapter one,** we observed different expression level of genes *cryptochrome 2* (*cry2)*and *Par domain protein 1 isoform1* (*Pdp1*$_{iso1}$) in the guts of individual held under different photoperiodic conditions (long and short day conditions). We detected, that these expression patterns are under control of juvenile hormone (JH) signalization. Direct involvement of JH receptor *Methoprene tolerant*(*Met*) and circadian clock genes *cycle* (*cyc*) and *Clock*(*Clk*) in expression regulation of *cry2* and *Pdp1*$_{iso1}$ in the gut were proved. Further, we exclude several other transcription factors containing bHLH PAS binding domain. *cry2*and *Pdp1*$_{iso1}$ are in reciprocal relationship and negatively influence expression of each other in the gut. In addition, genes *cry2* and *Pdp1*$_{iso1}$ act like a transcription factors in the gut directing expression of several target genes with relevant impact on gut function typical for reproductive or diapause state. The whole mechanism is self-autonomous and works independently from central clock mechanism signals from central nervous system. Revealed mechanism is based on circadian clock genes regulation of the peripheral tissue physiology in photoperiod responding manner. Although, the system was well established, there were still some uncovered elements influencing the system. In particular, expression level of *cry2* is not the same in short day conditions and after experimental disruption of JH signalization in long day conditions. We suggested that it is due to missing of some positive element, which stimulates expression of *cry2* in short day conditions (summarized in Fig. 5).

Photoperiod induced regulation of gut physiology, mentioned above, is further analyzed in **chapter two.** In this part, we characterized involvement of neurosecretory cells of *pars intercerebralis*(*PI*) and neuroendocrine gland *corpus allatum*(*CA*) in transduction of photoperiodic signal to peripheral tissues in *P. apterus*. Chirurgical extirpation of both of these tissues influences level of JH hormone in hemolymph, which results in changes in expression of *cry2*/*Pdp1*$_{iso1}$ expression ratio in the gut. This change influences expression of the previously documented target genes. Based on the presented data, we suggest that the predicted positive signal, activating *cry2* expression in short day conditions, is produced by cells of *PI*. The molecule carrying that signal still remains unknown (summarized in Fig. 5).

Subsequent, **chapter three,** concerns JH hormone signalization in *P. apterus*. This hormone is the main product of neuroendocrine gland *corpus allatum* and was associated with regulation of several physiological functions. In collaboration with other two groups, we interconnect results from studies concerning influence of JH hormone in ecdysis and metamorphosis, in gut physiology regulation, and in ovary maturation. We compared the mechanisms of JH regulation in these three distinct tissues and physiological processes in the one species. We found, that common component of JH signalization in all analyzed tissues is *JH-receptorMet*. The *Met* is through JH presence stimulated to bind another bHLH PAS transcription factor and in heterodimer regulate expression of tissue specific genes.While *Met* is necessary component of JH signalization in all analyzed tissues, there is tissue specificity in another bHLH PAS transcription factor. Based on these data we propose, that JH hormone released from *corpus allatum* can regulate distinct molecular mechanisms through JH receptor *Met* and its tissue specific partner *(Taiman- Tai*,*Clk,*and*cyc,* or another yet unidentifiedbHLH PAS transcription factor). The tissue specific selection of *Met* partner can explain diverse tissue specific response on the same signaling molecule (JH) (summarized in Fig. 5).

Understanding the mechanism described in chapter one and two residing in the gut and mechanism of JH signal diversification led us to an idea compare expression patterns of *cry2* and *Pdp1*$_{iso1}$ genes and their regulation in two different tissues (gut and fat body (**chapter four**). From preliminary results we expected that the expression will be fluctuating during a day, so we measured their daily expression profiles. While dial expression pattern of both characterized genes shows no daily fluctuations in the gut, but differ between long day and short day regimes, the situation in the fat body is different. Expression profile of both genes is fluctuating with high amplitude in the fat body, while photoperiodic regime has eitherweak (*Pdp1*$_{iso1}$) or no (*cry2*) effect. These experiments implicate, that both genes have different roles in these two tissues. In addition, results from RNAi studies of *cry2* and *Pdp1*$_{iso1}$ transcription factors (*cyc*, *Clk*, *Met*) reveal, that expression pattern of *cry2* in the fat body is not dependent on JH signalization, and expression in both tissues is regulated by circadian clock genes *cyc* and *Clk*. Both these transcription factors are necessary for correct expression pattern, but regulate expression differently in the gut and in

the fat body. Although it was established that *cyc* and *Clk* are necessary for maintenance of photoperiod induced difference in the gut, in the fat body are both necessary for sustaining of cyclic expression profile.

The last part of this work is article published in collaboration with AlenaKubelkova PhD (**chapter five**). In this chapter, we investigated differences between *wild type* (*wt*) and *non-diapause induction* (*npd*) mutant flies of *Chymomyzacostata*. We observed significant difference in expression profiles of several circadian clock genes in heads between *wt*and *npd* flies. In addition, we further analyzed differences in *timeless* (*tim*) regulatory sequence which were previously described. By using luciferase reporter assay and artificial modification of *tim* promoter sequence, we determined minimal regulatory sites necessary for correct expression of *tim* gene. These results suggest involvement of *tim, per, and Clk*genes in regulation of photoperiodic signal perceiving in the central nervous system.

Despite seemingly diverse topics of presented chapters, most of the work can be integrated into the one model deciphering transduction mechanism of photoperiodic signalization to the peripheral tissues and circadian clock gene involvement in tissue specific regulation in photoperiod respective manner. On the presented scheme (Fig. 5), we demonstrate suggested model of this mechanism. The model includes neurosecretory cells of *pars intercerebralis* and *corpus allatum* as the main components of transduction of photoperiodic signalization in the central nervous system. Further there is well documented hormonal signalization through juvenile hormone (JH) to peripheral tissues. This JH signal can be divided into several branches. These particular systems responding to JH signalization have the common component in JH receptor and various components in its binding partners. This system regulates expression patterns of circadian clock genes in the gut which further determine expression of target genes and functional physiology leading to reproductive or diapaus state. Although this mechanism has been well documented in the gut, it is obvious that the same genes are involved in another mechanism in the fat body and probably play a different function there. Parallel branches of JH signalization lead to regulation of larval metamorphosis and regulation of vitellogenesis. JH receptor Met is essential factor for this signalization in all three described processes, while the other bHLH PAS transcription factors (*cyc*, *Clk*,*tai*) play role always fundamentally in the one of these mechanisms.

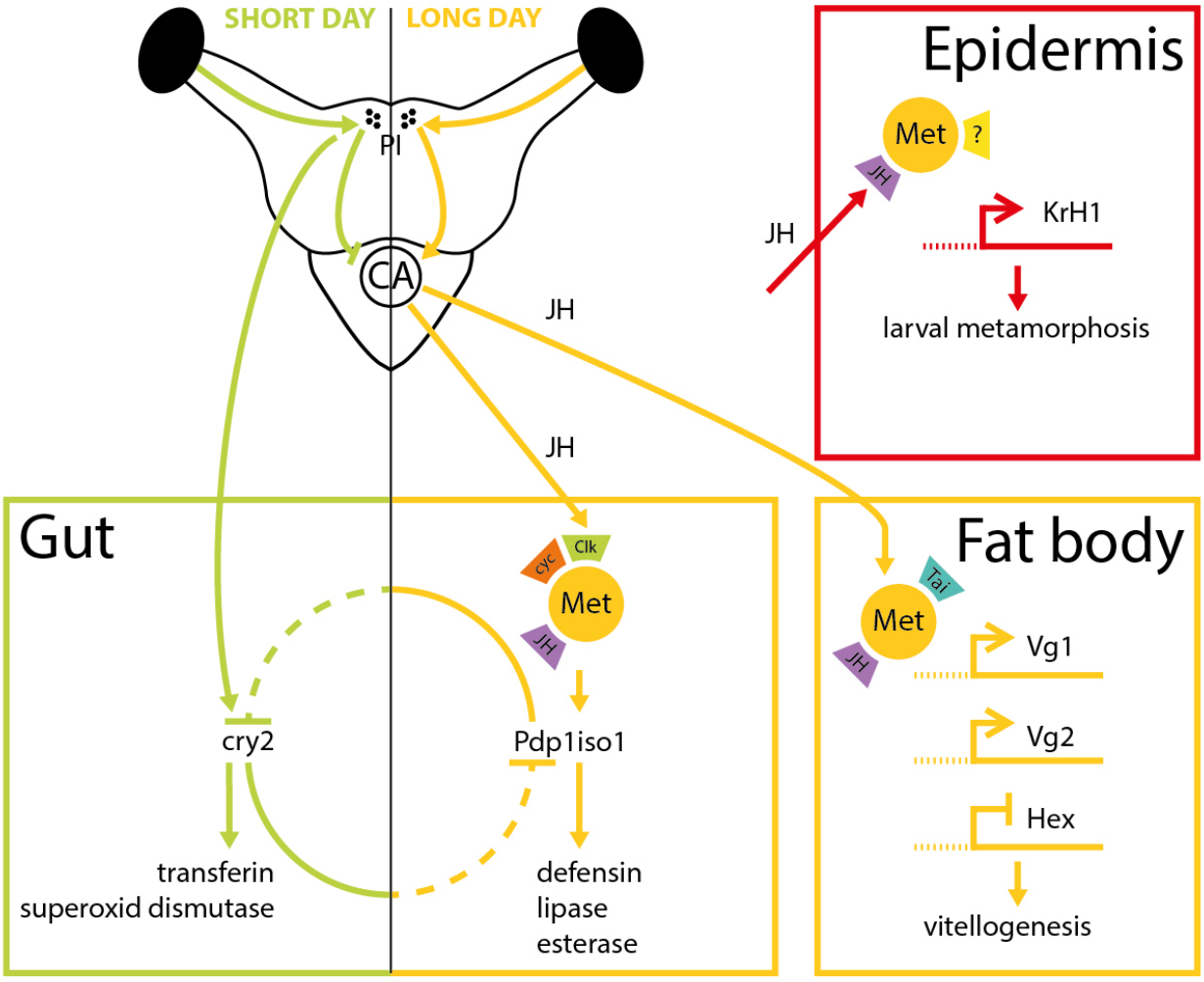

Fig. 5 Schematic overview of novel findings presented in this work. Left part displays processes typical for short day (SD) photoperiodic conditions (in green color). Cells of *pars intercerebralis* (PI) repress juvenile hormone (JH) production and release from *corpus allatum* (CA). Beside this effect, cells of PI directly stimulate expression of *cry2* in SD photoperiodic conditions in gut. The PI effect together with missing JH signal influence *cry2*/*Pdp1*$_{iso1}$ratio (*cry2* is six time more abundant) and trigger expression of genes typical for diapause state. Right part displays processes typical for long day (LD) photoperiodic conditions (yellow color). Cells of *pars intercerebralis* positively influence synthesis and release of JH from CA. JH then bind to JH receptor (*Methoprene tolerant – Met*) and tissue specific binding partner(s). *Met* together with *cycle* (*cyc*) and *Clock* (*Clk*) stimulates expression of *Pdp1*$_{iso1}$ in the gut. This effect together with no *cry2* stimulation from PI influence *cry2*/*Pdp1*$_{iso1}$ratio (*Pdp1*$_{iso1}$ is six time more abundant) and trigger expression of genes typical for reproductive state. Beside this effect, the JH binds to Met in fat body and epidermis. In the fat body *Met* binds to *taiman* (*tai*) and stimulates synthesis of *Vitellogenins*(*Vg1*, *Vg2*) which are necessary for vitellogenesis (ovary maturation). The same heterodimer repress expression of *Hexamerins* (*Hex*), typical storage hemolymph proteins. The last part (in red color) documented JH signalization regulating larval development. JH binds to *Met* which stimulates (probably together with still undefined partner) expression of *Krüppel homolog1*(*Krh1*), which is important transcription factor in directing of larvalecdysis metamorphosis. This process is photoperiod independent.

Further research direction

It was mentioned many times, that the non-model specie *P. apterus* can enable us to study several physiological functions and molecular mechanisms which cannot be investigated in the main insect model species*Drosophila melanogaster*. Elucidating molecular mechanism of each particular research objectives is very hard, because of missing regulatory and coding sequences of investigated genes. Recently emerging next generation sequencing techniques allow us to get genomes and transcriptomes from non-model species, but assembling and annotation of these sequencing data require researcher to be patient and well educated in bioinformatics. Despite these facts, the transcriptome comparison can reveal many interesting results which should be subsequently observed and experimentally proved.

Another experimental directions, is introduction of techniques such asgenome editing. Novel methods like TALENs with high efficiency of transgenic individuals production can enable us to make individuals with knockout of gene of interest, introduce and observe genetic interaction with genes from other species, or quantify expression *in situ* and in real time by using luciferase or GFP reporters. These techniques in combination with already well-established neurophysiological and behavioral methods should help us to elucidate mechanism of photoperiod clock system in central nervous system as well as photoperiodic regulation on tissue specific level.

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