Note:

Parts of this study containing or referring to unpublished data were omitted with permission of the Head of the Department of Molecular biology of the Faculty of Science, University of South Bohemia – prof. RNDr. Julius Lukeš, CSc.

University of South Bohemia in České Budějovice

Faculty of Science

Master Thesis

CIRCADIAN CLOCK OF TWO INSECT MODEL SPECIES – *Drosophila melanogaster* and *Tribolium castaneum*

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Annotation:

[redacted] The second part of this study deals with the expression pattern (both temporal and spatial) of two core clock factors known from *Drosophila*, *period* and *timeless*, in the central nervous system of the red flour beetle, *Tribolium castaneum*.

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I hereby declare that I have written this thesis by myself, on the basis of my own results and with the help of the literature resources listed.

I declare that in accordance with the § 47b of the Act No. 111/1998 Sb., as amended, I agree with publishing of the electronic version of this Master thesis in its abridged form. I agree that this electronic version will be placed in the STAG database administered by the University of South Bohemia at its websites. I am aware that this part of the STAG database is open to public.

5.1. 2010, České Budějovice ……..

Contents

1. Introduction

The research summarized in this thesis can be divided into two more or less independent parts but has one common denominator – the circadian clock system of insects. One part of this research is focused on a new insect model species: the red-flour beetle, *Tribolium castaneum.* The major aim of this study was to perform the basic analysis of the beetle's circadian clock; more specifically my task within this project was to describe both the temporal and spatial expression of the two core clock components known from *Drosophila*, *period* and *timeless*. This would give us the information necessary for further studies that should involve genetic manipulations, gene silencing and other approaches.

As to the model organisms used in this study, *Drosophila melanogaster* probably needs no long introduction. It is a principal model species that has been used in all possible kinds of modern-day biological research (particularly in physiology, genetics and developmental biology) for decades and there is no other animal for which such a wide range of research techniques and methods has been established. There is a number of reasons that predestined the fruitfly to become the organism of choice for biological research: It is small and easy to breed at quite low cost, it has a short generation time (about 10 days at room temperature) and high fecundity (females lay up to 100 eggs per day), males and females can be readily distinguished. A fruitfly has only four pairs of chromosomes (three pairs of autosomes and one sex-chromosome pair), giant polytene chromosomes can be seen in the larval salivary glands. Males do not show meiotic recombination, which facilitates genetic crossing. So called balancer chromosomes (artificially prepared chromosomes with multiple inversions) can be used to keep lethal alleles in stock in a heterozygous state without the risk of losing them due to recombination. Last but not least, the complete genome of *Drosophila* was sequenced and published in 2000 (Adams *et al.* 2000).

Much of the aforementioned applies for *Tribolium* as well: It is also easy to breed and very fertile with short generation time (aprox. 32 days at 30°C). Similarly to *Caernohabditis elegans*, RNA interference, a powerful method for gene silencing, is systemic in *Tribolium* meaning that the silencing signal spreads throughout the animal causing gene knockdown even

in tissues far from the original site of introduction of the dsRNA, it can even be passed to the offspring (Bucher *et al.*, 2002). The genome of *Tribolium* was sequenced and published in 2008 (Tribolium Genome Sequencing Consortium, 2008).

Tribolium ranks to the major pest species of stored dry goods such as grains. It causes enormous economical losses all around the world and has demonstrated resistance to all kinds of insecticides used against it so far.

1.1. Circadian clock

Although we perceive the unidirectional flow of time from past to future, our life is largely organized into 24-hrs sections of day and night. This is of course caused by the Earth's rotation around its own axis. The cyclical changes in light and temperature thus created have been influencing the living organisms ever since the life first appeared on the face of Earth. No wonder that the organisms have not only learned to react to these changes but more importantly, to anticipate them. And that is what the circadian clock system is actually used for: to help the animal plan its activities for the most appropriate time of the day and get ready for them before the actual need. This enables the animal to exploit all the possibilities to feed and mate to the fullest. The fact that such a system is very widespread, inherent to organisms ranging from bacteria to humans, suggests that its adaptive value must be high. Several experiments supporting the positive influence of biological clocks on fitness have been published so far (e.g. Ouoyang *et al.* 1998; Beaver *et al.*, 2002).

The phenomenon of the circadian (circa – about, dies – day) clocks is rather loosely defined by its three most outstanding properties: First, they are endogenous and able to 'free-run' with a period close to 24 hrs even in the absence of environmental cues (e.g. in constant darkness); second, they can be resetted or entrained by changes in environmental conditions to maintain synchrony with local time (that is why we don't experience a perpetual jetlag after a transmeridian flight). Under normal condition such entrainment occurs daily via master oscillations in the environment, particularly the solar or temperature cycle. Third, they posses a property called temperature compensation, meaning that the circadian clock keeps the same period length over a wide range of biologically relevant temperature – a feature rather unusual for a system relying on biochemical interactions.

It is probably the year 1971 that represents the milestone on the quest for describing the mechanism underlying the biological timekeeping in insects. It was then the authors Konopka and Benzer published the results of their attempts to obtain mutant fruitflies (*Drosophila melanogaster*) with abnormal circadian rhythm characteristics. They isolated three different

strains: two displayed either long (29 hrs) or short (19 hrs) period of locomotor activity as well as eclosion rhythms[1] and one was behaviorally arrhythmic. All these mutations mapped to the same site on X-chromosome and were called *per (period)*^{L}, *per*^{S} and *per*^{0}, respectively. At this point, the circadian timekeeping finally got concrete genetic foundations.

Since that time a lot of information about the circadian clockwork has been gathered and we have now quite a detailed knowledge of factors contributing to the clock system in several insect model species. As always the bulk of the information comes from the experiments on *Drosophila*, though, as I will show later, it is not easily applicable to other insect species.

1.1.1. Circadian clock in *Drosophila*

The current model of *Drosophila* circadian clock involves two interconnected transcriptional and translational feedback loops the CLOCK (CLK) and CYCLE (CYC) loop and the PERIOD (PER) and TIMELESS (TIM) loop.

At the core of the clock lie the proteins CLK and CYC that interact via their dimerizing PAS domains to form a heterodimer. In the nucleus, this heterodimer binds to the E box sequences in the promoters of several other clock components genes and thus drives their expression. Among the genes controlled in this way are also the key players from the second loop, the *period* and *timeless* genes. The PER and TIM proteins then accumulate in the cytoplasm, form a heterodimer and translocate to the nucleus where they interfere with the CLK/CYC complex and thus repress their own transcription.

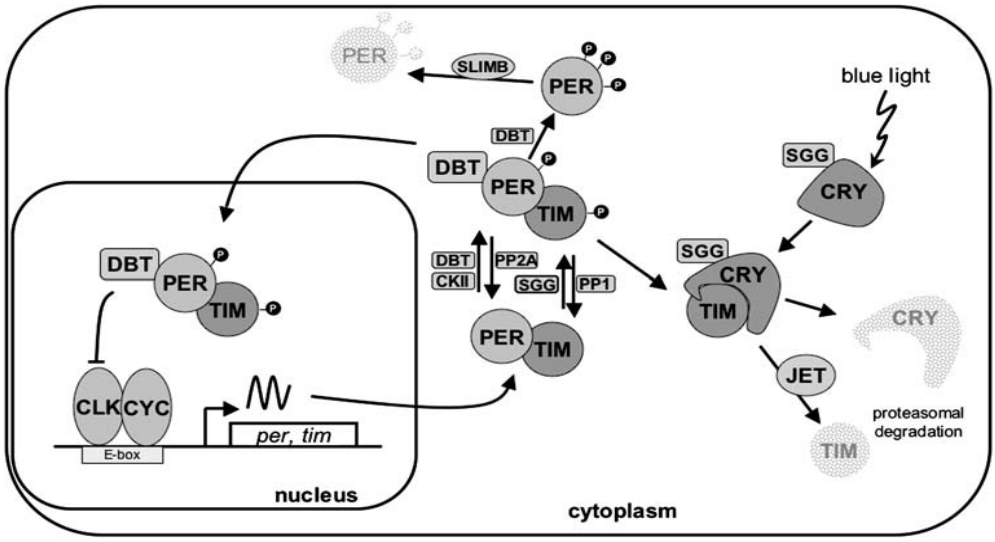


Fig. 1. A diagram depicting the regulatory actions that constitute the circadian clock system of *Drosophila*. Taken from Dubruille and Emery, 2008. The loop controlling the expression of clock gene was omitted for simplicity. See text for details.

[1] Although adult eclosion is a 'once in a lifetime' event it displays a robust population rhythm – the emergence from the pupal case is carefully gated to the early morning to protect the newly eclosed adults from desiccation.

1.1.1.1. The basic clock components

Now that I have outlined the general idea of the circadian clock work in *Drosophila*, I will try to introduce the key players in a little more detail.

The first putative clock component that was cloned is *period*, a locus known to be involved in biological timekeeping from the forward genetics experiments by Konopka and Benzer. Several reports on this topic were published almost simultaneously (e.g. Reddy *et al.* 1984, Bargiello *et al.,* 1984; Zehring *et al.,* 1984) and they shared the common approach: to correlate the pieces of putative *period* DNA with complementary mRNAs and then to use the obtained sequence in a rescue experiment on arrhythmic per^0 flies. Some of the transgenic lines showed partial restoration of the rhythms though somewhat mediocre. As became clear later the stretches of DNA used for the rescue didn't contain the whole *period* gene thus rendering faulty results. The final cDNA sequence (and the aminoacid sequence inferred from it) provided little insight into the protein's mode of action as no known domains or patterns could be identified, apart from the about 40 residues long threonine-glycine repeat that lead the scientist to a false belief that PERIOD could be a proteoglycan (Jackson *et al.,* 1986; Reddy *et al.,* 1986). A stretch of about 260 aa with sequence similarity to SIM and ARNT proteins was also identified: later, it became to be called the PAS domain[2], which stands for the initial letters of these three proteins.

The immunohistochemical experiments then revealed a striking difference in signal intensity between samples (adult heads) collected during the daytime and those taken in the middle of the night (Siwicki *et al.,* 1988), adding another dimension to the *period* issue. The PERIOD antigen was found in the photoreceptive cells in the compound eyes as well as in the brain cells, and it was shown to be present in the nucleus (e.g. Liu *et al.*, 1992). When it was shown that *per* mRNA levels from adult heads also cycle, even under free-running conditions (constant dark and temperature), it became obvious that *period* really is a clock gene (Hardin *et al.,* 1990). The *per* mRNA reaches its peak level at ZT 14 – 16, few hours earlier than its corresponding protein (ZT 18 – 20). It also appeared that the expression of *period* gene should involve a negative feedback as overexpression of *per* in photoreceptor cells induced from a transgene suppressed the normal mRNA cycling in the eyes but had no effect on the *per*-expressing cells in the brain (Zeng et al, 1994).

Another clock component was identified with the help of P-element based mutagenesis that revealed a strain that did not show any preference for night or day emergence, in contrast to wild-type flies that emerge near dawn. The new mutation was called *timeless,* mapped to the

[2] PAS – Period protein, human Aryl hydrocarbon receptor nuclear translocator protein and *Drosophila*'s Single-minded protein

second chromosome and apart from the behavioral arrhythmicity appeared to have no morphological or developmental impacts. The central role of this novel mutation in the clock system was readily obvious when it was discovered that it abolishes the circadian oscillation in *per* mRNA abundance (Sehgal *et al.*, 1994). Moreover, the *timeless* mutation also changed the cellular localization of the PER protein that could not be detected in the nucleus at any time point (Vosshall *et al.*, 1994) and suppressed the oscillation of PER protein both in LD and in DD (Price *et al.*, 1995). Yet another interesting feature of the *tim* mutant was observed: in contrast to the situation in the wild-type flies, in the mutant flies exposure to constant light didn't show any effect on PER protein abundance, which was similar as in LD, suggesting that the effect of light and that of *tim* mutation might be related (Price *et al.*, 1995). Similarly to *per* expression pattern, the *tim* mRNA levels also oscillate (with parameters resembling that of the *per* mRNA) during the day even in the absence of light-dark cycle in wild-type flies, but this rhythmicity is lost in the absence of TIM protein, suggesting an autoregulatory feedback loop to be taking place here as well. Given the fact that mutation in *tim* gene results in abolished *per* cycling an vice versa, it appeared that PER and TIM work together to keep the feedback loop rolling (Sehgal et al, 1995).

In support of this assumption, PER and TIM proteins were shown to associate with each other by yeast two-hybrid experiments and also by a GST-pull down assay *in vitro* (Gekakis *et al.*, 1995) and later confirmed in fly-head extracts by co-immunoprecipitation (Zeng *et al.*, 1996; Lee *et al.*, 1996; Myers *et al.*, 1996). In addition, these studies also suggested a mechanism for the light-mediated resetting of the circadian clock – that is via its effect on TIM abundance. This question had been lying on the table since the discovery that absence of TIM protein due to the mutation in *tim* gene happens to have the same effect on PER protein levels as the exposure of flies to constant light (Price *et al.*, 1995).

Saez and Young have demonstrated that co-expression of PER and TIM in *Drosophila* S2 cells[3] results in nuclear localization of these proteins, both PER and TIM remain cytoplasmic unless they are transfected into the cultured cells together. The researchers also used a GST-pull down assay to determine which parts of PER and TIM proteins can physically interact. They found out that PER binds TIM via its PAS domain and another site called the CLD (cytoplasmic localization domain) (Saez and Young, 1996).

Another line of research focused on the way how the transcriptional repression is actually achieved, because it was probable it couldn't be done by direct interaction of PER and/or TIM with the pertinent DNA sequences as neither of these proteins possessed any known DNA

[3] The S2 cell line (also called Schneider cells) was derived from a primary culture of late stage *Drosophila melanogaster* embryos, possibly the macrophage-like lineage.

binding motifs. The scientists therefore assumed that this is more likely accomplished by PER/TIM interfering with an unknown transcriptional activator. This notion was further supported by the identification of a 69bp enhancer element within the promoter region that was capable of driving *per*-like circadian, developmental and spatial expression of a reporter gene. This enhancer contained a consensus "E-box" sequence (CACGTG), a known binding site for basic helix-loop-helix transcription factors. The E-box sequence was also proved to be *necessary* for *per* transcription (Hao *et al.*, 1997, 1999).

Candidates for this function were provided by the Clk^{Jrk} and cyc^{0} mutations. The *Jrk* mutation was obtained through screening chemically mutagenized flies for aberrant or abolished circadian locomotor activity rhythms. Besides the behavioral arrhythmicity, both PER and TIM levels are extremely low and noncycling in *Jrk* homozygous flies and cycling, yet with a much lower amplitude in the heterozygous ones. This mutation mapped to the left arm of chromosome 3. The researches reasoned that a candidate gene for *Jrk* phenotype might be a *Drosophila* homolog of a circadian clock gene already known from mice, a bHLH-PAS transcription factor *Clock* (*mClock*) (Antoch *et al.*, 1997). This was supported by *in situ* hybridization using probe derived from EST sequence homologous to the *mClock* and by sequencing the *dClock* encoding genomic DNA of the *Jrk* mutant that revealed a premature stop codon (Allada *et al.*, 1998).

In the same issue of the Cell journal the same group of researches also reported the identification, characterization and cloning of another *Drosophila* clock gene, *cycle* (*cyc*) (Rutila *et al.*, 1998). The mutation was mapped to the left arm of the third chromosome, similarly to the *Jrk* mutation, but it was confirmed that these mutations are not allelic to each other as they can be genetically separated by meiotic recombination. Homozygous *cyc* flies displayed both behavioral and molecular phenotype resembling closely that of the *Jrk* flies: they are behaviorally arrhythmic and exhibit weak *per* and *tim* transcription. The cloning of the *cyc* gene was much simplified by the assumption that it should encode a bHLH-PAS domain similarly to the *dClock* gene. It was known that bHLH-PAS transcription factors often function as heterodimers with other bHLH-PAS proteins, so it was probable that *cyc* locus could encode the partner for the just recognized dCLK protein. The gene identified in this way displayed a high sequence similarity with the mammalian gene *Bmal1* (Ikeda and Nomura, 1997) that was proposed to function as a partner of mCLK in activating clock-relevant transcription (Hogenesch *et al.*, 1998).

Just like the *Jrk* mutation, the cyc^{0} also contains a premature stop codon, which should eliminate about 60% of the resulting protein, thus it presumably represents a null mutation. The hypothesis that dCLOCK together with dBMAL1/CYC drive the transcription of *per* and *tim* genes was supported by the transfection assays and yeast two-hybrid experiments performed by

Darlington and colleagues which also demonstrated that PER/TIM or even PER alone can actually repress the transcriptional activation by CLK/CYC heterodimer (Darlington *et al.*, 1998). This was further supported by Rothenfluh *et al.* (2000a) who engineered a *per* gene lacking the CLD domain, which resulted in nuclear localization of the resulting PERΔCLD protein in cultured S2 cells even without co-expressed TIM. This deficient PER protein was able to significantly lower the expression from a CLK/CYC controlled *per* promoter. Expression of this transgene in flies though resulted in largely arrhythmic locomotor behavior even though the same flies still expressed their genomic wild-type forms of both *per* and *tim* genes (Rothenfluh *et al.*, 2000a).

Neither the *cyc* mRNA (Rutila *et al.*, 1998) nor the CYC protein (Bae *et al.*, 2000, Wang et al 2001) undergo any circadian oscillation, but *dClk* transcripts were shown to exhibit cyclic changes in abundance. Darlington *et al.* (1998) described the *Clk*'s oscillation as bimodal with maxima at ZT 23 and ZT 5. However another research team reported very different results: the *Clk* expression pattern is actually antiphase to *per* and *tim* mRNAs oscillations (Bae *et al.*, 1998), persists in DD and seems to require both TIM and PER as per^0 and tim^0 mutations bring the *dClk* transcript level to its minimum. The CLK protein was also reported to cycle in head extracts although it achieved its peak amount at times when *per* and *tim* transcripts are low, which is paradoxical given that dCLK is required for *per* and *tim* gene expression (Lee *et al.*, 1998; Bae *et al.*, 2000). However, when Houl *et al.* (2006) produced a new antibody against dCLK to investigate its cellular and subcellular localization they didn't find any signs of oscillation neither in the pacemaker cells nor in the non-oscillator ones. Later it was confirmed that the original results were biased by the use of unsuitable extraction method and that the overall amount of dCLK protein remains unchanged during the day (Yu *et al.*, 2006; Kim and Edery, 2006).

Surprisingly, Glossop *et al.* (1999) reported that *dClk* mRNA levels are constitutively high in both Clk^{Jrk} and cyc^0 homozygotes, although quite the contrary would be expected based on the previous studies (either Clk^{Jrk} or cyc^0 mutation leads to very low TIM or PER abundance and both per^0 and tim^0 genotypes which eliminate PER or TIM result in through levels of *dClk* mRNA). This implied the existence of a yet unknown repressor of (at least) *dClk* gene expression (Glossop *et al.*, 1999).

1.1.1.2. Postranslational regulation of PER and TIM proteins

There is another circadian feature of the PER and TIM proteins that I haven't addressed yet, and that is their changing phosphorylation status.

The suspicion that also a post-translational modifications might have a role in the timekeeping system had existed quite long before (Zwiebel *et al.*, 1991) it was actually confirmed

by the study of Edery *et al.* in 1994. Edery and colleagues found out that the apparent size of the PER protein on the gel was changing − that is, increasing in the samples taken during the night. This mobility shift could be removed by treating the samples with phosphatase and it persisted when the phosphatase inhibitor sodium phosphate was added. When the PER protein levels are lowered due to the exposure to constant light or in the tim^0 background, it also doesn't show the mobility shift to higher molecular weight form in the late night (Price *et al.*, 1995).

Attachment of phosphate moieties to proteins is a well known tool how to mark them for degradation. It is worth noting that for an oscillation in protein levels to take place it is not sufficient to just produce the corresponding mRNA in a cyclical manner. The resulting protein has to have a reasonably short half-life to keep the levels cycling.

This leads us to the *double-time* (*dbt*) gene that was identified with the help of three mutant strains of flies: two of them were isolated on the basis of their abnormal locomotor activity rhythm – either longer (dbt^L) or shorter (dbt^S) than in the wild-type. Both these mutations were genetically mapped to the right arm of the third chromosome. The third line (dbt^P) was obtained in a screen of *Drosophila* lines with P-element insertions in that region. It was the one that failed to complement both the dbt^S and dbt^L mutations (meaning that they all affect the same gene). Among other effects, the dbt^S and dbt^L advance or delay the appearance of the low-mobility hyperphosphorylated form of PER respectively. The dbt^P, however, has much more severe consequences: it is associated with pupal lethality and in the larval brain it completely abolishes the molecular rhythms of both *per* and *tim* transcripts, though in a very different manner: While *tim* mRNA as well as protein are barely detectable in the pacemaker cells upon transition to DD and later disappears completely, PER protein level is elevated and stable both in LD and DD and it is constantly hypophosphorylated. This high amount of PER cannot be assigned to higher gene expression though and rather reflects an increase in the stability of the protein (Price *et al.*, 1998). Furthermore, the DBT protein was shown to interact with PER both *in vitro* and in *Drosophila* cultured S2 cells by GTS-pull down and co-immunoprecipitation. Based on its nucleotide and protein sequences, the *dbt* seems to be related to the human *casein kinase 1ε* (Kloss *et al.*, 1998). All these results combined with the previous work on *tim* and *per* mutants suggest that DBT regulates the circadian period by decreasing the stability of monomeric PER, which thus fails to accumulate in the absence of TIM and translocate to the nucleus (Vosshall *et al.*, 1994; Price *et al.*, 1995). In accordance with this hypothesis, it was shown that TIM inhibits phosphorylation of PER by DBT in S2 cells further supporting the notion that TIM protects PER from degradation (Ko *et al.*, 2002).

Several S2 cells studies revealed though, that PER can translocate into the nucleus and repress CLK/CYC activity without TIM (Chang and Reppert, 2003; Nawathean and Rosbash, 2004) and Shafer *et al.* (2002) was even able to detect nuclear PER before TIM in clock neurons, which would suggest sequential transfer of these proteins into the nucleus. On the other hand, results of Cyran *et al.* show, that PER can indeed enter nucleus of pacemaker neurons without TIM, but only if the function of DBT is impaired so that it cannot phosphorylate PER. In tim^0 background (with functional *dbt* gene) PER was not detected in the nucleus (Cyran *et al.*, 2005). A solution for this discrepancy might be offered by the study of Ashmore *et al.* (2003) that demonstrated that TIM can be detected in the nucleus in per^0 background if inhibitors of nuclear export and proteasome are added. It is therefore possible that TIM normally shuttles between nucleus and cytoplasm, which would account for its delayed accumulation even if PER and TIM do enter the nucleus as a complex (but some TIM is exported, to recruit more PER perhaps, or it is unstable and degraded in the nucleus).

How exactly is PER degraded after the phosphorylation by DBT was investigated by two research teams: Grima *et al.* (2002) and Ko *et al.* (2002) demonstrated a gene called *slimb (slmb)* that encodes an F-box/WD 40-repeat protein functioning as a part of the ubiquitine-proteasome pathway[4] to be involved in the regulation of PER levels. Although mutations at this locus are lethal at early larval stage, Grima and co-workers succeeded in bringing the *slimb* mutants to adulthood by a controlled expression of *slmb* from a transgene. The adult mutant flies were completely arrhythmic in DD but their behavior could be restored to a nearly wild-type pattern by expressing *slmb* in pacemaker neurons. The *slmb* mutation influences the stability of hyperphosphorylated PER (and TIM) that were erroneously present at all times of the circadian day in head extracts from flies kept in constant dark. Both these proteins cycle normally under LD conditions. The SLMB protein was co-immunoprecipitated by both anti-PER and anti-DBT antibodies (Grima *et al.*, 2002). Ko *et al.* reported that significantly more SLMB co-purified with DBT-phosphorylated PER from S2 cells extracts and the degradation of hyperphosphorylated PER protein was specifically enhanced by constitutively high expression of *slimb* in cell cultures (Ko *et al.*, 2002). All these results taken together suggest that phosphorylated PER is indeed a physiological substrate for the SLMB protein.

As to the regulation of the *dbt* itself, there is no evidence to suspect that it should be under circadian control or that other clock components should influence its expression (Kloss *et al.*, 2001). The subcellular localization of DBT exhibits a circadian rhythm though. The co-

[4] More specifically these F-box proteins are known to be responsible for substrate recognition by the SCF (Skp1/Cullin/F-box protein) ubiquitine E3 ligase complex.

immunoprecipitation studies revealed that DBT physically interacts with PER protein as well as with the PER/TIM heterodimer (not with TIM alone) and is carried together with these proteins into the nucleus in the middle of the night (ZT 18-22), where it possibly promotes the phosphorylation of PER again, once the TIM protein disappears from the complex at dawn (Kloss *et al.*, 2001).

TIM is also a substrate for one or more kinases (though it seems to be phosphorylated to a lesser extent than PER) (Zeng *et al.*, 1996): The use of tyrosine kinase inhibitor led to a block in the degradation of TIM in response to light, whereas the serine-threonine kinase (a class of kinases where DBT belongs) inhibitors did not (Naidoo *et al.*, 1999). In 2001, Martinek *et al.* identified the locus *shaggy* (that encodes the *Drosophila* ortholog of glycogen synthase kinase 3) as capable of shortening the circadian period of locomotor activity when overexpressed in the pacemaker cells by means of *tim*(UAS)-GAL4 system. The following immunohistochemical analysis revealed that under the free-running conditions the *sgg* overexpression shifted the nuclear transfer of both TIM and PER immunoreactivity toward earlier time compared to the wild-type. Overexpression of *sgg* in the per^0 background (where, according to its mobility, TIM is largely hypophosphorylated) showed that SGG is responsible for the phosphorylation pattern of TIM (Martinek *et al.*, 2001). The possibility that SGG could affect PER as well as TIM was deemed unrealistic on the basis of findings of Cyran *et al.* (2005) who tested this possibility by overexpressing *sgg* in tim^0 background but failed to find any differences in the stability, localization or phosphorylation of PER compared to tim^0 mutants without the overexpression of *sgg* (Cyran *et al.*, 2005).

It thus seems that DBT and SGG have opposing roles in the control of PER/TIM nuclear translocation, with DBT retarding the transfer and SGG accelerating it.

Another clock component was identified on the basis of two different mutant strains *Timekeeper* (Lin *et al.*, 2002) and *Andante* (Akten *et al.*, 2003): the serine-threonine kinase *casein kinase 2* (*ck2*). The mutation in the *Tik* locus affected the alpha catalytic subunit of the enzyme whereas the *And* locus encodes the regulatory beta subunit. Both these mutations lengthened the period of locomotor activity and are expressed in the pacemaker neurons in the brain. On the molecular level, the two studies offer little different results: They both report an increase in PER (and TIM, Akten *et al.*) protein abundance but while Lin *et al.* claim to have observed a modest, yet significant increase in the hypophosphorylated form of PER, Akten *et al.* report no difference in the phosphorylation status of neither PER nor TIM. What is certainly affected however, is the timing of the nuclear translocation of PER (in a complex with TIM) that is significantly delayed, which corresponds to the observed behavioral effects of these mutations.

(Lin *et al.*, 2002; Akten *et al.*, 2003) It seems probable though that CK2 is involved in the regulation of nuclear entry of PER/TIM complex, it may influence the transfer by phosphorylating either PER (possible at least *in vitro*, Lin *et al.*, 2002) or some other clock component like DBT or SGG. Later experiments using the whole CK2 holoenzyme with both α and β subunits identified the CK2 phosphorylation sites on PER and their role for phosphorylation *in vitro*. Lin and colleagues identified three CK2 consensus sites in the *per* gene sequence, mutated the serines within these sites into alanines, which significantly blocked phosphorylation of PER by CK2 holoenzyme. The investigators then used these mutated transgenes in a rescue experiment on arrhythmic per^0 flies. In most cases robust rescue of rhythmicity was observed; the mutations in serines resulted in locomotor activity period lengthened by ~2 hrs compared to controls rescued with wild-type *per* genomic fragment. The PER protein levels seemed to be rather unaffected by these modifications and so was its phosphorylation status, which would agree with the observations made by Akten *et al.* (2003). Furthermore, the nuclear entry of PER in pacemaker neurons is also delayed by about 2 hrs in comparison to controls, correlating nicely with the behavioral response of the mutants (Lin *et al.*, 2005).

To make the whole image even more complicated, it has been shown that also some specific phosphatases play a role in the circadian network: Sathyanarayanan *et al.* (2004) reasoned that protein-phosphatase 2 known from the *wnt* pathway could be involved in the circadian clock system as well. To learn this they first assessed the role of phosphatases by an assay in S2 cells and found that in the absence of phosphatase inhibitor PER was fairly stable, while upon addition of the inhibitor it became rapidly degraded (unless the cells were co-transfected with *tim* construct as well). Using the RNAi method the authors then knocked-down the expression of all regulatory subunits of the PP2 holoenzyme (which are responsible for the substrate specificity) encoded in the *Drosophila* genome in a stepwise manner. Only silencing of the genes *twins* and *widerborst* resulted in a reduction in PER levels, TIM levels were also affected but only slightly, suggesting that PER is the primary target for PP2. In wild type flies transcripts from the genes *tws* and *wdb* cycle over the course of the day and these oscillations are eliminated in the cyc^0 mutants implying that they might be under the circadian clock control. Overexpression of either of these genes or the gene coding for the catalytic subunit of PP2, the *mutagenic star* (*mts*) resulted in altered locomotor activity. The nuclear translocation was affected as well: in flies over-expressing the wild-type *mts*, the PER protein levels didn't cycle and PER was nuclear at all times, on the other hand it was barely detectable in the presence of dominant negative form of MTS suggesting that PP2 is required for PER stability and cycling (Sathyanarayanan *et al.*, 2004).

After the discovery that PER is regulated not only by kinases but also by a phosphatase the researchers prompted to find out, whether the same holds true for its partner TIM as well. The suspicion descended on the *protein phosphatase 1* as it is a ubiquitous eukaryotic enzyme involved in many important cellular processes including metabolism, cell cycle, muscle relaxation and others (Ceulemans and Bollen, 2004). This suspicion was only reinforced when it was found that TIM protein actually does possess a PP1 binding motif[5]. Fang *et al.* (2007) carried out a number of experiments that show a role for PP1 in the regulation of TIM stability. Expression of a specific PP1 inhibitor, NIPP1, reduced the levels of TIM by more than half in S2 cells and resulted in lengthened locomotor activity period when expressed in the pacemaker neurons in the brain of wild-type flies. Though the onset of nuclear translocation of PER seem to be unaffected by the NIPP1 presence, the immunoreactive signal seemed to be much weaker and less condensed compared to controls. The only distressing outcome of these studies is that the authors were unable to observe any changes in the phosphorylation-induced mobility-shifts of TIM in the protein gels as one would expect (Fang *et al.*, 2007).

1.1.1.3. Regulation of the dCLK/CYC feedback loop

As to the dCLK/CYC part of the model, I mentioned before that the expression of the *cycle* gene is not circadianly regulated on neither transcriptional nor translational level, but the *clock* mRNA abundance does oscillate during the day. Though it seems that the dCLK protein levels don't cycle after all, it is, however, regulated by a changing phosphorylation pattern on the post-translational level.

Let us consider the control of the *dClock* mRNA production first. As stated above, *dClk* transcript cycles with a phase opposite to that of *per* and *tim* mRNAs (peak – around ZT12; through – around ZT0) and its expression is regulated by other factors than just PER or TIM proteins. The potential repressor of *dClk* expression would appear to be the CLK/CYC dimer itself or some unknown factor activated by dCLK/CYC in a similar fashion as *per* or *tim* (Glossop *et al.*, 1999). The latter was found to be true by another study of Glossop and colleagues (2003) who reasoned that the previously identified clock component *vrille* (*vri*) (Blau and Young, 1999) would be a good candidate for the function of CLK/CYC repressor. This assumption was based on several characteristics of the expression of the *vri* gene: It is a clock controlled gene, whose transcript oscillates with a phase and amplitude comparable to that of *tim* and it is also regulated by the dCLK/CYC complex: Its mRNA level is constitutively low in both Clk^{Jrk} and cyc^{0} mutants; a

[5] A conserved PP1c-binding motif, so-called RVxF motif: [R/K]–X_{0-1}–[V/I]–{P}–[F/W] where X denotes any residue and {P} any residue except proline.

dCLK/CYC binding motifs, the E-box sequences CACGTG, were found within its promoter; and its expression is activated by dCLK/CYC in cultured S2 cells. Also the spatial expression of *vrille* gene supports its role in the circadian clock, both *in situ* hybridization and antibody detection found the *vri* products to be present in pacemaker cells of the adult *Drosophila* brain. Overexpression of *vri* in larval oscillator cells leads to low/absent *per* and *tim* mRNA levels. Finally, misexpression of *vri* gene results in aberrant circadian behavior (Blau and Young, 1999). Glossop *et al.* (2003) found that VRI protein also oscillates with opposite phase to that of *Clk* mRNA (it reaches its peak levels during the early night, ZT 13 – 17, the through occurs in the early day, ZT 1 – 5). Furthermore, VRI can physically interact with *Clk*-derived regulatory sequences and it is also phosphorylated. VRI displays effects consistent with its putative role as *Clk* repressor also *in vivo*: It causes a reduction in *dClk* mRNA abundance when (over)expressed both in wild-type and cyc^0 backgrounds (Glossop *et al.*, 2003).

Having resolved the obscurities related to the *dClk* repression, another questions have immediately arisen – the problem of *dClk* activation. A possible answer to this issue was provided by the study of Cyran *et al.* (2003). The authors of this study assumed that the activator of *dClk* should share some homology with its presumptive repressor – VRILLE, given that they should have the same target. They searched the *Drosophila* genome and found only one candidate gene, *PAR domain*[6] *protein 1* (*Pdp1*). *Pdp1* was previously identified as a direct target of dCLK/CYC by microarray analysis of adult wild-type and *Clk* mutant flies' heads (McDonald and Rosbash, 2001) and it is expressed rhythmically, which was shown not only by the microarray data but also by real-time PCR (Ueda *et al.*, 2002).

Pdp1 (as well as *vri*) encodes basic zipper transcription factors, a total of six *Pdp1* isoforms are known to be expressed *in vivo* – generated through alternative splicing and use of four alternative promoters (Reddy *et al.*, 2000). Only one of these though, the isoform *Pdp1ε*, exhibit a circadian oscillation in RNA levels. *Pdp1ε* and *vri* transcripts cycle with similar phases to one another, but *Pdp1ε* levels peak 3-6 hrs later than *vri* transcript does, this may reflect different strength of their promoters and/or different half-lives of their mRNAs. The pattern of expression in various clock-gene mutants (per^0, tim^0, cyc^0 and Clk^{Jrk}) is consistent with *Pdp1ε* transcription being regulated in a similar manner to *per*, *tim* and *vri* transcription. The expression on the level of proteins largely reflects that of mRNAs for both PDP1 and VRI, PDP1 peaks at about ZT 18 and VRI approximately 3 hrs earlier. A mutation deleting the whole *Pdp1* locus results in a moderate lengthening of the period of behavioral rhythms in heterozygous state (homozygotes usually survive to the adulthood), an effect comparable in magnitude to that inflicted by

[6] PAR domain proteins contain a Proline- and Acidic amino acid-Rich region.

heterozygous null mutation of *vri,* which causes period shortening (Blau and Young, 1999). To find out whether the repression of *dClk* by VRI is dependent on TIM or PER protein, Cyran *et al.* (2003) overexpressed *vri* in per^0 background, in which TIM is cytoplasmic and PER is absent (Allada *et al.*, 2001). This manipulation further decreased the *dClk* transcript levels (low as they were already), showing that nuclear TIM or PER are not necessary for *vri* mediated repression of *dClk*. Levels of *dClk* mRNA were significantly reduced (but still cycling) also in the heterozygous *Pdp1* mutant adults and almost undetectable in homozygous mutant larvae. Finally, PDP1 and VRI proteins were shown to specifically bind the same region in the *dClk* promoter, suggesting they could be competing for this binding site and therefore it would be the ratio between these two proteins that decides wheter the dClk gene expression is activated or repressed (Cyran *et al.*, 2003). In sharp contrast to these findings, Benito *et al.* (2007) reported that silencing of the *Pdp1* gene in pacemaker neurons of adult *Drosophila* flies does not appreciably change neither the rhythm nor the amplitude of *dClk* mRNA. The same holds true also for the overexpression of *Pdp1* in clock cells: not even this manipulation resulted in disrupted *dClk* mRNA cycling. On the other hand constant high or low levels greatly influenced the locomotor activity of the flies rendering them largely arrhythmic, although the pacemaker cells responsible for circadian behavior (LN_vs) seemed to contain functional oscillators. The authors therefore argue for a role of PDP1 in the circadian output rather than in the clock circuit itself (Benito *et al.*, 2007).

Another clock regulator *clockwork orange (cwo)* was originally revealed as a rhythmically expressed gene in genome-wide microarray studies (McDonald and Rosbash, 2001; Ueda *et al.*, 2002) and this was subsequently verified by real-time PCR as well. The *cwo* transcript cycles both in LD and DD with a phase close to that of *per* and *tim.* It contains several E-box sequences within its promoter region, a bHLH DNA binding motif and an ORANGE domain commonly present in transcriptional repressors. It is apparently a direct target of CLK/CYC-mediated regulation based both on genetical evidence (temporally flat through and high levels of *cwo* mRNA in Clk^{Jrk} mutants and per^0, respectively) and assays in S2 cells. Flies carrying a transposon inserted into the first intron of *cwo* gene display lengthened locomotor activity period but the rhythm dampen quite soon in DD and a substantial portion of these flies are completely arrhythmic. Similar period-lengthening effect was observed when researchers knocked-down the *cwo* expression in pacemaker neurons using RNA interference. CWO negatively influences expression of all known CLK/CYC target genes (*tim*, *per*, *vri* and *pdp1*) in cell cultures. Furthermore CWO was found to strongly repress its own promoter in *lucipherase*-reporter assay suggesting that it forms an autoregulatory negative feedback loop. Matsumoto *et al.* (2007) monitored the expression pattern of *per*, *tim*, *vri*, *Pdp1* and *cwo* also in *cwo*-RNAi transgenic flies

revealing a severe reduction in the amplitude of their oscillations both in LD and DD. (Lim *et al.*, 2007; Kadener *et al.*, 2007; Matsumoto *et al.*, 2007)

A more complicated mode of action has been recently proposed for CWO by Richier *et al.* (2008): Based on their observation that the newly engineered (and very probably null) mutation in *cwo* gene, cwo^{B9}, does not influence much the through levels of transcripts of the CLK/CYC targets *per*, *tim*, *vri* and *Pdp1*, but strongly decreases their peak levels in DD, the authors assumed that CWO functions as an activator of these genes necessary for accumulation of the relevant mRNAs during the subjective night. On the other hand, CWO apparently functions as a repressor of its own expression implying its dual role in the circadian network (Richier *et al.*, 2008).

All these little obscurities aside, there is a one important issue left with the CLK/CYC transcriptional feedback loop: We still don't know what purpose it should actually serve in the first place if it's not the control of dCLK protein levels. To my knowledge, answer to this question remains also unknown.

Even though dCLK protein is not circadianly regulated in its abundance, it is regulated by phosphorylation (Lee *et al.*, 1998, Kim *et al.*, 2002). The slowly migrating (that is the hyperphosphorylated) form of dCLK peaks in abundance during the late night and hypophosphorylated form peaks during the late day and this oscillation persists also in DD. This rhythm was abolished in both per^0 and cyc^0 mutants (the hyperphosphorylated form of dCLK is lacking), suggesting a role of PER in the dCLK protein phosphorylation as neither of these mutants express *per* gene. This in turn brings DBT into consideration, as it is carried into the nucleus together with PER and could easily influence the stability of CLK too since they are present in the same complex. Consistent with this hypothesis, the levels of CLK protein were significantly higher in S2 cells treated with *dbt* RNAi and also in the head extracts from dbt^{P}/dbt^{ar} mutant flies (Yu *et al.*, 2006). Another study on this topic showed that DBT phosphorylates CLK in S2 cells, but it is phosphorylated to some degree even if DBT is knocked down (the same results were obtained also in the previously mentioned report) proposing a role for another kinase(s) and possibly also phosphatase(s) (Kim and Edery, 2006).

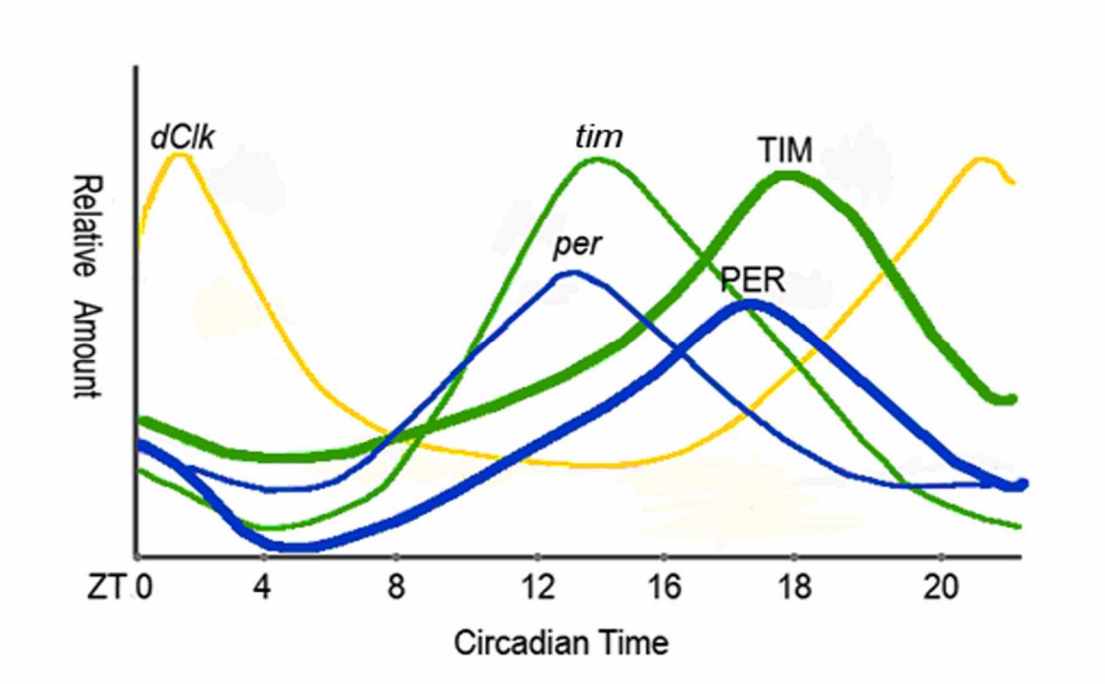


Fig. 2. Expression pattern of the cycling core components of the *Drosophila* circadian clock

1.1.1.4. Mechanism of entrainment of the circadian clock

Circadian clocks can be entrained by a number of signals – light and temperature, obviously, but food availability or even social experience has been proved capable of resetting the circadian rhythmicity (Levine *et al.*, 2002a).

Light-mediated entrainment

Light is generally regarded as the most prominent entraining signal; its effects on the clock system have been studied the most and consequently are the best-understood at the moment.

The key step in the photic entrainment of the circadian clockwork is undoubtedly a rapid degradation of the TIM protein along with its consequences on the PER protein levels − this has been known for quite some time (e.g. Zeng *et al.*, 1996). TIM is degraded via the common ubiquitine-proteasome pathway with phosphorylation playing an important role in this process (see above) (Naidoo *et al.*, 1999).

Although compound eyes and the canonical visual pathway are known to function to some degree in relaying the photic information to the core of the circadian machinery, another clock-specific pathway has been discovered.

Exposing flies to a series of monochromatic light pulses revealed that TIM is maximally degraded in response to illumination within the blue range of light, 450-500 nm, with little effect at

or above 600 nm, corresponding nicely to the maximal behavioral response too (Suri *et al.*, 1998). An accompanying study went further to demonstrate that TIM response to light does indeed correlate with the entrainment of the behavioral rhythms (Yang *et al.*, 1998). Given the action spectrum, the family of photolyases/cryptochromes was a logical choice to search in for a circadian photoreceptor; besides a plant blue light photoreceptor belonging to this group was already known. A candidate gene was found through a BLAST search and named *cry* (*cryptochrome*). It was discovered that the *cry* transcript undergoes a circadian changes in abundance (peak – ZT 1, through – ZT 17) and this rhythmic expression is abolished in mutants for all major clock factors (tim^0, per^0, Clk^{Jrk}, cyc^0). The CRY protein cycles during the day, but this oscillation doesn't persist in constants dark, the protein accumulates throughout the subjective day and night. Flies overexpressing *cry* gene were observed to be behaviorally hypersensitive to light pulses, especially at low light intensities (Emery *et al.*, 1998). An independent study also linked *cryptochrome* to the circadian photoreception but this time by the means of a genetic screen for mutants altering the *per* expression pattern. This recessive mutation mapped to the third chromosome within the ORF of a *cryptochrome* encoding gene and was named $cry^{b(aby)}$. Its effects on both *per* and *tim* expression seemed to be profound: the mRNA levels as well as the protein levels from head extracts were stable during the day. However, in the pacemaker cells in the brain the levels of both TIM and PER were observed to cycle, although with somewhat reduced amplitude and not in all neurons in comparison to wild-type. Temporally constitutive signals were observed in the eyes, explaining the contradictory results from Western blots. The behavioral effects of this mutation also were not as severe as one could expect. The cry^b mutant flies failed to respond to short light pulses delivered at times when they should induce phase shift in the locomotor activity. Yet, they were rhythmic in both LD and DD conditions with periods of about 24 hrs and they were also able to shift from one LD cycle to a different regime in which the lights came on 4 hrs later and were changed to dim blue light (Stanewsky *et al.*, 1998). To assess the role of extraocullar photoreception, cry^b mutant flies were crossed with *norpA* mutants (*no retinal potential* − loss-of-function mutation of phospholipace C, which causes the compound eyes and ocelli to be completely unresponsive to light, Pearn *et al.*, 1996). Oddly enough, not even these double mutants were completely blind in terms of circadian entrainment, though their ability to entrain to LD cycle and re-entrain to a shifted-over one was significantly decreased especially in dim blue light (Stanewsky *et al.*, 1998). The most intriguing characteristic of the cry^b mutants was discovered later: the fact that they remain rather robustly rhythmic in constant light (Emery *et al.*, 2000a), making it obvious that *cryptochrome* does have a major influence on the circadian

timing. The defects in behavior caused by the *cry^{b}* mutation can be partially rescued by overexpressing *cry^{+}* only in specific subgroup of brain cells, the LN_v cells (Emery *et al.*, 2000b).

The fact that combining two mutant alleles that should eliminate or at least impair both the visual and non-visual phototransduction pathways still did not cause the flies to be absolutely blind raised a question, whether some other potential photoreceptors could have been left intact by these mutations. One such structure that could be theoretically involved in circadian photoreception is the Hofbauer-Buchner (H-B) eyelet which is located beneath the compound eye and projects to the pacemaker center in the brain. To explore this possibility, Helfrich-Förster *et al.* (2001) and colleagues crossed flies bearing the *cry^{b}* mutation with *glass^{60J}* mutants. The *glass* mutation removes all eye structures together with the H-B eyelets. This, finally, resulted in a more or less circadianly blind fly strain: the doubly mutant flies failed completely to entrain and re-entrain to LD cycles and most of them exhibited free-running rhythmic behavior regardless of the light-dark regime and its changes. The constant light had also no effect on their behavior. Interestingly, a bump of locomotion induced by the light-off was observed even in these double mutants when they were subjected to high-intensity LD cycle, showing that they still *do* somehow respond to changes in illumination (Helfrich-Förster *et al.*, 2001). However, the mysterious H-B eyelets were shown to express the *norpA* gene as well along with *Rhodopsin* so the *norpA, cry^{b}* double mutants should have all the photic routes impaired, provided both these mutations are null (and no unknown norpA-independent opsins are present) (Malpel *et al.*, 2002). Besides, Klarsfeld *et al.*, demonstrated that the *glass^{60J}* mutation alone causes significant decrease in the overall light-sensitivity (likely due to the loss of group of per-expressing DN1 neurons that are also removed by the *gl^{60J}* mutation), which greatly compromises the use of this mutant as a meaningful circadian research tool (Klarsfeld *et al.*, 2004).

Contrary to the original belief, *cry^{b}* was later found not to be a complete null mutation, but merely a heavily hypomorphic one. Stanewsky *et al.* (1998) first failed to detect CRY protein in the head extracts from the *cry^{b}* flies, but subsequent studies confirmed that these flies do produce low levels of CRY^{B} protein afterall. Moreover, this protein can bind TIM with similar strength as the wild-type CRY, which could account for the residual responsiveness to light stimuli observed in all aforementioned experiments (Busza *et al.*, 2004). This prompted the researchers to produce a real null mutation of *cryptochrome* gene to see whether it can abolish the ability of the system to react to photic signals. Dolezelova *et al.* (2007) created a novel *cry^{0}* mutation in which the whole coding sequence of the *cry^{+}* was replaced with mini-*white^{+}* by homologous recombination. When combined with the *norpA* mutation, the flies have severe problems to re-entrain from a certain LD cycle to a shifted one, though they still can make it. This observation lends credence to the

speculations that another *norpA* and *cry* independent photoreceptor might be functioning in the circadian clock system (Dolezelova *et al.*, 2007).

Let us now consider the molecular mechanism by which CRY should be relaying the photic information (from one source or another) to the central oscillator. In 1999, Ceriani *et al.* showed that CRY indeed mediates the photic information and its presence together with illumination blocks the negative effect of PER/TIM complex on dCLK/CYC's activity therefore allowing for the activation of *tim* promoter in S2 cells. CRY and TIM were also demonstrated to interact directly with each other by co-immunoprecipitation in this system (Ceriani *et al.*, 1999). In the same year, it was reported that light-induced degradation of TIM involves the ubiquitin – proteasome pathway, as the inhibitors of proteasome prevented the turnover of TIM in response to the light signal (Naidoo *et al.*, 1999).

As CRY was shown to be part of the central circadian clock in mammals, the researchers went on to describe in detail its interactions with clock components also in *Drosophila*. In the experiments performed by Ceriani *et al.* that were mentioned above, the researchers failed to detect association between just CRY and PER. On the other hand Rosato el al. (2001) reported a strong interaction between CRY and fragments of PER both in yeast two-hybrid (Y2H) system and S2 cells, although they failed to detect interaction between CRY and full-length PER in yeast cells, which was assigned to the poor expression of the full-length protein in this system. The researchers tried to further specify the regions needed for this interactions by challenging the CRY (bait) with overlapping fragments of PER (prey) in the Y2H assay and found that CRY interacted with the C domain of PER (while TIM associates with PER via the latter's PAS domain), suggesting that PER, TIM and CRY could be theoretically found in the same complex even if CRY would be interacting with both these proteins directly (Rosato *et al.*, 2001) (see Fig. S1 in Supplements for overview of the protein domains). To resolve this apparently conflicting results regarding the possible CRY:PER association, Busza *et al.* (2004) studied the CRY, TIM and PER interactions directly in flies. They demonstrated that CRY proteins binds both TIM and PER in response to a light pulse but not in the dark (at least not beyond the strength of the background signal). The interaction between CRY and PER though is only secondary, because unlike TIM, PER cannot bind CRY alone (based on experiments with tim^0 flies as well as S2 assays). These results imply that TIM is the primary target of CRY after light activation (Busza *et al.*, 2004).

Quite recently, another factor involved in the light-induced degradation of TIM has been discovered. The mutation that led to its discovery lies in the locus called *jetlag*, which encodes an F-box protein that is a putative component of a ubiquitine ligase complex. Its behavioral effects

are reminiscent of those of *cry* mutants – they can be entrained to LD cycles, but they take longer to re-entrain to a shifted schedule and above all, they are rhythmic in constant light though they exhibit normal behavior in DD (as compared to wild-type flies). The *jet* mutants also respond poorly to phase-shifting light pulses. On the molecular level, it was shown that this mutation substantially reduce light-dependent degradation of TIM in *jet* flies and this defect can be reversed by expressing a wild-type JET from a transgene. Experiments on embryonic S2 cells showed that JET causes a rapid turnover of TIM upon light exposure but it requires co-expression of CRY to do so. It exerts its function by ubiquitination of TIM, perhaps through direct association (Koh *et al.*, 2006). This hypothesis was recently supported by a series of experiments performed by Peschel *et al.* (2009) who demonstrated a direct interaction between JET and TIM in S2 cells by co-immunoprecipitation, this interaction is greatly facilitated by CRY. *Jet* is also suspected to influence the stability of CRY protein – homozygous *jet* mutants display an increased level of CRY and enhanced degradation of CRY in the presence of JET was observed in S2 cells (Peschel *et al.*, 2009).

Yet another role, besides its function in photoreception, has been proposed for CRY in *Drosophila*. Krishnan and colleagues (2001) measured the olfactory response of both wild-type and cry^b mutants under LD and DD conditions using so called electroantennogram (EAG). They found out that contrary to the wild-type flies there is no peak in sensitivity of the antennae to food odorants that would persist under free-running conditions, suggesting that cry^+ is required for this output. To describe the state of peripheral oscillator in the antennae, the researcher took advantage of the transgenic *per-/tim-lucipherase* reporter strains and found out that cry^b flies bearing the *luc* reporter displayed largely arrhythmic luminescence signal both in LD and DD. These effects indicate that CRY might function as an integral part of the antennal oscillator, because if its role was limited to photoreception the EAG rhythms should free-run in constant dark. To completely bypass any influence of light, flies were reared in constant dark throughout their development and were entrained to temperature cycles. Not even these cry^b flies showed any circadian response in EAG rhythms (Krishnan *et al.*, 2001).

Temperature entrainment

The fact that circadian rhythms in behavior can be entrained also by temperature cycles has been known since 1960's when Zimmerman *et al.* (1968) published their findings derived from experiments on *Drosophila pseudoobscura*. Unfortunately, not much of an improvement has been achieved in this field since that time and the molecular mechanism underlying the temperature entrainment of circadian clock system is only poorly understood.

Adult wild-type flies can be entrained in their locomotor behavior to TC cycles (thermophase/cryophase) in the absence of light signal and this rhythm is maintained even if the temperature cycle is also removed, proving that we are dealing with a *bona fide* entrainment mechanism and not just a passive response of the organism (Busza *et al.*, 2007). Temperature can actually drive the circadian entrainment even under light conditions that normally induce arrhythmicity (constant light), but although flies do exhibit anticipation of the temperature transitions, the locomotor rhythms are lost when the temperature cycles are stopped (Yoshii *et al.*, 2005).

On the molecular level, both TIM and PER oscillate during the entrainment to TC cycles in constant dark and these oscillations persist also in the free-running conditions following the temperature entrainment, showing that in the end both light and temperature probably act on the same clock components (Glaser and Stanewsky, 2005; Stanewsky *et al.*, 1998). Interestingly the TC cycles under LL conditions are capable of restoring also the molecular rhythms in PER and TIM abundance meaning that the CRY-mediated light degradation of TIM must be somehow blocked (Glaser and Stanewsky; 2005).

To my knowledge only one gene locus *nocte* (*no circadian temperature entrainment*) has been implicated in functioning specifically in the temperature entrainment so far. This mutation was recovered from a screen for mutants with altered luminescence rhythms caused by expression of the reporter *period-luciferase* fusion gene. It caused a severe reduction in the luminescence rhythm, but this effect was restricted to temperature cycles only – under LD conditions the mutants showed robust oscillation in luminescence. Mapping experiments placed this locus on the X chromosome (but to a region far from both *per* and *norpA* gene). In contrast to wild-type flies (see above) in *nocte* mutants the temperature cycles administered under LL failed to drive the oscillation in abundance of PER and TIM proteins, the former being constitutively high and the latter constitutively low (Glaser and Stanewsky; 2005).

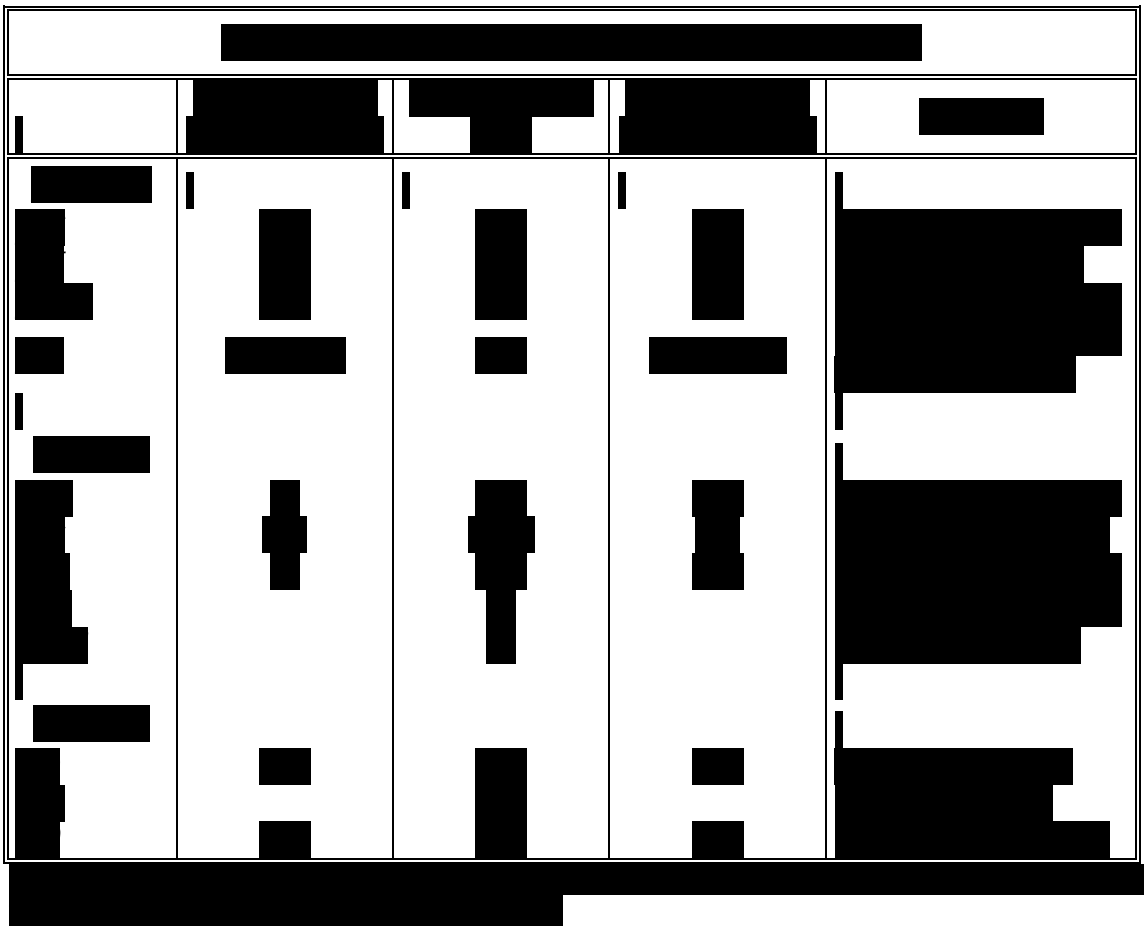
The notion that CRY is somehow involved also in the temperature mediated entrainment was supported by the recent study of Kaushik *et al.* (2007) who detected a physical interaction between CRY and PER/TIM also after a heat-pulse and flies bearing the severely hypomorhic cry^b mutation were unable to adjust their behavior after a phase-shifting heat pulse.

Another interesting factor connected to temperature (and temperature compensation) is that several of the clock gene mutants turned out to be temperature-sensitive with respect to their free-running periods. The per^L mutation causes the behavioral periodicities to lengthen with the rising temperature (from about 25 hrs at 15°C to almost 32 hrs at 29°C; e.g. Kaushik *et al.*,2007). Another per alleles cause the clock to run faster as the temperature is raised: per^S, per^T and

per^{SLIH} although for some of these mutants the temperature dependency is not very dramatic (reviewed by Hall, 2003).

Also the *tim* alleles were shown to be involved in the temperature sensitivity issue: for example *tim*^{rit} flies exhibit almost normal periods at 15°C but 35 hrs long at 30°C (Matsumoto *et al.*, 1999).

[redacted]



1.1.1.5. Anatomy of the clock

Per together with *tim* and other clock genes are expressed in about 150 neurons in the fly brain and they form about six major groups that were named according to their anatomical location. There are three clusters of dorsal neurons (DN1s, 2s, 3s), the ventrolateral neurons (LN_vs) and the dorsolateral neurons (LN_ds). Quite recently, lateral-posterior neurons (LPNs) have been described (see Fig. 1)

The LN_vs can be further subdivided in three groups: four small LN_vs (s-LN_vs) and four to five large LN_vs (l-LN_vs). Both these groups express the clock marker neuropeptide pigment-

dispersing factor (PDF) and are considered to be the main pacemaker cells responsible for the circadian behavior. Loss of these neurons (with pdf-GAL4 combined with UAS sequence fused to a cell killer factor) results in arrhythmic behavior in DD. In the vicinity of the lateral neurons there is a single cell that expresses PER but not PDF (the fifth s-LN_v).

The dorsolateral neurons (LN_ds) consist of six cells but only three to four of them express detectable amount of CRY which is also expressed in a subset of DN1s. (reviewed by Sandrelli *et al.*, 2009; Dubruille and Emery, 2008)

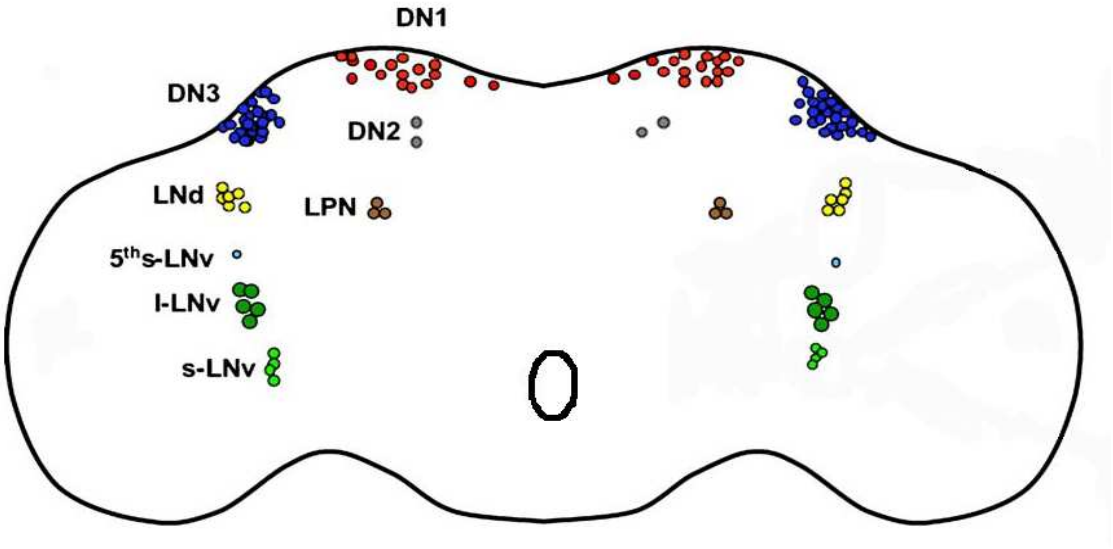


Fig. 3. The main clusters of cells expressing the clock factors in adult *Drosophila* brain (Modified from Dubruille and Emery, 2008)

1.1.2. Circadian clock in other insect species and mammals

Although *Drosophila* is undoubtedly the best-studied insect species from the viewpoint of (not only) biological timekeeping, it is already quite clear that it is not the best representative of the insect class as far as circadian clock system goes (and not only that). But before I get to this issue I think it would be beneficial to briefly introduce the mammalian-type of circadian clockwork first.

1.1.2.1. Mammals – the mouse

One of the most evident differences of the mammalian clocks, as opposed to the *Drosophila*'s system, is the redundancy of the clock components caused by gene duplication. So the key clock components are the *period* genes (*per1* and *per2*), the *cryptochrome* genes (*cry1*

and *cry2*) and the genes *Bmal1* (homolog of *Drosophila*'s *cyc*), *rev-erbα* and *Clock.* Mammalian *timeless* actually corresponds to the fly paralogue *timeout/tim2*.

The heterodimer BMAL1/CLK provides the positive drive to the cycle by binding to the E-box sequences in the *per*, *cry* and *rev-erbα* genes thus activating their expression. The PER and CRY proteins accumulate and subsequently dimerize in the cytoplasm and transfer to the nucleus to inhibit the activity of BMAL1/CLK and consequently their own transcription. The Rev-ERBα acts to inhibit the expression of *Bmal1* (which substitutes for *dClk* as the cycling component of the second feedback loop in this system), this repression is in turn counteracted via the PER/CRY interfering with the positive influence of BMAL1/CLK on the expression of the *rev-erbα* gene. Another element RORA functions as an activator of the *Bmal1* gene expression.

Apparently the overall logic behind the timekeeping system is the same both in mammals and *Drosophila*, but the construction details are curiously different. Especially notable is the switch in the role of cryptochromes which don't function as photoreceptors at all in the mammalian system and rather act as transcriptional repressors. Mammalian type of cryptochromes forms a different phylogenetic line than the *Drosophila* CRY and they are light insensitive. Interestingly, a second *cry* gene was recently discovered in several insect species which groups with the vertebrate-type of cryptochromes and in accord with its origin it does not respond to photic stimuli. (reviewed by Guilding and Piggins, 2007; Yuan *et al.*, 2007; Sandrelli *et al.*, 2008)

1.1.2.2. Other Diptera – the housefly

The most detailed study of circadian timekeeping in Dipterans other than *Drosophila* is probably the one performed on the common housefly, *Musca domestica*. Parameters of expression were determined for all the key clock factors known from Drosophila: *per*, *tim*, *vri*, *Clk*, *cyc* and *cry*. As expected, the first four exhibited a robust cycling in the levels of their corresponding mRNAs both in LD and DD with *md-Clk* oscillating in the opposite phase to the other three transcripts. Oddly enough, the expression of *cryptochrome* did not display any appreciable rhythms. Although the Western blot analysis as well as the initial immunohistochemistry using an enzymatic way of signal detection failed to reveal any cycling in the clock proteins PER and TIM (neither any shifts in mobility attributable to the posttranslational modifications of the proteins were observed), a more sensitive immunodetection method employing fluorescent antibodies disclosed oscillation in the subcellular localization of both TIM and PER in several neuronal groups. (Codd *et al.*, 2007)

In all, apart from the peculiar loss of rhythmic expression of the *dm-cry* gene, the reported results are in good agreement with the data obtained from experiments on *Drosophila*.

1.1.2.3. Lepidoptera

The molecular mechanism underlying the circadian timekeeping has been studied to some detail in three lepidopteran species – the giant silkmoth *Antherea pern*yi, the domestic silkmoth *Bombyx mori* and the monarch butterfly *Danaus plexippus*.

Homologs of all major clock components (*per*, *tim*, *Clk* and *cyc*) known from *Drosophila* have been identified also in these species (Sandrelli *et al*., 2008) and both the mammalian and the *Drosophila* type of *cry* genes were found in the genomes of the three Lepidopterans (Zhu *et al.*, 2005; Yuan *et al.*, 2007)

Both *per* and *tim* mRNAs appear to cycle in their abundance in head extracts and the rhythm persist also in DD (Reppert *et al.*, 1994, Froy et al., 2003, Iwai *et al.*, 2006)

The main pacemaker neurons seem to reside in the dorsolateral protocerebrum (four large cells in each hemisphere) but curiously neither PER nor TIM proteins were ever detected in the nuclei of these neurons (Sauman *et al.*, 2005; Sehadova *et al.*, 2004; Sauman and Reppert, 1996). In *Antherea* the cytoplasmic signals of both these proteins display rhythmic changes in intensity over the day (Sauman and Reppert, 1996), similar situation was observed also in the monarch butterfly (Sauman *et al.*, 2005, Zhu *et al.*, 2008) on the other hand, in *Bombyx* only the bmPER signal was found to oscillate but nevertheless it still remained stubbornly cytoplasmic (Sehadova *et al.*, 2004).

The failure to observe any of the clock components in the nucleus of course meant that no feedback loop that could control the circadian expression in a manner similar to *Drosophila* or mammals could be identified. This puzzling situation was finally resolved (at least for the monarch butterfly) in 2008 when Zhu and co-workers localized the vertebrate type of CRY protein (CRY2) in the nucleus of the pacemaker neurons in the early to middle night that is at times when repression of *dp-per* gene expression should occur. The dpCRY2 protein (but not dpPER) was proved to be able to inhibit the dpCLK/dpBMAL1-mediated transcription in a monarch embryonic cell line, while dpCRY1 (the Drosophila type of CRY) mediates the light-induced degradation of dpTIM. Interestingly a vast majority of monarch PER was localized in the nucleus of these embryonic cells, unfortunately the researchers were unable to confirm this pattern for the pacemaker cells in the brain due to high background staining produced by the available anti-PER antibody (Zhu *et al.*, 2008).

Information on the circadian clock system from other species is very scarce but yet it can offer some interesting insights. Judging by the searches through the genome sequences, both the honeybee (*Apis mellifera*) and the red flour beetle (*Tribolium castaneum*) lack the *Drosophila* type of *cryptochrome* and posses only the vertebrate type of this gene. Expression analyses of the clock components in *Apis mellifera* revealed similar robust pattern in cycling of the *am-cry* and *am-per* transcripts and a consistent oscillation in *am-cyc* mRNA levels that was almost antiphase to those of *cry* and *per*. No statistically significant and persistent rhythms in abundance were measured for either *am-Clk* or *am-tim* (Rubin *et al.*, 2006).

Given the evolutionary relations among the organisms mentioned above it seems likely that the ancestral circadian clock system included two different cryptochromes – one was the main clock repressor and one was relaying the photic information from the outside world to the core oscillator. It is also probable that the original main positive element in the transcriptional feedback loop was the protein BMAL1/CYCLE rather than CLOCK (Yuan *et al.*, 2007; Zhu *et al.*, 2008; Sandrelli *et al.*, 2008).

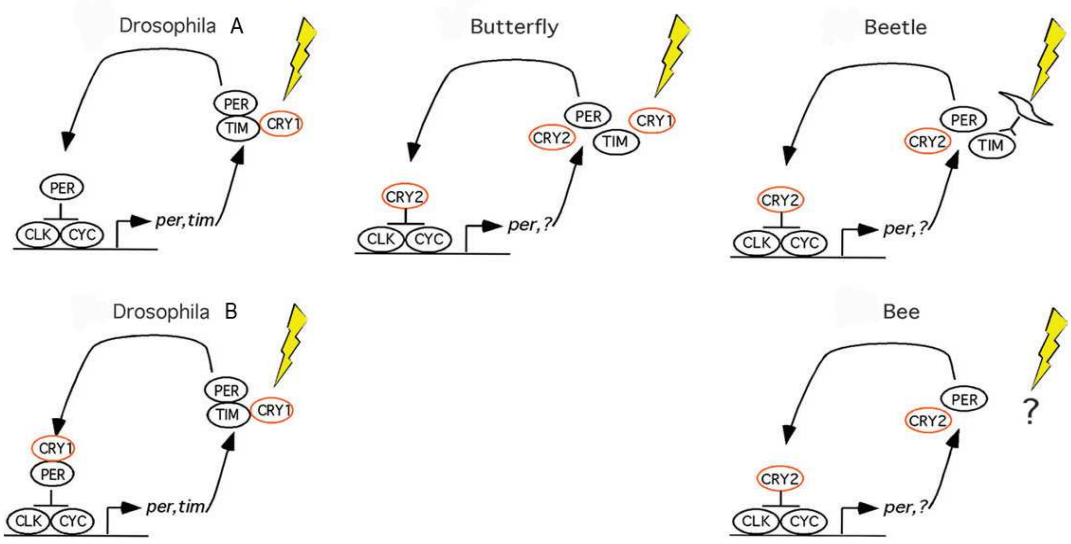


Fig. 4. Models of circadian timekeeping mechanisms as proposed by Yuan *et al.*, 2007. According to current hypotheses the butterfly model should represent the ancestral state of the clockwork and those of *Drosophila* and the beetle (*Tribolium*) and bee the derived versions with some of the original components missing and some adopting different roles. Drosophila A depicts the organization of the circadian clockwork in the central pacemaker cells in brain and Drosophila B in the peripheral oscillators.

2. Materials and Methods

2.1. *Drosophila* locomotor activity monitoring

2.1.1. Breeding and crossing of *Drosophila melanogaster*

Flies were raised on a standard cornmeal-agar medium supplemented with treacle and yeast at 25 °C.

[redacted]

balancer	marker gene	phenotype
TM6B	*humeral (Hu)*	extra humeral bristles are formed
CyO	*curly (Cy)*	wings curled upward
	stubble (Sb)	bristles of Sb/+ less than one-half normal length, somewhat thicker than wild type
	sternopleural (Sp)	sternopleural bristles increased in number

Tab. 2. Phenotypic display of the marker genes employed in this study

page 32 was omitted

2.1.2. Measurement of the locomotor activity periods and data analysis

To determine the locomotor activity periods of relevant fly strains, male flies 1 to 4 days old were placed individually into glass tubes containing food supply (sacharose, agar and water) which were than set into the Drosophila Activity Monitors - DAM2 (TriKinetics, Inc. (Waltham, USA) boards and placed into incubator units with defined light and temperature regimes. These boards were connected to a computer where the information about the locomotor activity of the individual flies was acquired and stored.

The data were analyzed using the flytoolbox (Levine *et al*., 2002b) of Matlab software to perform several different analyses: autocorrelation, MESA and periodogram. Since the periodogram analysis seemed to be the most reliable and accurate one, the free-running periods summarized in the Results were derived from this method only.

The results presented in the next chapter are based on data pooled from at least three independent experiments for each temperature condition.

2.2. Expression of *period* and *timeless* in *Tribolium castaneum*

2.2.1. Breeding of *Tribolium castaneum*

The beetles were raised on a medium made of whole-grain flour and yeast supplemented with antimycotic fumagilin B. Animals were reared under an LD cycle 12:12 (12 hrs of dark and 12 hrs of light) while the temperature was held constant at 26°C.

2.2.2. Real-time RT PCR

Sixty larval or adult heads were collected on dry ice at appropriate times (ZT 0, ZT 4, ZT 8, ZT 12, ZT 16, ZT 20 and ZT 24; ZT 0 and 24 – lights on, ZT 12 – lights off). Total RNA was then isolated from these samples using the TRIzol reagent (Sigma) following the manufacturer's recommendations. The quality of the extracted RNA was checked on denaturizing RNA agarose gel containing formaldehyde.

Three micrograms of extracted totRNA were used in the subsequent reverse transcription reactions using the SuperScript II or III reverse transciptases from Invitrogen and oligo(dT) primers. The conditions of these reactions were set according to the protocol supplied by the manufacturer.

The quantitative PCR itself was performed on a microwell plate-based cycler from Roche Diagnostics GmbH, the LightCycler 480 system. A total of 5 µl of cDNA (diluted 50 times) was used in each 20 µl-reaction mix (LightCycler 480 SYBR green I Master mix, 1x conc.; primers 400 nM each and milliQ water). Amplifications were carried out following this program: pre-denaturation, 95°C for 10 min; amplification – denaturation 95°C, 10 sec, annealing 58°C, 10 sec, elongation 72°C, 20 sec – 35 cycles.

Real - time RT PCR primers			
gene transcipt	**primer**	**primer sequence**	**length of amplicon (bp)**
period isoA	per fwd1	5' GAGAGTTTTCAGTTGAACCAAAAG*ATG 3"	351
	per rev1	5' GATCAG*CCAATGCTCGTAAAC 3'	
period isoB	per fwd2	5' CCAGTGTGGCCAATAAG*ATG 3'	344
	per rev1	5' GATCAG*CCAATGCTCGTAAAC 3'	
timeless isoA	tim fwd1	5' CCTGTCATCTACTACTTTGCTC*TAC 3'	395
	tim rev1	5' CCTT*ACCGAATTCGACTGAATGG 3'	
timeless isoB	tim fwd1	5' CCTGTCATCTACTACTTTGCTC*TAC 3'	385
	tim rev2	5' CCCTT*TGCTCATCCTCTTTCG 3'	
rp49	rp 49 fwd1	5' CGTTATGGCAAACTCAAA*CGC 3'	189
	rp 49 rev1	5' CAAGG*AACTGGAAGTGTTGTTG 3'	

Tab. 3. Information on primers used for the real-time PCR experiments. The asterisk (*) denotes the position of an intron

Clock genes and the housekeeping gene *rp 49* were amplified in separate wells in triplicates, similarly to the controls (no-template control or reactions with genomic DNA or plasmid containing a known *tim* or *per* isoform sequence as substrates).

The relative quantification mode was chosen for this study meaning that the amount of a specific target molecule is determined by relating it to the amount of another (reference) transcript whose expression does not change during the day (a housekeeping gene). The quantification method takes advantage of the correlation between fluorescence (in this case generated by binding of SYBR green I dye to double-stranded DNA molecules) and the amount of PCR product. The crossing point (Cp) is then determined for each transcript. The Cp value corresponds to the exponential phase of the PRC reaction and represents the cycle at which the fluorescence exceeds background. The amount of PCR products at this point should be the same in every reaction. Obviously, the higher is the initial number of template copies; the sooner reaches the PCR reaction the crossing point. Therefore, comparing the Cp values of target and reference genes for a specific sample (and taking into account differences in the amplification efficiencies of each template) yields relative proportions of these gene products in this sample.

Data obtained from the real-time PCR experiments were analyzed with the LightCycler 480 software to determine the Cp value for each target. The reaction efficiency for each substrate

was derived from a standard curve based on a series of dilutions (10x, 20x, 30x, 50x, 75x and 100x) of the appropriate cDNA. The standard curves were constructed for three independent experiments using both larval and adult cDNA samples. Since the resulting efficiencies for particular transcripts did not substantially differ; the mean efficiency was used for subsequent data analyses. The quantification results were normalized to the ZT 0 samples.

2.2.3. *In situ* hybridization

We decided to employ the non-radioactive method of mRNA detection using single-stranded RNA probes labeled with digoxigenine (DIG) attached to the uracil base. Such a probe can be then detected with an *anti*-DIG antibody.

Probe generation

A sequences of about 1500 bp in length that were chosen for the probes construction were amplified from a cDNA sample by a standard PCR using specific primers (listed in Table 4) and ExTaq Hot Start polymerase (Takara). The PCR products were then cloned into pGem-T*easy* cloning vector (Promega) following the manufacturer's recommendations. Because the pGem vector lacks the T3 promoter site which is better suited for *in vitro* transcription than the Sp6 site contained in the vector sequence, the gene fragments were cut out from the pGem-T*easy* plasmid using appropriate restriction enzymes and cloned into pBluescript KS (-) vector. The orientation of the cloned sequence was verified by PCR and sequencing.

***In situ* hybridization primers**			
gene transcipt	**primer**	**primer sequence**	**probe length (bp)**
period	per fwd0	5' CACACCATGACTACTACGACAG 3"	1595
	per rev1	5' GATCAG*CCAATGCTCGTAAAC 3'	
timeless	tim fwd0	5' TCGTTACGTACTTCCTCAAATTCG 3'	1597
	tim rev1	5' CCTT*ACCGAATTCGACTGAATGG 3'	

Tab. 4. Information on primers used for the *in situ* hyybridization experiments. The asterisk (*) denotes the position of an intron

For the *in vitr*o transcription itself, the relevant fragment was amplified from the plasmid vector using the M13 universal primers and the PCR products were subsequently transcribed by T3 or T7 RNA polymerases resulting in *antisense* or *sense* RNA probes. So called cold reaction (containing no labeled nucleotides) was run along with the labeling ones and this cold reaction was then separated in 1% denaturizing agarose gel to assess the length and quality of the newly made probes.

The quality of the generated probes were further examined by Spot-blot method on a membrane to make sure the probes were properly labeled with the DIG-U nucleotides and can be thus detected by *anti*-DIG antibody.

Sample preparation

Whole heads of adult beetles were dissected at ZT 8 (the timepoint identified by the real-time PCR analysis as the one that corresponds to the highest expression rate of target genes), immediately put into the fixing solution (4% formaldehyde in PBS, pH 7,5) and left there for 18-20 hrs at 4°C. The tissues were then brought through a dehydrating ethanol series and chloroform (70% EtOH – 90% EtOH – 100% EtOH – chloroform) into paraplast. The samples were incubated in the melted paraplast in a vacuum oven overnight at 58°C to ensure its complete penetration into the tissue. After positioning and cooling at RT the samples were cut to 9 µm thick vertical sections and attached on SuperFrost ULTRA Plus microscopic slides (Thermo/Menzel Glässer).

In situ hybridization procedure

The mRNA locator kit from Ambion was used for *in situ* hybridizations - the manufacturer's protocol was generally followed, apart from several modifications. The samples were first deparaffinized in xylene and then brought through a rehydrating ethanol series (100% EtOH – 90% EtOH – 80% EtOH – 70% EtOH) into distilled water and washed in PBS (pH 7,5), in some cases a post-fixation step (3,7 % formaldehyde/ 20-5 min at RT) was inserted after the washing. The slides were then deproteinized in 0,2 N HCl; 20 min, RT and/or Proteinase K solution. Sections were subsequently treated with triethanolamine and acidic anhydride solutions, washed in PBS and then incubated in pre-hybridization solution for 4 hrs at 58-60°C.

The probes were then diluted in the hybridization solution (1:50 – 1:200) and denaturized at 65-90°C. The slides were incubated with the diluted probes O/N at 58-60°C in a water bath. Next day, the slides were washed several times in 4x and 2xSSC (sodium chloride, tri-sodium citrate dehydrate) solutions of decreasing concentration at 58-60°C followed by RNase A treatment (30 min, 37°C). After this treatment the samples were washed again in even less concentrated (0,1x) SSC and subsequently in PBS supplemented with 0,3% detergent Triton X-100 (PBS-Tx). To prevent unspecific binding of the anti-DIG antibody, the tissues were blocked in 10% normal goat serum in PBS-Tx (30 min at RT) and then incubated overnight at 4°C with sheep-anti-DIG antibody conjugated to alkaline phosphatase, AP (Fab fragments; Roche Diagnostics), diluted 1:500 in PBS-Tx. Next morning, the slides were washed several times in PBS-Tx and the signal was visualized using the BCIP/NBT substrate (Perkin Elmer), the reaction was stopped in distilled water.

Stained sections were dehydrated again through an ethanol series (70% EtOH – 96% EtOH − 100% EtOH), brought to xylene and then mounted in DPX mounting medium (Fluka). Samples were examined and photographed using the Zeiss Axioplane 2 microscope equipped with CCD camera.

2.2.4. Northern blot

The Northern blot method was performed to verify the ability of *per* and *tim* probes to bind their target sequences.

The total RNA isolated with TRIzol reagent from adult heads was mixed with formaldehyde load dye and denaturized at 65°C for 15 min, the samples were then separated in 1% denaturizing agarose gel. The RNA was then transfered onto positively charged nylon membrane (SuPerCharge Nylon) using the TurboBlotter capillary transfer apparatus (Schleicher&Shuell). The membrane was cross-linked by exposure to UV light and prehybridized in the ULTRAhyb solution at 68°C for 1 hr (NorthernMax kit, Ambion). After prehybridization treatment either the *sense* or *antisense* probe was added into the ULTRAhyb solution and the hybridization was left to proceed overnight at 68°C in a hybridization oven. Next morning, the membranes were washed in low and high stringency washing buffers (NorthernMax kit, Ambion) and then in PBS-Tx and subsequently they were incubated with a blocking solution (5% nonfat dry milk, 5% bovine serum albumine, BSA, in PBS-Tx) for 2 hrs at RT. Following the blocking step, the enzyme-conjugated antibody (either the sheep-anti-DIG-AP or the goat-anti-DIG-horseradish peroxidase, HRP, both Roche) was added into the blocking solution (dilutions: 1:2500 for Sh-anti-DIG-AP and 1:1000 for G-anti-DIG-HRP) and the membrane was left in the antibody solution for another two hours (at RT). The signal was visualized by the reaction of the particular enzyme (conjugated to the antibody) and its corresponding substrate resulting either in a colored precipitate (AP+ BCIP/NBT substrate – Perkin Elmer) or light emission (SuperSignal West Dura – Pierce – or Immobilon Western – Millipore Corp. – chemiluminiscent substrates for HRP enzyme). The chemiluminiscent signal was detected using the Fujifilm LAS 3000 luminoimager.

2.2.5. Western blot analysis

Protein extraction

Whole heads were dissected from CO_2-anesthesized animals on dry ice at ZT 20 (this timepoint should correspond to high protein levels) and placed immediately into an ultra-low-temperatures freezer and stored at -80°C. Upon use, the tissues were homogenized in a triple-detergent lysis buffer (50 mM Tris-Cl, pH 8.0; 150 mM NaCl; 0.1% SDS; 1.0% Nonidet P- 40; 0.5% sodium deoxycholate and 0,02% sodium azide) supplemented with Roche cocktail of protease inhibitors (sample/buffer volume ratio: 1:1 After adding appropriate volume of 5x dyed gel loading buffer, the samples were boiled for 10 min, sonicated (1.5 min at full power) and

centrifuged for 5 min at 14 000g and 4°C. The sample was loaded on the gel (5 µl/well if not stated otherwise) or stored at -80°C.

Gel running conditions

A standard SDS/PAGE procedure and buffers were employed in this study: 12% SDS/polyacrylamide gel was run using Tris-Glycine-SDS buffer under constant current of about 15mA. To estimate the protein molecular weights either the Chemiluminiscent BlueRanger marker (Pierce) or the Dual color marker (Biorad) was run on the same gel.

Tank electroblotting

After electrophoresis, the proteins were transferred by wet tank electroblotting onto a PVDF membrane. The transfer was performed in the Mini Trans-Blot cell (Biorad) using the 25 mM Tris, pH 8.3, 192 mM glycine, with 20% MeOH and 0.1% SDS transfer buffer. The electrotransfer was left to proceed for about 1 hr under constant voltage of 100V (current of about 350 mA).

Immunostaining

After immunoblotting, the membrane was blocked with 5% non-fat dry milk, 5% BSA diluted in PBST, overnight at 4 °C. Next day, the blocking was followed by incubation with primary antibody diluted in the blocking buffer for 2 hrs at RT. After washing in PBST (5 times for 10 min) the membrane was incubated with HRP-conjugated secondary antibody (Pierce Stabilized secondary antibody diluted 1:1,000) for 2 hrs at RT. Subsequently, the membrane was washed in PBST again (5 times for 10 min) and then treated with the SuperSignal West Dura chemiluminiscence substrate (Pierce); the reaction was then visualized and photographed using Fujifilm LAS 3000 Intelligent Dark Box luminoimager.

2.2.6. Immunocytochemistry on paraplast sections

Sample preparation

Whole heads were dissected from CO_2-anesthesized animals in chilled Ringer's solution at ZT 20, ± 0,5 hr (this timepoint should correspond to high protein levels). The tissue was immediately submerged into a modified Bouin-Hollande fixing solution (0.7% mercuric chloride, no acetic acid) and incubated for 12 to 24 hrs at 4 °C. The fixed tissues were then brought through a dehydrating ethanol series and chloroform (70% EtOH – 96% EtOH – 100% EtOH – chloroform) into paraplast. To ensure its complete penetration into the tissue the samples were incubated in a melted paraplast in a vacuum oven overnight at 58 °C. After positioning and cooling at room temperature (RT) the samples were cut into 7-10 µm thick sections and attached

onto microscopic slides. The sections were then dried on a hot plate (45°C) for at least 48 hrs and used immediately or stored refrigerated until use.

ICC procedure

The slide were first deparaffinized in xylene and then brought through a rehydrating ethanol series (96% EtOH – 70% EtOH) into distilled water. To remove residual heavy metal ions from the fixing solution the slides were treated with 'de-Zenker' solution (70% EtOH containing 0.5% iodine) and subsequently with 7.5% sodium thiosulfate. Next, the slides were washed in distilled water and phosphate-buffered saline supplemented with 0.3% Tween 20 (PBST). The slides were then blocked in 10% goat normal serum in PBST for 30 min at RT in order to prevent unspecific antibody binding. After this, the samples were incubated with a primary antibody (guinea pig-*anti-tribolium castaneum* TIM; Gp-*anti-tc*PER; rat-*anti-tc*TIM and rat-*anti-tc*PER – all diluted 1: 50, 100 or 200 in PBST) in a humidified chamber overnight at 4°C. Following day, the slides were rinsed thoroughly with PBST and than incubated with goat *anti*-Gp or *anti*-rat IgG secondary antibody conjugated to a fluorochrome (both Invitrogen, diluted 1:750 in PBST) for 1 hr at RT. Subsequently the slides were washed in PBST again. Stained sections were dehydrated through an ethanol series (70% EtOH – 96% EtOH – 100% EtOH), treated with xylene and then mounted in DPX mounting medium (Fluka). Samples were examined and photographed using the Zeiss Axioplane 2 microscope equipped with Nomarski (DIC) optics and a CCD camera.

3. Results

3.1. *Drosophila* locomotor activity monitoring

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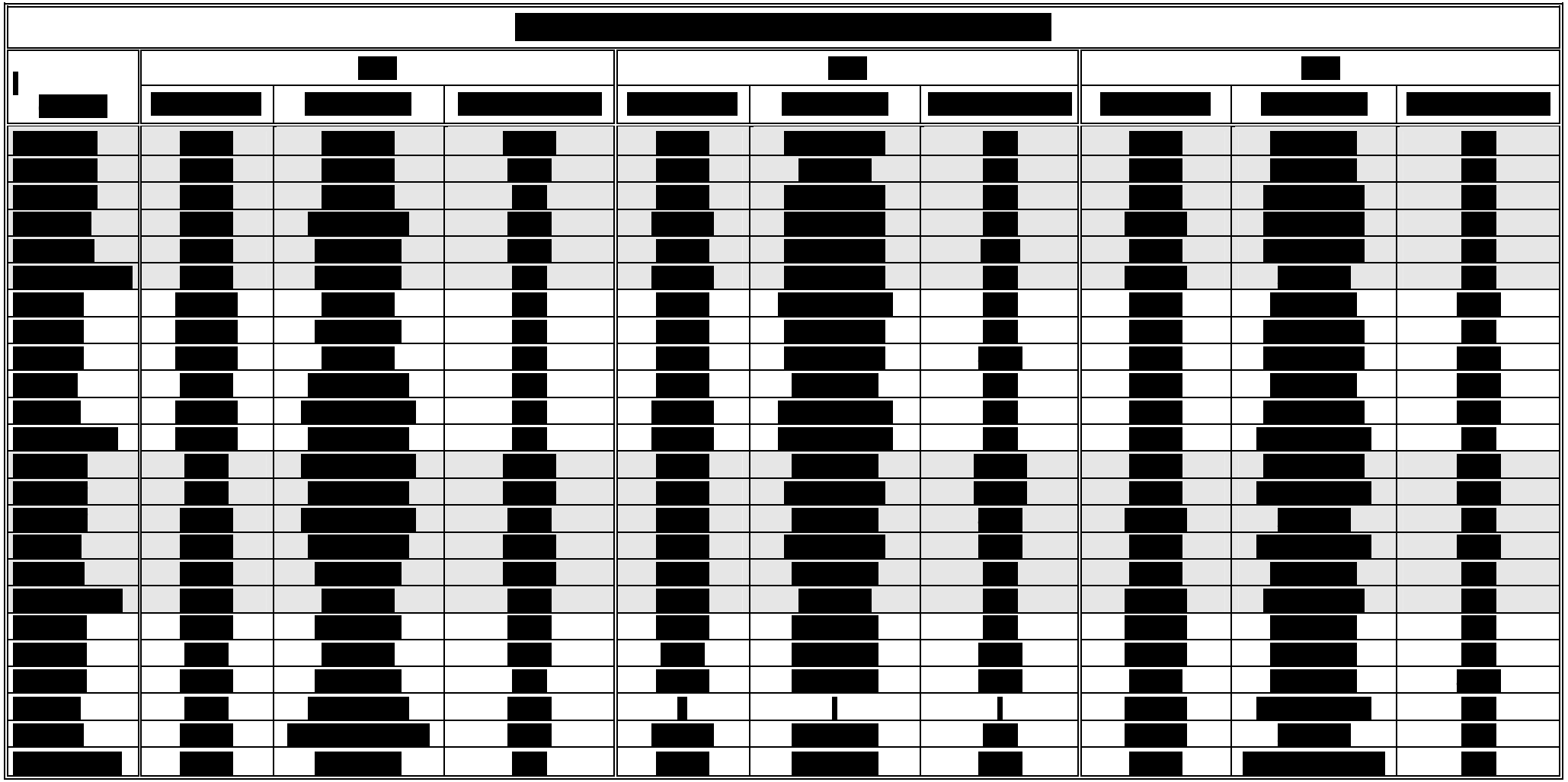

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3.2. Expression pattern of *period* and *timeless* in the beetle *Tribolium castaneum*

A search through the *Tribolium* genomic database revealed that all the major clock components known from *Drosophila* are also present in the genetic information of *Tribolium*. The sequences of *Clock, cycle, period, timeless, cryptochrome* and *clockwork* seem to be well conserved and there is no obvious indication that their expression or function should be impaired in any way.

3.2.1. Real-time RT PCR

Over the course of this study I found that both *timeless* and *period* genes give rise to at least two different splice variants of mRNA. These splice variants differ by one short exon in the 3' region − the *perA* isoform includes an extra exon at positions 2804-2922 and *timA* isoform contains an exon at positions 3021-3104 that is lacking in the *timB* variant.

Expression of both of these variants for each gene was measured in larval or adult head extracts from samples collected at 4hrs-intervals for a period of 24 hrs.

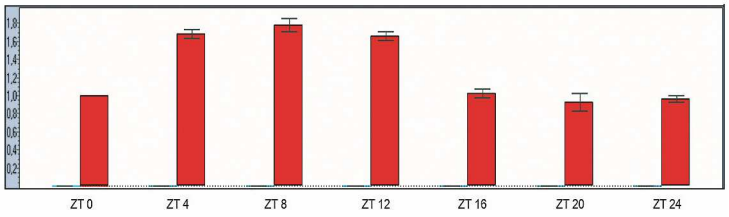
We decided to assess the abundance of the relevant transcript by comparing it to the expression of a housekeeping gene *ribosomal protein 49 (rp49)* which should be stable throughout the day.

Figures 8 - 9 show typical expression pattern obtained for all four transcripts in adult and larval head extracts. Only the variant *perA* seem to display significant oscillation in daily abundance; which was apparent especially in adult samples (Fig. 8).

In larvae, the expression of *per* as well as *tim* seems to be much lower than in adults; especially the expression of *perA* seem to be beyond the sensitivity range of our assay. While in larvae the typical Cp value (indicates the number of cycle in which the intensity of fluorescence exceeds the background level) fluctuates around 30 (see tables in Fig. 8-9 for details), in adults this indicator usually reads a value of 21-22 (26 for *perA*). This huge difference cannot be assigned to overall low expression rates in larval samples as the expression of *rp49* is quite comparable (19-21 in larvae versus 18-20 in adults).

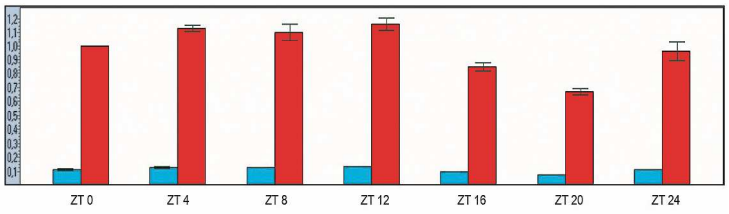
From all the transcripts in question the *perA* isoform is by far the least abundant; one can speculate that this might be caused by its expression being restricted to only few cells, supposedly the core pacemaker neurons.

A



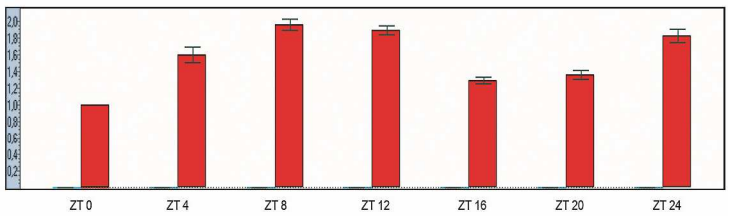
	Name		Target	Ref.	Ratios	
Sample	Target	Ref.	Mean Cp	Mean Cp	Target/Ref	Norm.
zt 0	*perA*	*rp49*	26,66	18,54	3,12 E-3	1,000
zt 4	*perA*	*rp49*	25,83	18,49	5,25 E-3	1,683
zt 8	*perA*	*rp49*	26,06	18,82	5,54 E-3	1,776
zt 12	*perA*	*rp49*	26,09	18,74	5,17 E-3	1,657
zt 16	*perA*	*rp49*	26,84	18,76	3,20 E-3	1,026
zt 20	*perA*	*rp49*	27,02	18,79	2,90 E-3	0,9298
zt 24	*perA*	*rp49*	26,82	18,64	3,01 E-3	0,9633

B



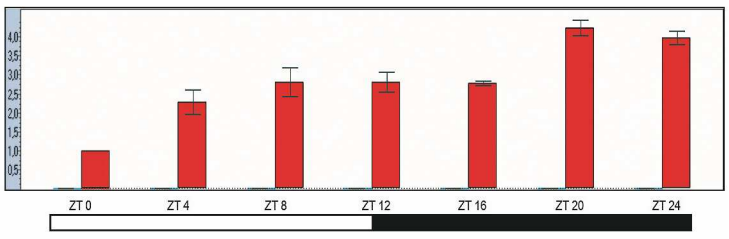
	Name		Target	Ref.	Ratios	
Sample	Target	Ref.	Mean Cp	Mean Cp	Target/Ref	Norm.
zt 0	*perB*	*rp49*	20,75	18,54	0,1147	1,000
zt 4	*perB*	*rp49*	20,60	18,57	0,1295	1,129
zt 8	*perB*	*rp49*	20,85	18,80	0,1265	1,103
zt 12	*perB*	*rp49*	20,60	18,62	0,1333	1,163
zt 16	*perB*	*rp49*	21,02	18,57	9,70 E-2	0,8456
zt 20	*perB*	*rp49*	21,46	18,67	7,68 E-2	0,6694
zt 24	*perB*	*rp49*	20,88	18,61	0,1096	0,9561

C



	Name		Target	Ref.	Ratios	
Sample	Target	Ref.	Mean Cp	Mean Cp	Target/Ref	Norm.
zt 0	*perA*	*rp49*	35,00	20,63	4,75 E-5	1,000
zt 4	*perA*	*rp49*	35,00	21,36	7,60 E-5	1,599
zt 8	*perA*	*rp49*	35,00	21,67	9,29 E-5	1,956
zt 12	*perA*	*rp49*	35,00	21,62	8,99 E-5	1,891
zt 16	*perA*	*rp49*	35,00	21,03	6,16 E-5	1,296
zt 20	*perA*	*rp49*	35,00	21,11	6,47 E-5	1,361
zt 24	*perA*	*rp49*	34,48	21,02	8,63 E-5	1,816

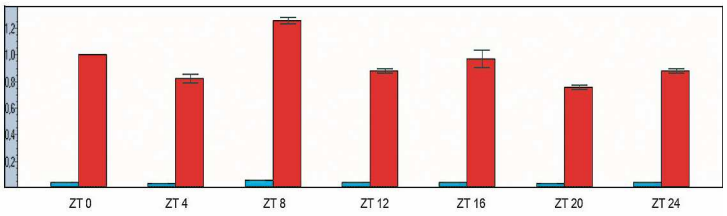
D



	Name		Target	Ref.	Ratios	
Sample	Target	Ref.	Mean Cp	Mean Cp	Target/Ref	Norm.
zt 0	*perB*	*rp49*	31,72	20,70	2,67 E-4	1,000
zt 4	*perB*	*rp49*	21,24	21,46	6,07 E-4	2,268
zt 8	*perB*	*rp49*	31,08	21,62	7,53 E-4	2,814
zt 12	*perB*	*rp49*	31,14	21,69	7,53 E-4	2,817
zt 16	*perB*	*rp49*	30,78	21,29	7,45 E-4	2,787
zt 20	*perB*	*rp49*	30,08	21,21	1,13 E-3	4,233
zt 24	*perB*	*rp49*	30,05	21,09	1,07 E-3	3,986

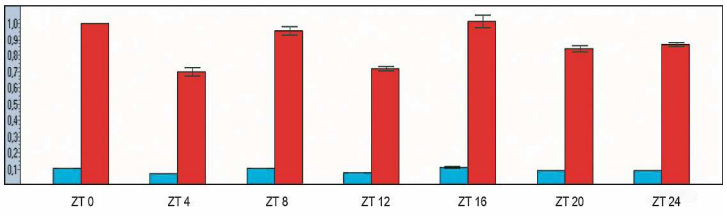
Fig. 5. Typical real-time PCR outcomes for the *period* isoforms A and B in adult larval head extracts and the corresponding Cp values
A – *perA* variant in adult samples; B – *perB* variant in adult samples; C – *perA* variant in larval samples; D – *perB* variant in larval samples
The bar denotes the light regime (white – lights on, black – lights off)

A



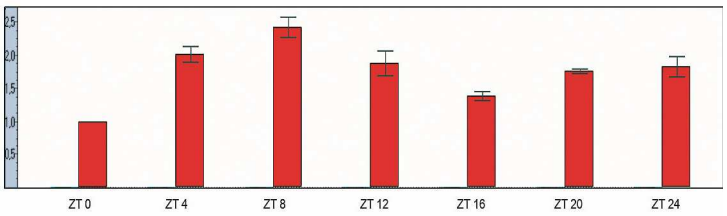
	Name		Target	Ref.	Ratios	
Sample	Target	Ref.	Mean Cp	Mean Cp	Target/Ref	Norm.
zt 0	*timA*	*rp49*	22,43	18,44	4,30 E-2	1,000
zt 4	*timA*	*rp49*	22,71	18,43	3,53 E-2	0,8208
zt 8	*timA*	*rp49*	22,21	18,56	5,42 E-2	1,259
zt 12	*timA*	*rp49*	22,85	18,68	3,79 E-2	0,8812
zt 16	*timA*	*rp49*	22,57	18,54	4,18 E-2	0,9722
zt 20	*timA*	*rp49*	23,10	18,70	3,25 E-2	0,7555
zt 24	*timA*	*rp49*	22,86	18,68	3,79 E-2	0,8805

B



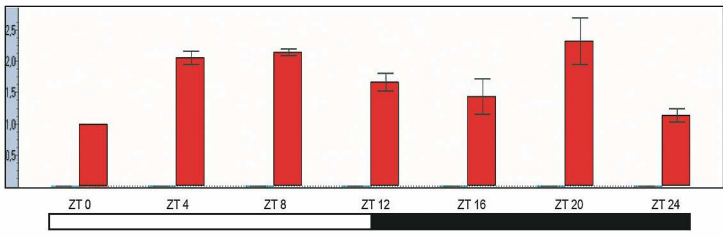
	Name		Target	Ref.	Ratios	
Sample	Target	Ref.	Mean Cp	Mean Cp	Target/Ref	Norm.
zt 0	*timB*	*rp49*	20,96	18,00	0,1076	1,000
zt 4	*timB*	*rp49*	22,16	18,66	7,52 E-2	0,6993
zt 8	*timB*	*rp49*	21,87	18,86	0,1030	0,9574
zt 12	*timB*	*rp49*	22,22	18,77	7,73 E-2	0,7588
zt 16	*timB*	*rp49*	21,98	19,06	0,1087	1,011
zt 20	*timB*	*rp49*	22,94	19,75	9,01 E-2	0,8376
zt 24	*timB*	*rp49*	22,22	19,07	9,33 E-2	0,8675

C



	Name		Target	Ref.	Ratios	
Sample	Target	Ref.	Mean Cp	Mean Cp	Target/Ref	Norm.
zt 0	*timA*	*rp49*	30,88	19,50	3,01 E-4	1,000
zt 4	*timA*	*rp49*	29,97	19,63	6,03 E-4	2,005
zt 8	*timA*	*rp49*	29,96	19,92	7,29 E-4	2,425
zt 12	*timA*	*rp49*	30,60	20,18	5,65 E-4	1,878
zt 16	*timA*	*rp49*	30,65	19,77	4,19 E-4	1,392
zt 20	*timA*	*rp49*	30,34	19,81	5,29 E-4	1,760
zt 24	*timA*	*rp49*	29,93	19,45	5,51 E-4	1,834

D



	Name		Target	Ref.	Ratios	
Sample	Target	Ref.	Mean Cp	Mean Cp	Target/Ref	Norm.
zt 0	*timB*	*rp49*	32,68	20,56	2,05 E-4	1,000
zt 4	*timB*	*rp49*	32,38	21,38	5,17 E-4	2,062
zt 8	*timB*	*rp49*	32,52	21,59	5,39 E-4	2,152
zt 12	*timB*	*rp49*	32,91	21,60	4,20 E-4	1,675
zt 16	*timB*	*rp49*	32,73	21,17	3,59 E-4	1,435
zt 20	*timB*	*rp49*	31,80	20,97	5,83 E-4	2,328
zt 24	*timB*	*rp49*	32,88	20,95	2,84 E-4	1,132

Fig. 6. Typical real-time PCR outcomes for the *timeless* isoforms A and B in adult larval head extracts and the corresponding Cp values

A – *timA* variant in adult samples; B – *timB* variant in adult samples; C – *timA* variant in larval samples; D – *timB* variant in larval samples

The bar denotes the light regime (white – lights on, black – lights off)

3.2.2. *In situ* hybridization and Northern blot

After determining the temporal pattern of *timeless* and *period* expression, we wanted to look into its spatial expression in adult head sections.

Unfortunately, not much of a success has been achieved in this matter. I tried to detect the *per* and *tim* expressing cells by a specific DIG-labeled RNA probes but I was unable to reveal any cells where the obtained signal could be considered as reliably specific.

The target cells where *anti-per* and *anti-tim* probes hybridized were confined to two main regions – the suboesophageal ganglion and distal parts of lateral protocerebrum. The number of cells stained in the SOG was suspiciously high (tens of neurons) and the same region was marked also by the sense probes. The same holds true for the protocerebral region as well though the number of positive cells was much lower (5-10 in each hemisphere) and somewhat variable. This staining pattern turned out to be quite persistent as it didn't change much under different experimental conditions I have tried so far.

The overall background staining was also quite high, however diluting the probes did not help to significantly decrease the background − it seemed to just lengthen the time needed for the signal to develop.

To make sure that the probes I was using are actually capable of detecting its target I performed the Northern blot analysis. The anti-*per* probe worked just fine, it revealed a single faint band of approximately the right size and no signal was observed for the sense probe (Fig. 10). As to the *tim* probes I was unable to detect any signal for neither the *antisense* nor the *sense* probe with none of the detection methods described in the Materials and Methods section. This could be caused by the fact that the concentration of the total RNA, which was used as a substrate for *tim* mRNA detection was somewhat lower than the one used for the experiments on *period*.

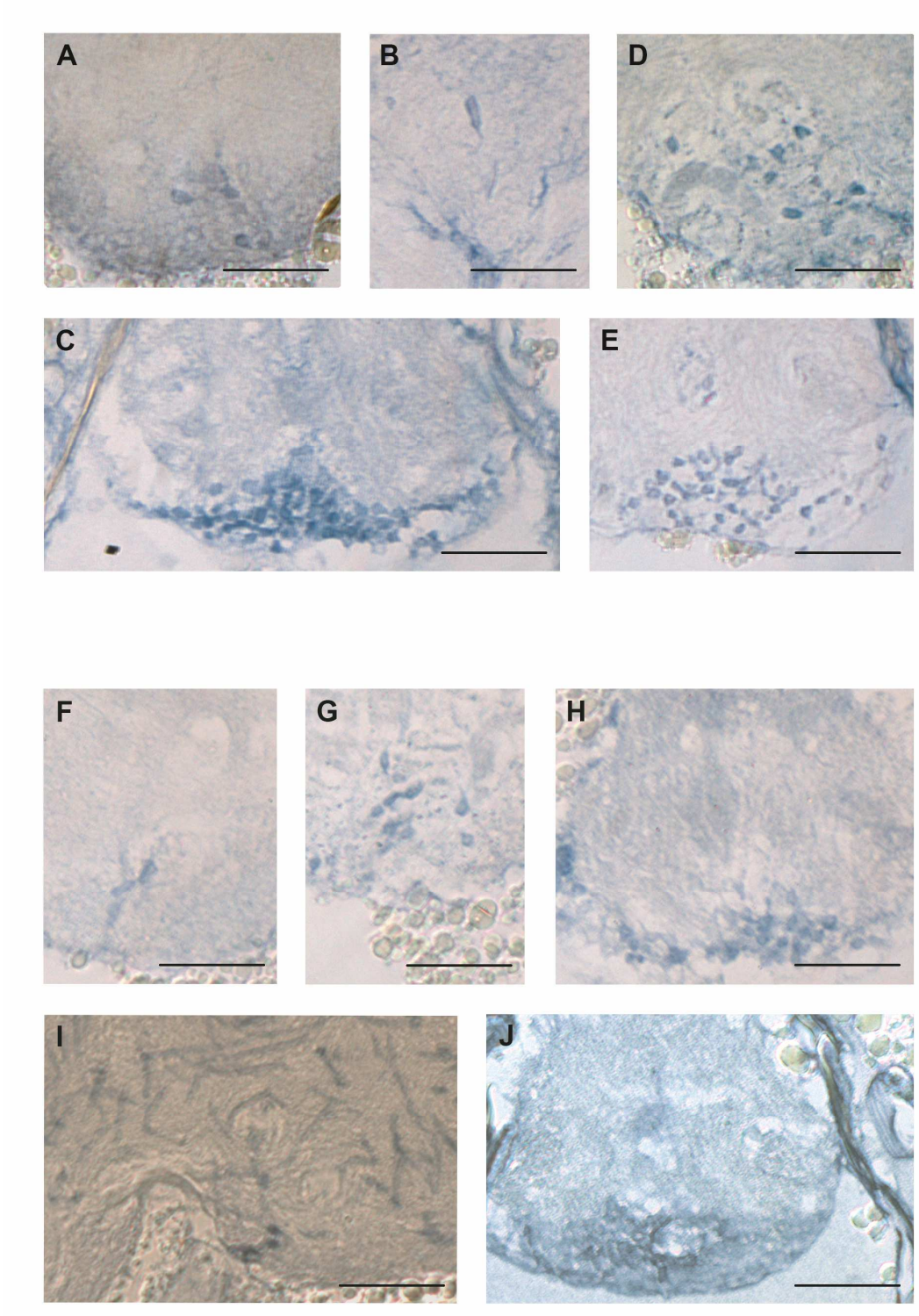


Fig. 7. The *in situ* hybridization results.
A-E – typical pattern obtained with *timeless* probes. A-C – *tim* sense probe; D+E – *tim antisense* probe.
F-J – typical pattern obtained with *period* probes. F-H – *per sense* probe; I+J – *per antisense* probe
A,B,D,F,G,I – cells in lateral protocerebrum; C,E,J – signal in suboesophageal ganglion
Scale bars – 50 µm

3.2.3. Immunodetection

I also tried to localize the TIMELESS and PERIOD proteins in the *Tribolium* head sections and on the Western blots using custom-made antibodies raised against short synthetic peptides.

Given the fact that all the antibodies employed in this study are polyclonal and therefore there was a good chance they should work on the denaturized proteins I first attempted to asses their specificity on Western blots. Out of the total of four antibodies only the Gp-*anti*-TIM produced a distinct signal. This antibody revealed single band of about 75 kDa – that is, however, much less than would be expected based on the expected protein sequence (124 kDa). The rest of the antibodies produced no significant staining, only very faint background.

I tried these antibodies on paraplast tissue sections too though these experiments should be regarded as just an initial testing of the antisera. The most consistent signal was produced by the aforementioned Gp-*anti*-TIM – four cells were repeatedly detected in the *pars intercerebralis*. However, similar pattern was observed also in samples treated with the pre-immunization serum that was used as a control.

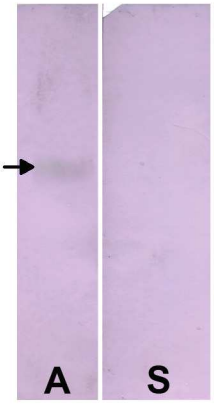


Fig. 8. Northern blot results obtained with the *per*-targeted probes
A – *antisense* probe; S - *sense* probe
The band marked by the arrow had about 3500 bp in size

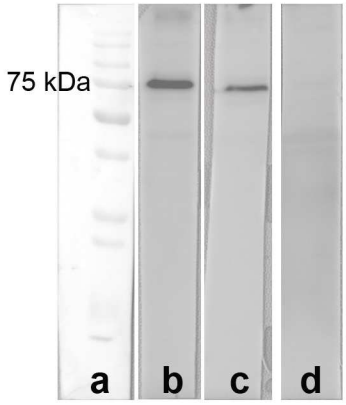


Fig. 9. Western blot results obtained with the Gp-*anti*-TIM and Gp-*anti*-PER antibodies
a – Dual Color marker (Biorad)
b+c – Gp-*anti*-TIM diluted 1:400 (b) and 1:800 (c)
d – Gp-*anti*-PER diluted 1:400

4. Discussion

4.1. *Drosophila* locomotor activity monitoring

4.2. Expression of *timeless* and *period* genes in the beetle *Tribolium castaneum*

I have attempted to specify both the temporal and spatial expression profiles of two of the core clock components known from the well-established circadian research model *Drosophila melanogaster* in the new emerging insect model species *Tribolium castaneum*.

I have succeeded only partially in this matter: While we obtained some information on the relevant mRNA abundances in time (at least for the splice variants known so far), my attempts to localize the *per*- and *tim*-expressing neurons in the adult brains have failed.

As to the real-time PCR experiments, two major observations have been made: First, except for the *per A* splice variant, none of the transcripts under study displayed significant circadian oscillation. Second, expression of all four isoforms of *per* and *tim* is much lower in larval than in adult heads.

It is known that in *Drosophila* both *per* and *tim* produce (at least) two splice-variants of their corresponding mRNAs. As yet, more information has been gathered on the splicing of the *period* mRNA. The splice variants A and B differ by one exon (no. 8) in the 3' region. The ratio between the spliced and unspliced variants is dependent on the ambient temperature and is implicated in the mechanism of temperature compensation of the circadian clock system. What is important to note however, is that both these variants oscillate with similar phase in *Drosophila* head extracts (Majercak et al., 2004). The *tim* splice variants also differ by one exon that is more frequently retained at low temperatures (tim^{cold} transcript). Again, both these variants cycle in their daily abundance, but the phase of expression of the tim^{cold} variant is delayed relative to the expression of the spliced *tim* version as well as *per* transcript (reviewed by Dubruille et al., 2008). The question whether the alternative splicing of *Tribolium per* and *tim* gene products is somehow connected to temperature cannot be answered here as this was not the subject of this study and therefore the temperature was held constant in all experiments on *Tribolium* presented here. What I am trying to point out is that even if this was the case we would still expect (by analogy with *Drosophila*) both the splice variants to behave in a similar way. The fact that *timeless* gene expression is not cycling isn't actually that surprising, because it is presumed that it is

cryptochrome rather than *timeless* that should serve as the core circadian repressor in the circadian clock of the beetle (see Yuan et al., 2007). There is of course another possibility: since I used whole head extracts for the real time PCR analysis we have to keep in mind that the resulting signal might not come from the brain cells solely but also from the photoreceptors or other tissues. One can imagine that the cycling of *tim* and/or *per* variants in several core clock neurons might be easily masked by its stable (or shifted) production elsewhere, by this logic we would have to presume that the *per A* variant is primarily produced in the clock neurons leaving its circadian expression 'unmasked'. This possibility should have been resolved by the subsequent *in situ* hybridization experiments and immunodection but as this part failed to produce reliable results, all I can conclude is that *per A* variant seems to oscillate in abundance throughout the day and for the other transcripts measured in this study I cannot exclude the possibility that their cycling expression could have been masked. A somewhat less complicated explanation would be that the observed cycling in the *per A* variant is caused by other factors than circadian regulation and the lack of oscillation in the *per B* variant actually depicts the true expression profile of this gene. I am inclined to believe rather the first interpretation since the observed expression pattern for *per A* seemed to be quite consistent and there were no significant drops in the production of *rp 49* suggesting that not much of an error had been introduced by for example different extraction efficiencies among the samples or other methodological inaccuracies.

As I mentioned above the spatial expression of the *tim* and *per* genes would give us an information indispensable for accurate evaluation of the actual role of these two genes in the circadian clockwork of the beetle. Unfortunately this goal has not been achieved.

The *in situ* hybridization experiments gave highly suspicious outcome: enormous signal arising from the SOG, rather weak variable staining of cells in the lateral protocerebrum and hard-to-interpret uniform staining of the photoreceptors.

Obviously this method will require yet a lot of fine-tuning to produce reliable results. Though I have tried several approaches (denaturing the probe at higher temperatures, increasing the hybridization temperature and the stringency of the post-hybridization washings) the *in situ* hybridization protocol is a rather complex procedure with many variables and I certainly have not exploited all the potential adjustments (such as hydrolyzing the probe to produce shorter fragments that should penetrate the tissue more easily).

Particularly disturbing though is the strong and apparently identical signal arising from the *sense* probes which did not seem to change much under any of the experimental conditions I have tried so far. Although one can suspect this could be a result of contamination of sense probe by the *antisense* one I highly doubt that. Not only it is unlikely that this contamination should

occur in both *tim*- and *per*-targeted probes on two independent occasions but even if this was so, one would still expect the signal arising from the *sense* probe to be significantly weaker than the one from the *antisense* probe and this did not happen. Therefore I think it will be necessary to reconsider also the fixation conditions. Due to the extensive loss of tissue sections (especially from the central part of the head) during the ISH procedure I assumed the samples might be fixed insufficiently because the fixative could have penetrate poorly through the cuticle protecting the head. For this reason I employed a post-fixation treatment which actually seemed to improve the tissue adhesion a little bit but it turned out to worsen the appearance of the tissue no matter how briefly I dipped the sections into the post-fixation solution. This leads me to the conclusion that original fixation conditions (O/N at 4°C) are probably sufficient or even undue, which could lead to artifacts. That is why I would suggest trying shorter fixation times or even different fixing solutions. Also designing a new set of probes targeted against different part of the relevant mRNAs might help to improve the results for there is also the possibility that the native mRNAs adopt rather stable secondary structures and thus the probes might have troubles binding it (in this case hydrolysis of the probes should also help).

Although it might be beneficial to produce a different set of probes, the Northern blot experiments proved that at least the *per*-targeted *antisense* probe employed in this study is able to specifically localize its target mRNA. Unfortunately, this method failed in the case of *tim* probes. The amount of total RNA used in those experiments though was lower than in the experiment on *per* detection and since the proportion of mRNAs in the total RNA extracts is generally very low (around 2%), it is probable that the detection methods employed were simply not sensitive enough to visualize the signal. In future, using isolated mRNA rather than total RNA might improve the outcomes. Nevertheless, the positive results of the Northern blot analysis on *per* strongly suggests that the design and production of the probes were unflawed and the failure of the *in situ* hybridizations have another cause.

Considering the immunodetection experiments no success has been achieved in this field either. We had the total of four antibodies made – two for each PER and TIM -- that were raised either in guinea pig or rat against short synthetic oligopeptide (about 20 aa). None of these antibodies recognized protein band of expected molecular weight on Western blots – except for the Gp-*anti*-TIM antiserum they did not specifically mark any bands at all. Although the chance that polyclonal antibodies work on both denaturized (Western blot) and native proteins (immunohistochemistry) is usually quite high it is nevertheless possible that the antisera employed in this study recognize only proteins in their native state. I therefore performed several experiments on the fixed tissues as well and although I certainly cannot offer final conclusion, the

results I have obtained are not very promising. The antibodies seem to produce no consistent and specific signal. Especially in the case of Gp-antisera, I have observed strong background staining and many dyed axons attributable to the secondary antibody. This might suggest that our standard blocking procedure (30 min-incubation in 10% goat normal serum at RT) is not sufficient in this case or that different secondary antibody should be used in future.

The worst scenario that the antisera we ordered simply do not recognize the target proteins at all would not actually be that improbable. It is a well known fact that production of antibodies and especially those raised against synthetic short peptides is somewhat risky business and rather small portion of antibodies produced this way usually work well.

To sum up, many questions regarding the expression of *timeless* and *period* genes in *Tribolium castaneum* remain to be resolved but all these problems finally amount to nothing compared to our worst finding: Although both I and my supervisor David Doležel have tried several different approaches, we have not been able to identify any useful circadian phenotype in the beetle. *Tribolium* seems to lack any circadian rhythmicity in either locomotor activity or eclosion and although it does (on the population scale) exhibit increased activity during the daytime this pattern does not persist in constant dark (D.D. personal communication) rendering this trait absolutely useless for circadian research. It is obvious that until we find a reliable circadian phenotype that can be measured reasonably well further circadian research on this animal would be rather questionable.

5. Summary

The results presented in this thesis can be summarized as follows:

[redacted]

B. Expression of timeless and period in Tribolium castaneum

1. Both *period* and *timeless* locus give rise to at least two different splice variants of the relevant mRNAs.
2. The relative abundances of all four isoforms were determined in both adult and larval head extracts with a 4 hrs-resolution.
3. Out of these four splice variants only the *perA* isoform displayed robust and consistent circadian oscillation.
4. Expression of both *per* and *tim* genes is significantly lower in larvae than in adults. The *perA* splice variant is the least abundant isoform in both cases.
5. The attempt to localize the *per* and *tim*-expressing cells in head section using both RNA probes and antibodies failed.
6. No useful circadian phenotype could be identified for *Tribolium*, which is seriously compromising its aspirations to become the new insect model for circadian research.

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7. Supplements

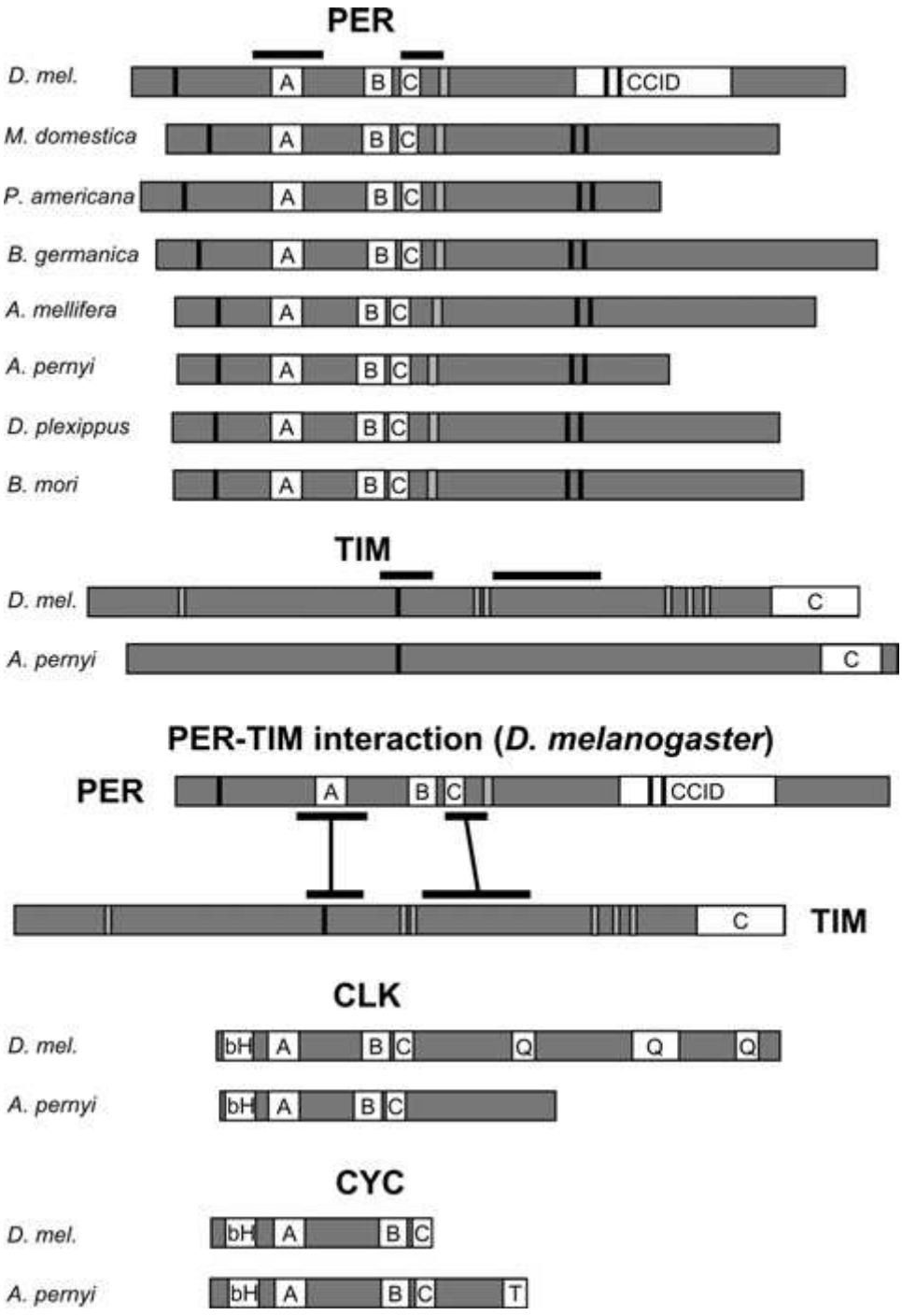

Fig. S1. Schematic representation of PER, TIM, CLK and CYC of different insect species depicting the conserved protein domains (white boxes) - 'bH', basic helix–loop–helix (bHLH) domain; A, B, PAS-A and PAS-B motifs; C, CLD domains; Q, poly-glutamine stretches; T, transcriptional activating domains.
Black boxes represent NLS sequences and light-grey boxes NESs.
Grey bars represent primary amino acid sequences of each protein to scale.
Taken from Helfrich-Förster, 2005.

Fig. S2. The *sternopleural (Sp)* phenotype; source: http://staff.aist.go.jp

Fig. S3. The *stubble (Sb)* phenotype source: http://cgslab.com/phenotypes

Fig. S4. The *curly (Cy)* phenotype source: http://arrogantscientist.files.wordpress.com

pages iii-xxv were omitted