Faculty of Science
University of South Bohemia
České Budějovice

Ph.D. Thesis

Circadian rhythms and photoperiodism in insects

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České Budějovice 2008

Stehlík J., 2008: Circadian rhythms and photoperiodism in insects. Ph.D. Thesis, in English – 60 pages, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

Annotation:

In this thesis we present experimental data and discuss circadian rhythm regulation in the housefly, *Musca domestica*. Our findings suggest that, although differences in the mechanisms of circadian rhythm regulation between *Musca domestica* and *Drosophila melanogaster* are not as fundamental as was originally expected, they still provide interesting insight into the evolution of biological clocks. We also studied possible involvement of one of the circadian clock genes, *timeless*, in photoperiodic induction of diapause in a drosophilid fly, *Chymomyza costata*. We found the transcription of *tim* gene to be strongly disrupted in CNS of *npd*-mutant (non-photoperiodic-diapause) larvae. Analysis of genomic structure of *tim* gene revealed that the promoter of $timeless^{npd}$ allele carries a large deletion, a possible cause of disruption of photoperiodic calendar function in *npd*-mutant larvae of *C. costata.*

Financial Support:

This work was supported by the Grant Agency of the Czech Republic (Grant No. 204/01/0404, Ivo Šauman; Grant No. 204/07/1032, Ivo Šauman), Czech Science Foundation (Grant No. 206/05/2085, Vladimír Košťál), Ministry of Education of the Czech Republic (Project No. LC07032, Ivo Šauman), Academy of Sciences of the Czech Republic (Project No. Z 50070508, Ivo Šauman and Vladimír Košťál) and by Grant Agency of University of South Bohemia (GAJU 56/2003/P-BF, Jan Stehlík; GAJU 46/2005/P-BF, Jan Stehlík).

Declaration:

"Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury."

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České Budějovice, 30.1.2008 Jan Stehlik

Aknowledgement:

I would like to thank to my supervisor, **Ivo Šauman**, for opportunity to work in his team, for his help every time I asked for it and for giving me a lot of freedom in my work. I would like to thank to **Vladimír Košťál** for guiding me trough the field of the photoperiodism, for constructive ideas and for huge help with our experiments and preparation of manuscript. I feel also very thankful to **David Doležel** for his permanent help with my research since we met; from tracing down species of our interest, teaching me many laboratory practices, to helping me with many experiments and preparation of our manuscript. I would like to thank **Radka Závodská** and **Kimio Shimada** for collaboration on diapause research.

I'm also grateful to **Alena Kobelková, Silvie Fexová and Roman Neužil** for their friendly help and for kind atmosphere in our lab. I would like to thank **Vaclav Brunhofer** for his help and inspiriting consultations and **Standa Vrba** for supporting me with material needed for some of my experimets. My thanks also belong to **Hana Albínová** and **Ivana Nýdlová** for their kind assistance.

Co-authors agreements:

Publication no. 1
Codd, V., D. Dolezel, J. Stehlik, A. Piccin, K. Garner, R. Seth, K. Straatman, E. Louis, R. Costa, I. Sauman, C. P. Kyriacou, and E. Rosato. 2007. Circadian rhythm gene regulation in the housefly, *Musca domestica*. Genetics 177: 1539-5107.

We hereby declare that Jan Stehlík contributed to this study to a great extent. He significantly participated on cloning and sequencing of six *Musca* clock genes homologues. He also performed by himself analysis of their expression at mRNA level using Real-Time RT PCR. He prepared figures showing outputs of the Real-Time RT PCR together with David Doležel.

…… ……
David Doležel Ivo Šauman

Publication no. 2
Stehlík J., R. Závodská, K. Shimada, I. Šauman, and V. Koštál. 2008. Photoperiodic induction of diapause requires regulated transcription of *timeless* in the larval brain of *Chymomyza costata*. J. Biol. Rhythms – *in press.*

We hereby declare that Jan Stehlík had a major contribution to this study. He did most experimental work: quantification of *tim* mRNA levels using Real-Time RT PCR and sequencing of *tim* gene in both wild-type and *npd*-mutant strains. He also prepared all of the figures except one, which was created by Radka Závodská.

…….. …….. ……..
Vladimír Košťál Ivo Šauman Radka Závodská

Contents (shortened version):

Chapter 1:

INTRODUCTION

Ever since life first appeared on this planet it has been subjected to daily cycles of light and dark, and also to seasonal cycles of climatic changes. Daily cycles of light and dark are caused by the 24-hour periodicity of the earth's rotation around its axis. Seasonal climatic changes are caused by the 1-year periodicity of the Earth's orbit around the Sun. Nearly every organism enhances its chances to survive in its niche by adapting to these predictable oscillations of the surrounding environment. In this thesis, I have focused on molecular mechanisms of the two most studied examples of such adaptations: circadian rhythms and photoperiodism.

Although both articles presented in this thesis have their own detailed introductions, this introduction was written to guide the reader through a bit wider background, describing the basis of circadian rhythms and photoperiodism and show their well-documented examples from everyday life of a variety of organisms.

Circadian Rhythms:

Circadian rhythms have evolved as a natural adaptation of organisms to daily cycles of light and dark. These "day-time specific" processes can be observed on molecular, physiological or behavioral levels. They are driven by an endogenous, genetically determined circadian clock. The presence of such an internal clock enables organisms to anticipate upcoming environment changes in advance. Circadian clocks exhibit three distinct characteristics common among all studied organisms (Pittendrigh and Daan, 1976). (1) The circadian clocks can "free-run" in constant conditions (e.g. constant darkness and temperature) without any external cues from surrounding environmental oscillations, with a period close to 24 hours. (2) The natural cycles of light and temperature, however, do serve to entrain and phase-control these endogenous oscillators so that, under natural conditions, their period matches exactly 24 hours. (3) The circadian clocks are temperature insensitive within a certain range of temperatures. This phenomenon is referred to as temperature compensation.

Although all living clocks display the above three basic timekeeping properties, their structural components may differ widely. However, every „circadian timing system" consists of three parts: the clock input pathway, the core oscillator, and the clock output pathway (Klein et al., 1991).

First, the clock input pathway provides the connection of the clock with the surrounding environment and is necessary for entrainment. Important part of the input system are photoreceptors. For example, in the cockroach and the cricket compound eyes are the exclusive photoreceptive organs for entrainment (Page, 1982; Loher, 1972). In several other studied insects species, photoreceptors for entrainment are distinct from the visual system. Such extraretinal photoreceptors were found in the silk moth, butterflies, flies and grasshoppers (Page, 2001; Plautz et al., 1997). The pineal gland and other extra-retinal photoreceptors, along with the retina, were found to serve for photoentrainment among several reptile (Foa, 1991; Foa et al., 1993; Janik et al., 1990; Falcon and Collin, 1989) and bird species (Norgren and Silver, 1989; Takahashi, 1987; Takahashi et al., 1989). In mammals, photic input for entrainment is provided exclusively by the retina (Yamazaki et al., 2002).

Second, the core oscillator represents the actual time-keeping device, the endogenous clock or pacemaker, for generation of daily rhythms. In many cases more than one pacemaker was identified in studied organisms. If such oscillators drive circadian rhythms all over the organism's body, they are usually called primary or central pacemakers and are commonly found somewhere in the nervous system. If pacemakers drive circadian rhythms that express only locally, in partial regions of an organism's body, they are called secondary or peripheral pacemakers and are found in a variety of non-neural tissues. For example, large basal retinal neurons of the eye serve as pacemakers and also as photoreceptors in the marine molluscs *Bulla gouldiana* and *Aplysia californica.* (Block and Wallace, 1982; Jacklet and Colquhoun, 1983; Blumenthal et al., 2001).

For insects, the primary pacemakers are found either in optic lobe sites of crickets, cockroaches and beetles or in cerebral lobe sites of moths and flies. The clocks located in the testes of gypsy moth, in the epidermal cells of cockroaches and in the prothoracic gland of the moth *Samia cynthia* represent well-documented examples of secondary pacemakers found in insects. They lie outside the central nervous system and appear to have their own photoreceptive cells (reviewed in: Page, 2001). The anatomical details of the circadian pacemaker in the fruitfly *Drosophila melanogaster* were revealed during the last decade (Helfrich-Forster et al., 2007; reviewed in Hall, 2003). Although several studies suggest that self-sustained oscillators and their photoreceptors are widely distributed in the body of the fruitfly, only a few dorsal and ventral lateral neurons between the lateral protocerebrum and

the medulla of the optic lobes appear to have the function of the primary pacemaker in this species (Plautz et al., 1997; Kaneko and Hall, 2000). Moreover, immunological studies have shown that some of these circadian pacemaker neurons in the insect brain can be labelled with an antibody to a crustacean pigment dispersing hormone (PDF). It was suggested that PDF immunoreactivity may serve as a marker of circadian pacemaker cells in insects (Helfrich-Forster and Homberg, 1993).

Primary circadian pacemakers in reptiles and birds often involve the pineal gland, the retina and the suprachiasmatic nucleus (SCN) (Foa, 1991; Tosini and Menaker, 1998; Norgren and Silver, 1989; Underwood et al., 1990, 2001). In mammals the only primary pacemaker is located in the SCN (Klein et al., 1991; Moore and Leak, 2001). Light is projected directly to the SCN from the retina via optic nerves (Meijer 2001; Yamazaki et al., 2002). Also, a variety of peripheral pacemakers outside the central nervous system have been demonstrated for vertebrates (Yamazaki et al., 2000; Buijs and Kalsbeek, 2001; Tosini and Fukara, 2002).

Third, the clock output pathway ensures the transfer of the timing information from the core oscillator to the rest of the organism. These output pathways include both neural and humoral signals. For insects, most of the output signals documented are involved in the regulation of various developmental events. For example, secretion of ecdysone, prothoracicotropic hormone and eclosion hormone is under the control of the circadian pacemaker (reviewed in Dunlap, 2004). One of the most important circadian output signals found in avian and mammal bodies is the secretion of the hormone melatonin (Arendt, 1995; Zatz, 1996; Korf et al., 1998). In mammals, melatonin is secreted from the pineal gland and the circadian rhythm of its secretion is driven endogenously by circadian clocks. Its concentration in plasma is low during the day and high at night (Shochat et al., 1997). Some well known biological effects of this hormone are its ability to induce sleep, control of seasonal photoperiodic responses and its potential to entrain circadian clock. However, the fact that its production depends on the light cycle means that it must have a modulatory role rather than a primary role (Reppert et al., 1994; Dollins et al., 1994). In humans, circadian output rhythm has been well documented also for another hormone, cortisol. This adrenal hormone is secreted primarily during the day to help mobilize energy and declines to a minimum at the onset of sleep (Krieger et al., 1971; Van Cauter, 1989).

The fact that circadian rhythms are found in virtually all studied organisms shows their evolutionary importance (reviewed in Dunlap, 1999). For example regulation of the photosynthetic machinery in the unicellular prokaryote *Synechococcus* (Cyanobacteria) shows circadian character (Johnson et al., 1996; Kondo and Ishiura, 2000). The eukaryotic unicell *Gonyaulax* (Dynophyta) exhibits a of flash bioluminescence rhythm (Hastings and Sweeney, 1959) and *Paramecium* (Ciliata) shows a mating reactivity rhythm (Miwa et al., 1987; Johnson and Kondo, 2001). Many species of higher plants possess circadian rhythms of photosynthesis, leave movement, blooming, releasing pollinator-atracting fragrances during day-light hours (numerous orchid species) and closing petals and dipping heads at night (DeMairan, 1729; Sweeney, 1987). Locomotor activity rhythms are well-documented, for example, in some rodents (DeCoursey, 1990; Riccio and Goldman, 2000), bats (DeCoursey and DeCoursy, 1964), chipmunks (DeCoursey et al., 2000), the fruitfly *Drosophila melanogaster* (Konopka and Benzer, 1971), cockroaches (Stengl and Homberg, 1994), moths (Keil et al., 2001), crickets (Reichle et al., 1965; Campbell, 1976) and beetles (Fondacaro and Butz, 1970). Some insects and birds are well known for expressing their communication rhythms, e.g. fireflies flash at twilight when males begin to court females (Copeland and Moiseff, 1997), crickets call at night during courtship (Loher, 1972) and many birds chorus at dawn (reviewed in Dunlap, 2004). Sleep-wake rhythms are well-documented in a number of rodents and other mammals. Most of them sleep at night, but rats and hamsters sleep mostly during the day (DeCoursey and DeCoursy, 1964; Pittendrigh and Daan, 1976; DeCoursey, 1990). Some of them lower their body temperature during sleep (Lyman et al., 1982).

Human circadian rhythms free-run in constant conditions with a period of approximately 24.3h and entrainment is brought by the light-dark cycle (Czeisler et al., 1981; Honma et al., 1987; Minors et al., 1991; Czeisler, 1995; Boivin et al., 1996; Rimmer et al., 2000). Widely studied human rhythms are the sleep-wake cycle and the core body temperature rhythm (Lewis and Lobban, 1957; Aschoff, 1965; Minors and Waterhouse, 1981; Moore-Ede and Sulzman, 1981; Refinetti and Menaker, 1991; Lack and Lushington, 1996; Middleton et al., 1996; Murphy and Campbell, 1997; Waterhouse et al., 1999; Baehr et al., 2000; Lavie, 2001; Edwards et al., 2002; Palmer, 2002). The core body temperature starts to fall in the evening in anticipation of sleep and rises in the early morning in preparation for the active phase. Also,

alertness, mental performance, physical activity (Folkard, 1990; Monk, 2001) and levels of the above-mentioned hormones melatonin (Lewy et al., 1980; Bojkowski et al., 1987A,B; Arendt, 1995; Schochat, 1997; Zeitzer et al., 2000) and cortisol (Krieger et al., 1971; Van Cauter, 1989) exhibit circadian patterns in humans. Everyone can experience the endogenous nature of the circadian clock when travelling across several time zones by plane, e.g. from Europe to USA. Such a person will wake up significantly earlier (several hours) than normaly, and this behavior will be entrained to normal after a few days. This so called “jet lag” will also be experienced when returning back home (Klein et al., 1972; Arendt et al., 2000). Jet lag effects on our overall condition and performance, resulting in suffered sport results, was also demonstrated (Recht et al., 1995).

For humans, the social environment is a major determinant of sleep-wake schedules, even though it is not necessarily in synchrony with the external natural light-dark cycle. This is why some individuals experience considerable difficulty synchronizing their sleep-awake schedules to these social schedules. For example, people exposed to circadian stresses at their workplaces, mainly shift workers in around the clock services, often experience acute disasters like fatique, sleepiness and reduced efficiency, sometimes resulting in a variety of accidents (Hamelin, 1987; Mitler et al., 1988; Moore-Ede, 1993; Rajaratnam and Arendt, 2001;). These people may also eventually develop some of the several chronic sleep-wake syndromes and mood disorders. For example, FASP (Familial Advanced Sleep Phase syndrome) is characterized by early sleep onset and offset (Jones et al., 1999; Toh et al., 2001). Patients with DSPS (Delayed Sleep Phase Syndrome) cannot get to sleep before 2:00 or 3:00 A.M. and they have difficulty waking before late morning (Uchiyama et al., 2000; Cole et al., 2002). SAD (Seasonal Affective Disorder) is a seasonal form of mood disorder and is characterized by periods of depression and periods of excitation (Wehr, 1990; Rosenwasser and Wirz-Justice, 1996). Even increased risk of heart disease (Knutsson and Boggild, 2000) and cancer (Anisimov, 2003) was observed among people exposed to circadian stresses.

Understanding that the circadian nature was the basis for some of above-mentioned chronobiological disorders helped in the development of effective treatments for them. Such treatments generally consist of strengthening exposure to an appropriate circadian synchronizer, light and melatonin being the most prominent (Arendt, 2000; Eastman and Martin, 1999; Skene et al., 1999). For example, timed

melatonin ingestion successfully stabilizes sleep onset and improves sleep quality in blind individuals (Sack et al., 2000). Because of the absence of light perception, their circadian rhythms free-run with a period slightly longer than 24 hours, these individuals suffer from a mismatch between their sleep-wake cycle and natural light-dark cycle. Melatonin has also been used to alleviate jet lag problems (Arendt, 1999).

Besides circadian rhythms, circadian clocks also play an important role in several other processes (Dunlap et al., 2004). Some animals use their biological clocks to ensure that critical developmental events take place at an appropriate time of the day. The birth of some mammals, the hatching of birds or eclosion of insect adults from their pupae are such circadian clock gated events. For example, eclosion of new adult fruitflies from their pupal cases always takes place near dawn (Pittendright, 1954). Another well studied clock controlled behavior is the hatching of moth larvae from eggs, which occurs in early morning and was studied in the silkmoth *Antheraea pernyi* (Sauman et al., 1996b). Time sense and time-place learning are functions that require continuous consultation of a circadian clock as well. Time sense in bees allows them to arrive daily at a feeding site only at the time of food availability (Bradbury and Vehrencamp, 1998). Time-place learning was documented, for example, in rats (Aragona et al., 2002) and several fish (Dittman and Quinn, 1996; Reebs, 1996) and several bird species (Biebach et al., 1991). Moreover, when migrating birds and insects use the sun as a cue for celestial orientation, they use an endogenous circadian clock to compensate for the daily changes in the sun's position (Hoffman, 1960; Dunlap et al., 2004). This is called time-compensated celestial navigation. Possible anatomical connections between circadian clock and light input were studied in monarch butterflies, which migrate every year with amazing precision from North America to Mexico and back (Sauman et al., 2005; Zhu et al., 2008). Circadian clocks are also involved in synchronization of individuals within the population of many species. For example rabbit pups anticipate the daily visit of the mother by opening the nest in order to facilitate their access to her when she arrives to feed them (Jilge, 1993).

Molecular mechanisms that underlie the function of circadian clocks have been studied in various model organisms including prokaryotic alga *Synechococcus* (reviewed Kondo & Ishiura, 2000), the filamentous fungus *Neurospora* (McWatters et al., 1999), plants (Millar et al., 1995a,b), mammals (reviewed Hastings & Maywood, 2000), insects (reviewed in Kaneko, 1998; Dunlap, 1999; Sauman and Hashimi, 1999)

and fish (Whitmore et al., 2000). Due to the power of its genetics, the fruitfly, *Drosophila melanogaster*, represented the premier species for elucidating the molecular basis of circadian rhythms.

While *Synechoccocus*, plants and *Neurospora* appear to use different molecular components of the central clocks, *Drosophila* clock gene homologs have been found in birds, mammals, fish and other insects (reviewed in Dunlap 1999; Stanewsky, 2002; Hall, 2003). This fact, together with detailed research including functional studies on mouse and zebra fish (*Danio*) (Whitmore et al., 1998), suggested that the molecular mechanism underlying the circadian rhythmicity among mammals, fish and insects would be conserved. This idea is further supported by the fact that some mutations originally described in the fruifly were also identified in mammals. For example, a single amino acid substitution causing FASP (Familial Advanced Sleep Phase syndrome) in humans, was localized within the *casein kinase I epsilon* binding domain of the human homolog of the *Drosophila* period gene (hPer2) (Toh et al., 2001), a gene previously known to be essential for the fruitfly circadian clock (Konopka and Benzer, 1971) including that PER phosphorylation by *casein kinase I epsilon* (Kloss et al., 1998; Price et al., 1998) is essential for *Drosophila* clock function.

The original *Drosophila* clock model is based on two interlocked transcriptional feedback loops containing the negative elements *period* and *timeless* (reviewed in Dunlap, 1999; Stanewsky, 2002). Genetic and molecular investigations disclosed distinct diurnal oscillations in the expression of *per* and *tim* genes (Hardin et al., 1990). Further research on the mechanism of regulatory oscillations revealed that the proteins PER and TIM form a heterodimer, which is translocated into the nucleus. There it interferes with the assembly of constitutive transcription factors CLOCK and CYCLE (also called BMAL) and thereby inhibits the expression of *tim* and *per* (reviewed by Sauman and Hashimi, 1999). The rate of assembly of the TIM/PER dimer, which is regulated by a kinase called double-time (DBT), sets in the circadian rhythm, and the degradation of TIM upon illumination synchronizes this system with the environmental alternations of light and darkness (Kloss et al., 1998; Price et al., 1998). The effect of light is mediated by the *cryptochrome* protein (CRY), which contains a flavonoid photosensitive pigment (Emery et al., 1998; Stanewsky et al., 1998). Fine-tuning and enhancing of the *Drosophila* circadian clock requires an orchestrated action of additional clock genes *vrille* (*vri*), *shaggy* (*sgg*), *slimb*, *pdp* and

ck2 (Blau and Young, 1999; Martinek et al., 2001; Ko et al., 2002; Cyran et al., 2003; Lin et al., 2002). The key parameters for the *Drosophila* clock model are (1) a temporal delay between the accumulation of both *per* and *tim* transcripts and their corresponding proteins, (2) stability of the PER and TIM products and (3) temporally controlled translocation of PER/TIM dimer to the nucleus (Saez and Young, 1996; Ruoff, 1998).

Initial molecular analysis of the circadian clock in the silkmoth, *Antheraea pernyi*, revealed striking differences between the moth and fruitfly timing mechanisms (Sauman and Reppert, 1998; Sauman et al., 1996a,b). The most notable differences are the absence of nuclear translocation of the silkmoth PER to the nuclei of the putative pacemaker cells and missing temporal delay between *per* transcription and translation, the two crucial features of the *Drosophila* circadian clock model. It was suggested that *A. pernyi* represents an exception in the evolution of the circadian clock mechanism in insects. However, detailed investigation of the regulation of the circadian clock gene expression in two hemimetabolous insects, the American cockroach *Periplaneta americana* (Sehadova et al., 2003, 2008), and the European linden bug *Pyrrhocoris apterus* (Syrova et al., 2003), also showed the same discrepancies with the *Drosophila* clock model. Moreover, several representative species from major insect orders were screened for PER subcellular localization in the adult central nervous system and the PER protein was never detected in the nuclei of *per* expressing cells (Zavodska et al., 2003). Thus, of all insects, the critical condition for the functional "negative feedback oscillator" model – the nuclear translocation of PER – appears to be true only in *Drosophila*. The question thus arises whether the "negative feedback oscillator" model in *Drosophila* is an exception rather than the generally accepted rule.

We focused on the housefly, *Musca domestica* in order to uncover a possible evolutionary flexibility in the way how the basic negative feedback loop underlying circadian rhythmicity can be constructed among closely related species. The housefly is a species phylogenetically closely related to *Drosophila*, as they both belong to the higher dipterans, the Cyclorrhapha group. *Musca's* homologues of *Drosophila* clock genes were cloned and detailed analysis of their expression at the mRNA and protein levels is presented in the first of the two papers covered in this thesis. Although our results indicate that the molecular mechanism underlying the circadian clock in these

two dipteran species is different, it turned out that these differencies are not as fundamental as originally appeared (Codd et al., 2007).

Photoperiodism and diapause:

Photoperiodism has evolved as a match to seasonal climatic changes resulting from the earth orbiting the Sun. Seasonal influences are particularly marked at extreme latitudes, where variations in external conditions are most pronounced. Plants and animals in these locations have adjusted their life strategies to seasonal variations in factors such as ambient temperature and food supply. The most widely used environmental cue for timing of life cycles is photoperiod (i.e. ratio of day length and night length), because it correlates perfectly with the time of year (reviewed in Dunlap et al., 2004).

By analogy to better known circadian systems, photoperiodic systems presumably involve photoreceptors, some kind of photoperiod measuring device and an output signal. In plants, the site of photoperiodic perception was located in the leaves (Thomas and Vince-Prue, 1997). Organized photoreceptors, such as compound eyes, are used in photoperiodism in relatively primitive insects. In contrast, extraretinal photoreception was found in more advanced groups, such as moths and flies (reviewed in Vaz Nunes and Saunders, 1999; Saunders, 2002). However, a central problem in photoperiodism of insects is the nature of photoperiod time measurement, as discussed later. Involvement of circadian clocks was suggested in many species, including the parasitic wasp *Nasonia vitripennis* (Saunders, 1969; Saunders et al., 1970), the flesh fly (Saunders, 1973), the spider mite (Hoy, 1975) and various other species of flies, butterflies and beetles (reviewed in Nunes and Saunders, 1999). On the other hand, findings not suggesting such involvement have been recorded for several moths and the green vetch aphid (Takeda and Skopik, 1997).

Birds and other non-mammalian vertebrates use diverse extraretinal photoreceptors located in the pineal gland as well in the brain (Philip et al., 2000; Dawson et al., 2001). Because photoperiodic time measurement in vertebrates is accomplished by a circadian clock, these two systems share several structural components (reviewed in Dunlap et al., 2004). For example, in mammals, the pineal

melatonin rhythms serve as an endocrine component of the photoperiodic mechanism, in addition to its function as a circadian output signal. The duration of each nightly episode of melatonin secretion codes for day length in all photoperiodic mammals (Elliot, 1976; Underwood and Goldman, 1987; Bartness and Goldman, 1989; Woodfill et al., 1994; Barrel et al., 2000; Goldman, 2001). In birds and lizards, however, pineal melatonin is not an essential component of their photoperiodic mechanism (Underwood and Goldman, 1987; Juss et al., 1993; Dawson et al., 2001; Underwood and Hyde, 1990).

The most obvious manifestation of photoperiodism in plants include seasonal rhythms in flowering and reproduction, induction of cold hardiness and bud dormancy (Garner and Allard, 1920; Lumsden and Millar, 1998). Photoperiodism is also more widely documented in some cold-blooded vertebrates (Underwood and Goldman, 1987; Underwood and Hyde, 1990). In birds it has been demonstrated that photoperiod length influences timing of such diverse functions as reproduction, molt and migration (Rowan, 1926; Baker, 1938; Gwinner and Wiltschko, 1980; Berthold and Querner, 1981; Dawson and Goldsmith, 1983; Gwinner, 1996). Many mammals show photoperiodic responses in reproduction, seasonal fattening, hibernation and other functions (Pengelley et al., 1976; Demas and Nelson, 1998; Lincoln and Richardson, 1998; Ruby et al., 1998; Goldman, 1999; Barrel et al., 2000; Yellon and Tran, 2002). For example, some high-latitude hibernating mammals have the reproductive and molting season restricted to a few months in summer because they hibernate for as long as 7 to 8 months each year. The presence or absence of photoperiodism in humans has not been established.

The most studied photoperiodic response in insects is diapause, an endogenously mediated form of dormancy. Diapause routes the developmental program away from direct morphogenesis into an alternative programme: energy reserves are stockpiled, metabolic rate drops and development is halted (reviewed in Denlinger, 2000; Kostal, 2006). Although diapause is best known as an over-wintering strategy for insects in the temperate zones, summer diapause, which occurs during the dry and hot season, is not uncommon in mediterranean and tropical zones (Masaki, 1980).

The stage of developmental arrest in which diapause proceeds may take very different forms, but for any single species the capacity for diapause is usually restricted to a single specific developmental stage (reviewed in Kostal, 2006). On one

side, there are various immobile stages such as diapausing eggs (e.g. the silkworm, *Bombyx mori;* Chino, 1958), cocooned mature larvae (e.g. the European corn borer, *Ostrinia nubialis;* Chippendale and Yin, 1979) and pupae (e.g. the flesh fly *Sarcophaga;* Denlinger, 1972), which do not accept any food and display deep metabolic supression. On the other side, diapausing free-living larvae and adults (e.g. the Colorado potato beetle, *Leptinotarsa decemlineata;* De Kort, 1981) can move and their metabolic supression is usually less deep.

In a few species, diapause is obligatory. In such cases, the initiation of diapause needs no external cues because it represents a fixed component of the ontogenetic programme and is expressed regardless of the environmental conditions (reviewed in Denlinger, 2000). It is most prevalent in species that complete only a single generation in a year (e.g. the gypsy moth, *Lymantria dispar;* Bell, 1989).

Most diapauses, however, are facultative (reviewed in Denlinger, 2000). This implies that the decision to enter diapause is determined by specific environmental cues. Such a decision is not an immediate response to environmental adversity but is rather programmed by environmental cues (most often photoperiod) received at an earlier stage of development. Facultative diapause is commonly found in species with several generations per year.

Insects that have a facultative diapause and live in the temperate zone rely almost exclusively on photoperiod (daylength) for programming of the diapause (reviewed in Denlinger, 2000; Kostal, 2006). Using the analogy of the man-made calendar, photoperiod length serves as an “insect’s calendar” when signaling for the coming deterioration of environmental conditions. The short days of late summer and early autumn are the most common cue used to program overwintering diapause. In the case of insects with summer diapause, long days serve as a signal to induce diapause.

When a population sample of individuals is exposed to a *critical photoperiod* at their sensitive stage, half of them will enter diapause and the other half will continue in direct development (reviewed in Denlinger, 2000; Kostal, 2006). Moreover, diapause is expressed only when an adequate number of inductive days have accumulated. The stage sensitive to the photoperiodic signal, the *photosensitive stage*, usually occurs well in advance of the onset of diapause. Such a photoperiodic response can usually be modified by other factors, mostly by temperature (Hoy, 1975;

Saunders, 1971), food content (Denlinger, 1986) and sex of the insect (reviewed in Denlinger, 2000).

Diapause is terminated not in response to “favorable” environmental conditions, but only after the process of diapause development has been completed (reviewed in Denlinger, 2000; Kostal, 2006). The rate of diapause development is often influenced by photoperiod and temperature (Tauber et al., 1986). Once diapause is terminated, the insect is able to resume its development as soon as the environmental conditions become permissive.

Currently, good information exists on the environmental regulation of diapause; also some of its hormonal regulators were already identified. Juvenile hormones, ecdysteroids and their regulatory neuropeptides (allatotropins, allatostatins and PTTH) are involved in diapause regulation (Yin and Chippendale, 1973; Yagi and Fukaya, 1974; Brown and Chippendale, 1978; De Loof et al., 1979; Bean and Beck, 1980; Bradfield and Denlinger, 1980; Briers et al., 1982; Denlinger et al., 1984; Denlinger, 1985; Lee et al., 1997). A unique neuropeptide that regulates early embryonic diapause was found in the silkmoth *Bombyx mori* (Yamashita, 1996). However, much less is known about the molecular mechanisms involved in diapause programming: a mechanism for distinguishing short days from long days, a mechanism that counts the number of inductive days and a mechanism how this information is stored until the insect reaches the correct developmental stage for expression of diapause (reviewed in Denlinger, 2000)

The involvement of clock genes in the programming of diapause response has been suggested. One of the best-known clock genes, *period*, does not appear to be involved, at least in the reproductive diapause of *Drosophila melanogaster* (Saunders et al., 1989; Saunders, 1990; Saunders et al., 1990). Despite that negative finding, investigation into the potential involvement of circadian clock genes in photoperiodic response resumed during the last decade. It has been shown that phases, amplitudes and/or levels of various clock gene expression were affected by photoperiod in several insect species (Goto and Denlinger, 2002a,b; Goto et al., 2006; Syrová et al., 2003; Mathias et al., 2005; Iwai et al., 2006). Recent studies in *D. melanogaster* (Sandrelli et al., 2007; Tauber et al., 2007) and pitcher-plant mosquito, *Wyeomyia smithii* (Mathias et al., 2007), indicated that, although circadian clocks and photoperiodic calendar appear as genetically distinct mechanisms/processes, the clock gene *timeless*

(*tim*) may affect the incidence of diapause directly, independently of its function in the central circadian oscillator.

In the second paper (manuscript *in press*) presented here, we have contributed to this effort by studying the role of the *timeless* gene in the photoperiodism of a drosophilid fly, *Chymomyza costata*. This study started back in the 1980's, when Riihimaa and Kimura (1988) selected a mutant strain of this fly that did not respond to a photoperiodic signal (NPD strain). Genetic linkage analysis confirmed that the larval non-photoperiodism and adult eclosion circadian arrhythmicity in the NPD strain were caused by a mutation in a single autosomal gene locus called *npd (non photoperiodic diapause)* (Riihimaa and Kimura, 1989; Riihimaa, 1996). The *tim* mRNA transcripts were not detectable by Northern blot analysis in the fly heads of *npd*-mutants, while they were detectable and showed typical daily oscillations in the wild-type strain (Pavelka et al., 2003). All these previous data strongly suggest that the product of *tim* gene represents a molecular link between circadian and photoperiodic clock systems in this fly.

However, diapause and photoperiodic sensitivity are expressed only in larval stages of *Chymomyza costata*. Hence, the main objective of this paper was to find whether there are any differences between the wild-type and *npd*-mutant larvae in the levels and circadian patterns of *tim* gene expression. The paper also presents the analysis of the genomic structure of the *tim* gene in both wild-type and NPD strains (Stehlik et al., 2008 - *in press*).

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Chapter 2:

ORIGINAL PUBLICATIONS

DOI: 10.1534/genetics.107.079160

Circadian Rhythm Gene Regulation in the Housefly *Musca domestica*

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Manuscript received July 23, 2007
Accepted for publication September 13, 2007

ABSTRACT

The circadian mechanism appears remarkably conserved between Drosophila and mammals, with basic underlying negative and positive feedback loops, cycling gene products, and temporally regulated nuclear transport involving a few key proteins. One of these negative regulators is PERIOD, which in Drosophila shows very similar temporal and spatial regulation to TIMELESS. Surprisingly, we observe that in the housefly, *Musca domestica*, PER does not cycle in Western blots of head extracts, in contrast to the TIM protein. Furthermore, immunocytochemical (ICC) localization using enzymatic staining procedures reveals that PER is not localized to the nucleus of any neurons within the brain at any circadian time, as recently observed for several nondipteran insects. However, with confocal analysis, immunofluorescence reveals a very different picture and provides an initial comparison of PER/TIM-containing cells in Musca and Drosophila, which shows some significant differences, but many similarities. Thus, even in closely related Diptera, there is considerable evolutionary flexibility in the number and spatial organization of clock cells and, indeed, in the expression patterns of clock products in these cells, although the underlying framework is similar.

Abstrakt:

Mechanismus cirkadiánních hodin se zdá být značně konzervovaný u druhu *Drosophila melanogaster* a u savců. Sestává z negativních a pozitivních zpětných vazeb, cyklování produktů řady genů a z regulovaného transportu do jádra několika proteinů. Jedním z těchto negativních regulátorů je gen PERIOD, jehož exprese je u druhu *Drosophila melanogaster* časově i místně regulována, podobně jako exprese genu TIMELESS. U blízce příbuzného druhu dvoukřídlého hmyzu, Mouchy domácí (*Musca domestica)*, výsledky „*Western blot*“ analýzy extraktů hlav překvapivě ukázaly, že hladiny proteinu PER, na rozdíl od proteinu TIM, necyklují. Ani pomocí immuno-cytochemické metody nebyl protein PER během dne lokalizován v jádře žádného z neuronů v mozku. Stejná situace byla před nedávnem pozorována i v jiných hmyzích řádech. Až prostřednictvím immunofluorescence a konfokální analýzy jsme získali výsledky, které nám umožnili porovnání buněk obsahujících proteiny PER a

TIM obou druhů *Musca domestica* a *Drosophila melanogaster*. Toto srovnání ukázalo řadu podobností mezi oběma druhy. Z toho je patrné, že dokonce i u blízce příbuzných druhů dvoukřídlého hmyzu je patrná flexibilita nejen v počtu a umístění jednotlivých buněk cirkadiánních hodin, ale také v expresi genů cirkadiánních hodin v těchto buňkách.

- článek otištěn v časopisu Genetics 177, stránky: 1539-5107.

- můj podíl na publikaci je 25%

Photoperiodic induction of diapause requires regulated transcription of *timeless* in the larval brain of *Chymomyza costata*.

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Abstract

Photoperiodic signal stimulates induction of larval diapause in *Chymomyza costata*. Larvae of NPD strain (*npd*-mutants) do not respond to photoperiod. Our previous results indicated that the locus *npd* could code for the *timeless* gene and its product might represent a molecular link between circadian and photoperiodic clock systems. Here we present results of *tim* mRNA (real time-PCR) and TIM protein (immunohistochemistry) analyses in the larval brain. TIM protein was localized in two neurons of each brain hemisphere of the 4-d-old 3rd instar wild-type larvae. In a marked contrast, no TIM neurons were detected in the brain of 4-day-old 3rd instar *npd*-mutant larvae and the level of *tim* transcripts was approximately 10-fold lower in the NPD than in wild-type strain. Daily changes in *tim* expression and TIM presence appeared to be under photoperiodic control in the wild-type larvae. Clear daily oscillations of *tim* transcription were observed during the development of 3rd instars under the short-day conditions. Daily oscillations were less apparent under the long-day conditions, where a gradual increase of *tim* transcript abundance appeared as a prevailing trend. Analysis of the genomic structure of *tim* gene revealed that *npd*-

mutants carry a 1855 bp-long deletion in the 5'-UTR region. This deletion removed the start of transcription and promoter regulatory motifs E-box and TER-box. We hypothesize that this mutation was responsible for dramatic reduction of *tim* transcription rates, disruption of circadian clock function and disruption of photoperiodic calendar function in *npd*-mutant larvae of *C. costata*.

Abstrakt:

Fotoperiodický signál indukuje diapausu u larev druhu *Chymomyza costata*. Larvy mutantního NPD kmene na fotoperiodický signál nereagují. Naše předchozí výsledky ukázali, že lokus *npd* pravděpodobně kóduje gen *timeless* a jeho produkt tak zřejmě představuje molekulární spojení mezi cirkadiánním a fotoperiodickým systémem. Zde prezentujeme výsledky analýzy exprese mRNA genu *tim* (kvantitativní PCR) a proteinu TIM (immuno-cytochemická analýza) v mozcích larev. V případě nemutantního kmene byl protein TIM detekován ve dvou neuronech v každé mozkové hemisféře larev třetího instaru. V případě mutantního NPD kmene nebyl žádný protein TIM v mozcích stejně starých larev detekován a také hladina mRNA genu *tim* byla přibližně 10x nižší než v mozcích larev nemutantního kmene. Ukázalo se, že denní změny v expresi genu *timeless* a v přítomnosti proteinu TIM v mozcích larev nemutantního kmene jsou pod fotoperiodickou kontrolou. V podmínkách krátkého dne byli u larev třetího instaru pozorovány denní oscilace transkripce genu *tim*. Tyto oscilace byly méně patrné v podmínkách dlouhého dne, kdy převládl postupný nárůst hladiny transkriptu genu *tim*. Analýza sekvence genu timeless ukázala, že mutantní *npd* alela nese ve svém promotoru deleci dlouhou 1855 párů bazí. Tato delece tak odstranila start transkripce a regulační sekvence *E-box* a *TER-box*. Tato delece je tak pravděpodobně příčinou dramatického poklesu hladiny transkripce genu *timeless* a narušní funkce cirkadiánního systému a fotoperiodického kalendáře u larev mutantního NPD kmene druhu *Chymomyza costata.*

- článek byl v listopadu 2007 přijat k otištění do časopisu *Journal of Biological Rhythms*, předpokládaný termín otištění: květen 2008

- můj podíl na publikaci je 75%

Chapter 3:

SUMMARY

In this work we present experimental data and discuss circadian rhythm regulation in the housefly, *Musca domestica*. We also studied possible involvement of one of the circadian clock genes in photoperiodic induction of diapause in a drosophilid fly, *Chymomyza costata*.

Circadian rhythm gene regulation in the housefly, *Musca domestica*

Summary of results:

In order to examine whether *Musca domestica* species shares the same circadian clock mechanism as the one described in detail for *Drosophila melanogaster*, we analysed the *Musca* circadian clock and its rhythmicity using several approaches. We have cloned several of the *Musca* homologues of the *Drosophila melanogaster* clock genes (*period, timeless, vrille, clock, cycle and cryptochrome*) and analysed their temporal and spatial expression in *Musca* heads. *Musca* individuals were reared under three different light regimes: LD = 12hours light/12hours dark, DD = continuous darkness and LL = continuous light. We obtained the following results:

- *Musca* rhythmic behavior:

- *Musca domestica* individuals showed a clear circadian rhythm of their locomotor activity and adult eclosion appears to be a circadian clock gated event

- we analysed mRNA levels of six cloned *Musca* clock genes in heads of *Musca* individuals reared under the three different light regimes (LD, DD and LL):

- *Mdper, Mdtim, Mdvri* and *Mdclock* mRNA levels clearly showed circadian patterns in LD conditions, their rhythms persisted even in the absence of external cues in DD conditions and were lost in LL conditions
- *Mdcyc* and *Mdcry* mRNA levels turned out to be expressed at constant levels

- we analysed the temporal expression of MdPER and MdTIM proteins in *Musca* heads (Western blot):

- MdTIM protein abundance showed a clear circadian pattern and this protein was immediately degraded in response to light
- MdPER protein abundance showed surprisingly no evidence for any cycling nor evidence for light-induced degradation on Westerns

- immunohistochemical (IHC) analysis of spatial distribution of MdPER protein in the *Musca* cephalic nervous system:

- PER-like immunoreactivity was detected in the cytoplasm of several lateral neurons of the protocerebrum

- immunofluorescent analysis of spatial co-expression of MdPER and MdTIM proteins in the whole-mount *Musca* brains:

- nuclear co-expression of both proteins was detected in several neuronal groups at Zt24: (1) small ventral lateral neurons, (2) PDF null lateral neurons, (3) dorsal lateral neurons, (4) dorsal neurons and (5) photoreceptors
- cytoplasmatic expression of MdPER protein and nuclear expression of MdTIM protein was detected in one group of large ventral lateral neurons at Zt24
- we could not detect any MdTIM and MdPER proteins at times earlier than Zt24 in the above-mentioned neurons, so we suggest that there is a cycle in the abundance of these two proteins
- strong arrhythmic expression of MdPER protein was detected in the cytoplasm of two additional neuronal groups: (1) medial neurons and (2) medio-lateral neurons

Conclusions:

We have cloned several *Musca* clock gene homologues. Most of them (*Mdper, Mdtim, Mdvri, Mdclock and Mdcyc)* appeared to have daily patterns of their mRNA abundances in *Musca* heads very similar to those found in *Drosophila*. The endogenous character of cycling found in case of *Mdper, Mdtim*, *Mdvri* and *Mdclock* suggests that these genes are not only structural homologues of *Drosophila* clock

genes, but also their functional homologues. Of special interest was that, in contrast to *Drosophila, Mdcry* mRNA levels showed a lack of any cycling in *Musca* heads.

No evidence for any cycling nor light-induced degradation of MdPER protein in *Musca* heads are in striking contrast with the situation described in *Drosophila*. Cycling of both PER and TIM protein abundances in *Drosophila* heads and their degradation in response to light are the key parameters for the *Drosophila* "negative feedback oscillator" model. Also, the fact that MdPER protein was detected exclusively in the cytoplasm of several lateral neurons by IHC suggest that the mechanism of circadian rhythm regulation in *Musca domestica* differs from the model described for *Drosophila*. Translocation of PER protein into the nucleus of pacemaker cells is necessary for the *Drosophila* "negative feedback oscillator" model.

However, immunofluorescence analysis of spatial expression of MdPER and MdTIM proteins in whole-mount *Musca* brains identified several groups of neurons that exhibit co-expression of both proteins. We considered these neurons to be putative clock cells in *Musca domestica*. In all of them, with the exception of the large lateral neurons, co-expression of MdPER and MdTIM proteins exhibit a key parameter of *Drosophila* model: translocation to the nucleus. The large lateral neurons are the only group of putative clock cells to show cytoplasmatic MdPER and nuclear MdTIM protein expression. This suggests that this neuronal cluster might have a special function, as suggested also previously for *Drosophila*. All identified putative clock cells in the *Musca* brain largely correspond to those described in *Drosophila*. We also suggest that there is a cycle in the abundance of MdPER and MdTIM proteins in *Musca* pacemaker cells.

The strong arrhythmic expression of MdPER protein detected in the cytoplasm of medial and medio-lateral neurons suggests that MdPER protein in these neurons may play a different role from that found in the putative clock cells.

Our findings suggest that, although differences in the mechanisms of circadian rhythm regulation between *Musca domestica* and *Drosophila melanogaster* are not as fundamental as was expected, they still provide interesting insight into the evolution of biological clocks.

Prospects of future research:

- it would be very helpful to use various genetically modified *Musca*, for example flies with *Md-period* or *Md-timeless* genes expression knocked-out, for functional studies or use them as negative controls for Western blots or immunoreactive assays to verify the specifity of the used antibodies

- to analyze the levels of *Mdcry* transcripts in brain tissue only (instead of whole heads) and localize these transcripts *in situ*

- to clone the full length CDS of other homologues of *Drosophila melanogaster* clock genes (*e.g. Pdp1, shaggy, double-time, casein kinase II, clock work orange, slimb*) and analyse their temporal and spatial expression in the *Musca* brain

- it would be interesting to see whether *double-time* kinase, which enmarks PER protein for degradation in *Drosophila*, is also located in neurons with strong arrhythmic cytoplasmatic expression of MdPER

Involvement of *timeless* gene in photoperiodic induction of diapause in *Chymomyza costata*.

Summary of results:

In order to examine whether the *timeless* gene is involved in photoperiodic induction of diapause in *C. costata,* we took up on the advantage of *npd*-mutant strain (see page 9 for its characteristic). We focused on analysis of *timeless* gene expression in the brains of both wild-type and *npd*-mutant strains larvae. Larvae were simultaneously reared at two different photoperiodic regimes: SD (short days) and LD (long days). In order to explain different expression levels, we compared genomic DNA sequences of the *timeless* gene in these strains. We obtained the following results:

- we examined expression of the *timeless* gene in the brains of 4 day old 3rd instar larvae reared under SD or LD conditions:

- in the wild-type brains we detected (1) relatively high levels of *tim* mRNA, (2) presence of TIM protein in two neurons in each hemisphere and found that (3) daily patterns of both *tim* mRNA and TIM protein levels differ between SD and LD photoperiodic regimes
- in the *npd*-mutant brains we detected (1) approximately 10-times lower levels of *tim* mRNA, (2) no TIM protein and (3) no daily changes of *tim* mRNA levels

- we analysed levels of *tim* mRNA in the brains of 2 to 8 day old 3rd instar wild-type larvae reared under SD or LD conditions:

- in the SD regime, clear daily oscillations of *tim* mRNA abundance were detected
- in the LD regime, cycling of *tim* mRNA abundance was less apparent and its gradual increase appeared as a prevailing trend

- we analysed levels of *tim* mRNA in the brains of wild-type larvae upon transfer of 4 day old 3rd instar larvae from one photoperiodic regime to another:

- upon SD->DD (continuous darkness) transfer, SD-characteristic daily oscillations of *tim* mRNA levels were only slightly expressed and 100% of the larvae followed their pre-transfer SD-programmed developmental destiny (= diapause)
- upon LD->DD transfer, LD-characteristic steady increase of *tim* mRNA levels were observed and 92% of larvae followed their pre-transfer LD-programmed developmental destiny (= pupariation)
- LD->SD transfer resulted in rapid adjustment of *tim* mRNA abundance from an LD-characteristic pattern to an SD-characteristic pattern; also 93.3% of larvae changed their pre-transfer LD-programmed developmental destiny and entered diapause

- comparison of genomic DNA of the *timeless* gene between both wild-type and *npd*-mutant strains revealed:

- presence of 14 exons and 13 introns in both strains
- positions of all introns were conserved in both strains
- CDSs (coding sequences) had equal length of 4071 nucleotides in both strains
- 57 nucleotide substitutions within their CDSs resulting in 37 amino acid substitutions in *npd*-mutant TIM protein
- 1855 bp long deletion was detected in the promotor of the *npd*-mutant *tim* gene. This deletion removed the start of transcription and all putative regulatory motifs (E-box and TER-box) found in the wild-type strain

Conclusion:

Wild-type larvae of *Chymomyza costata* enter diapause when reared under SD conditions, but not under LD conditions. We observed relatively high levels of *tim* mRNA, presence of the TIM protein in two neurons in each hemisphere and found that daily patterns of both *tim* mRNA and TIM protein levels differ between SD and LD photoperiodic regimes in the brains of wild-type larvae. An SD-characteristic pattern of *tim* mRNA levels showed clear circadian oscillation, although its endogenous component appeared to be weak. These data suggest that the *timeless*

gene analysed may be a functional part of the larvae circadian clock and its expression is under photoperiodic control in the wild-type strain.

On the contrary, *npd*-mutant larvae did not respond to a photoperiodic signal as they did not enter diapause when reared under SD conditions. Approximately 10-times lower levels of *tim* mRNA and no TIM protein were observed in the brains of *npd*-mutant larvae, suggesting that proper *timeless* expression may be necessary for larval photoperiodic sensitivity. This hypothesis is further supported by the fact that transfer from LD to SD conditions resulted in rapid adjustment of *tim* mRNA abundance from an LD-pattern to an SD-pattern and almost 100% of larvae changed their LD-programmed developmental destiny and entered the diapause.

A large deletion, 1855bp long, found in the promoter of the *timeless* gene in the *npd*-mutant strain, removed the start of transcription and cis-acting transcription regulatory motifs (e.g. E-box, TER-box) found in the wild type strain. E-box and TER-box are necessary for robust and cyclic transcription of the *timeless* gene in *Drosophila melanogaster*. We thus assume that the 1855bp deletion found in the promoter of the *timeless* gene in the *npd*-mutant strain represents a possible cause of the dramatically lowered levels of *tim* mRNA in this strain.

Our results indicate that the *timeless* gene may participate in seasonal time measurement in *Chymomyza costata* larvae. However, further detailed experimentation is needed in order to verify the role of the *timeless* gene in *C. costata* photoperiodism and elucidate how the 1855bp deletion affects the circadian and photoperiodic timing systems.

Prospects of future research:

New valuable insights into the topic of our interest, whether the *timeless* gene is involved in photoperiodic induction of diapause in *C. costata,* may possibly be obtained in further functional studies. Several different approaches may be used for such future research**:**

- DNA recombinant vectors carrying CDS of some detectable signal protein (e.g. GFP or Luciferase) under the control of the wild-type or *npd*-mutant *tim* promoter can be used for transfection of *Drosophila* S2 cell cultures to elucidate the effect of the 1855bp deletion on transcription; a similar kind of

vector may also be used for transformation of *D. melanogaster* flies to examine the effect of the 1855bp deletion on circadian rhythmic transcription

- DNA recombinant vectors carrying the wild-type *timeless* CDS under the control of wild-type *tim* promoter can be used for transformation of *npd*-mutant *C. costata* flies in order to see if such a vector will "rescue" the mutant phenotype (resulting in wild-type like phenotype = photoperiodic sensitivity) in the transformed mutant flies

- DNA recombinant vectors carrying antisense RNA of wild-type *timeless* CDS under the control of a relevant promoter (e.g. *actin, timeless*) can be used for transformation of wild-type *C. costata* flies in order to "knock-down" the *timeless* gene by RNA silencing mechanism to see if the photoperiodic sensitivity will be affected in transformants

Due to the fact that diapause is a regulated developmental arrest, which involves an arrest of cell proliferation and differentiation, *C. costata* may also represent a good model organism, in which relationships between the processes of circadian time control, cell proliferation and seasonal life-cycle patterning may be tested.

Chapter 4:

CONFERENCE PRESENTATIONS

ABSTRACT 1

TIMELESS: A link between circadian and photoperiodic clocks in the fly, *Chymomyza costata*? - The story goes on

Stehlik J., Zavodska R., Shimada K., Sauman I., and Kostal V.

A central question in our study is whether the structural homologue of the clock gene *timeless* may serve as a functional part of photoperiodic time-measuring system in the fly, *Chymomyza costata* (Diptera: Drosophilidae). A mutant strain of *C. costata* is available, in which both circadian rhythmicity of adult eclosion behaviour and photoperiodic induction of larval diapause were lost after mutation of a single autosomal gene locus *npd.* Our previous research revealed that *npd* could code for TIM protein. Here, we report about the cloning of 5′ untranslated region of *timeless* gene in *C. costata*, which revealed that *npd*-mutants carry a large deletion in the promoter sequence. Quantitation of *timeless* mRNA transcripts (real-time qPCR) in larval CNS confirmed the difference between the two strains. Clear diurnal rhythmicity was found in the wild-type CNS and the diurnal patterns differed between short-day (max at Zt16) and long-day (max at Zt24) photoperiodic regimes. Endogenous rhythmicity (under constant darkness) was detectable but relatively weak. Two neurons producing TIM protein were localized in each brain hemisphere of the wild-type larvae using specific anti-TIM antibody and the level of TIM immunoreaction showed a clear diurnal pattern. In contrary, very low transcriptional rates of *timeless* were observed in the *npd*-mutant's CNS and no diurnal pattern was found. Similarly, no TIM protein could be detected in *npd*-mutant's CNS. Our results indicate that *C. costata*'s *timeless* gene might be not only the structural homologue of the *Drosophila*'s *timeless* gene, but also the functional element of *C. costata*'s circadian clock. In addition, mutation in the promotor region of *timeless* gene could cause the loss of both adult circadian rhythmicity and larval photoperiodism and thus, TIM protein might represent a molecular link between circadian and photoperiodic clock systems in this fly.

The Fifth International Symposium on Molecular Insect Science
May 20 - 24, 2006, Tucson, Arizona USA

TIMELESS: A link between fly's circadian and photoperiodic clocks? - the story goes on

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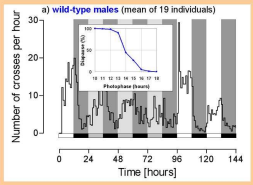

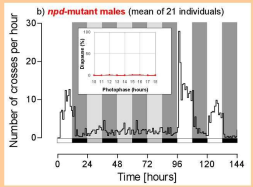

Fig. 1: Locomotion activity measured in individual adult male flies of the wild-type (A) and *npd*-mutant (B) strains. Males of both strains displayed clear diurnal rhythms in locomotion under the photoperiodic regime (short-day, L12:D12). Endogenous rhythmicity under continuous darkness was seen only in the wild-type flies (though the rhythm was dampening relatively rapidly). The insets in Figures show photoperiodic responses in larvae: while the wild-type larvae respond to sub-critically short-days by entering larval diapause, no photoperiodic response is observed in the npd-mutants.

INTRODUCTION

Circadian clocks allow organisms to anticipate daily changes in environmental factors and appropriatelly tune their physiological state. Significant progress in understanding the molecular details of circadian clocks has been achieved in *Drosophila melanogaster*. The photoperiodic clocks perceive seasonal change of day/night lengths, which signals for seasonally cycling deterioration of conditions for life. Physiological mechanisms of photoperiodic clocks remain unknown. **A central question is whether the known molecular elements of circadian clocks may serve as functional parts of photoperiodic clocks.** Here we present a continuation of our study on this topic using the drosophilid fly, *Chymomyza costata* as a model.

A mutant strain of *C. costata* is available, in which both circadian rhythmicity of adult behaviour and photoperiodic induction of larval diapause were lost after mutation of a single autosomal gene locus *npd* (Fig. 1A, B). Our previous research has been focused on adult flies (Pavelka et al., J. Eur. Entomol. 100, 255-265, 2003) and revealed that *npd* locus could code for TIMELESS protein. Recently, we have extended our attention to the larvae, a stage which is sensitive to photoperiodic signal.

RESULTS

1. The *npd*-mutants show a significant change in the promoter region of *timeless* gene. The structures of coding sequences are similar in the wild-type and *npd*-mutant strains.

Almost complete genomic structures of the *timeless* gene were elucidated in both fly strains. Several nucleotide substitutions that change the amino acid sequence of the protein were found within the *timeless* coding sequence of *npd*-mutants (Fig. 2). The most apparent difference between the two strains, however, was found in the 5'-leader region of cDNA and in the promoter sequence. The *npd*-mutants carry a completely different promoter sequence lacking the TER-box and E-box motifs, which are critically needed for correct transcription of *tim* in *D. melanogaster*.

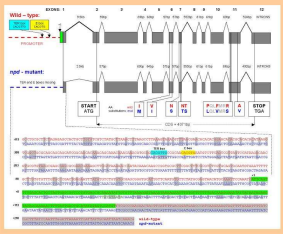

Fig. 2: Genomic structures of *timeless* gene significantly differ between the wild-type strain and *npd*-mutants. The coding sequences (CDSs) are almost identical in the two strains; the differences in amino acid translations are highlighted. Eleven introns were found. The first intron, which is positioned within the 5'-leader sequence, is very long and still not completely identified (the length was only estimated by PCR). Structure of the promoter sequence is completely changed in the *npd*-mutants (see lower part of the Figure).

2. Transcription of *timeless* gene is suppressed in the larval CNS of *npd*-mutants.

2.1. The diurnal cycling-patterns of *tim* mRNA abundance differ between the long-day and short-day reared wild-type larvae.

On day 16 of larval age (day 4 of the 3rd instar), which is the stage with maximum sensitivity to photoperiodic signal, the diurnal pattern of relative abundance of *tim* mRNA transcripts in the CNS were measured using quantitative real time PCR (qRT-PCR) technique. Generally, the abundances were very low, and without any diurnal pattern, in the *npd*-mutants. In the wild-type flies, the abundances were relatively high and the diurnal patterns differed between the larvae reared at long-day conditions (LD, 16L:8D) (larvae destined to pupariation) and those reared at short-day conditions (SD, 12L:12D) (larvae destined to enter diapause) (Fig. 3).

The diurnally cycling pattern of *tim* mRNA abundance was apparent throughout the 3rd instar in the SD-reared wild-type larvae (Fig. 4, blue line). The cycling-pattern was not clearly observable in the LD-reared larvae, probably because it was "masked" by the overall increasing trend of the *tim* mRNA abundance during the 3rd instar (Fig. 4, red line).

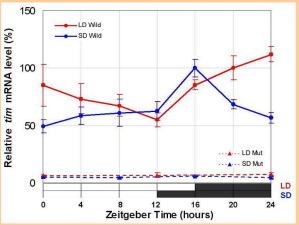

Fig. 3: Diurnal changes in the relative abundance of *tim* mRNA transcripts in the CNS dissected from larvae of *C. costata* when they were 16-d-old. Larvae were reared at either long-day (LD) or short-day (SD) conditions. Ten CNS were dissected into RNAlater (Qiagen). Three independent replications of each sample were made. Total RNA was isolated using RNA Blue (TopBio, Czech Rep.) and was used for cDNA synthesis (RT system, Promega). cDNA was used as a template for qRT-PCR reactions (Rotor Gene RG3000, Corbett Research), which were primed using *tim* gene-specific primers designed to span exon-intron boundaries. The abundance of a reference transcript, *ribosomal protein 49* (*rp49*) was estimated in parallel in each sample. The points show means ± S.D. ($n = 3$) relative abundances of *tim* mRNA transcripts at different Zeitgeber times.

CONCLUSIONS

- The promoter of *timeless* gene shows significant structural change in the *npd*-mutants of *Chymomyza costata*. Because no TER-box and E-box sequences were found upstream of the start of 5'-leader sequence of cDNA, transcription of the gene could be severely impaired in the *npd*-mutants.
- Very low levels of *tim* mRNA transcripts and no expression of TIM protein were seen in the larval CNS dissected from *npd*-mutants. In contrast, *tim* mRNA reached relatively high abundance and two TIM-expressing neurons were found in each brain hemisphere in the wild-type larval CNS.
- In the wild-type larvae, differing patterns of *tim* mRNA abundance development during the 3rd instar characterized the larvae reared at long-days and short-days. Clear diurnal oscillations of *tim* mRNA abundance were found in the short-day larvae (destined to diapause). A weak endogenous component seems to support such diurnal cycling.
- Upon transfer of 3rd instar larvae from long- to short-days, the *tim* mRNA abundance pattern rapidly (within 1 day) entrained to new photoperiodic conditions. This was accompanied with gradually ceasing cell proliferation in the larval brains and entrance of the larvae into diapause state.
- **Collectively, supporting data were obtained, which suggest that the TIMELESS protein could be functionally involved in both circadian and photoperiodic clocks of the *C. costata* fly.**

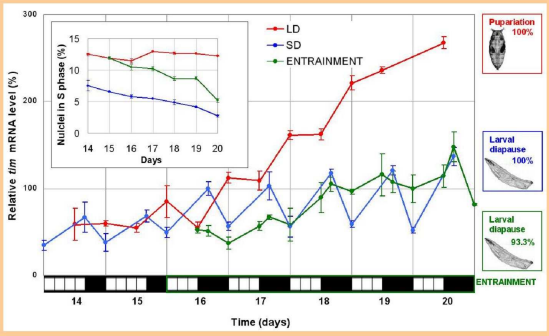

Fig. 4: Diurnal changes in the relative abundance of *tim* mRNA transcripts in the CNS dissected from larvae of *C. costata* during the 3rd instar development. Larvae were reared at either long-day (LD) or short-day (SD) or they were transferred from LD to SD when 15-d-old (see legend of Fig. 3 for more details). The inset in Fig. 4 displays the changes in relative proportions of nuclei in synthetic phase (S phase) of the cell cycle. The nuclei were prepared from larval CNS (ten CNS per sample, 2-3 replications (Cycle Test Plus DNA Reagent Kit, Becton Dickinson) and measured on FACScalibur flow cytometer (Becton Dickinson) (5 x 10 000 nuclei were measured in each replication).

2.2. Two neurons in each brain hemisphere express TIM protein in the wild-type larvae. No TIM-expressing neurons were found in the *npd*-mutants.

Using the *C. costata* specific rabbit anti-TIM antiserum, two neurons were stained in each brain hemisphere in the CNS of wild-type larvae (Fig. 5A, B). No immunoreaction was observed in the ventral ganglion. The CNSs were dissected from 16-d-old larvae reared at either LD or SD conditions. Similarly positioned neurons were consistently found in the LD- and SD-brains dissected at four different Zt times (Zt 2, 8, 14, 20). The same antiserum (equally applied in a parallel experiment), failed to detect any TIM-positive neurons in the CNSs dissected from *npd*-mutants (Fig. 5C).

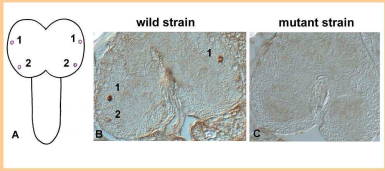

Fig. 5: TIMELESS expression in the brain neurons of wild-type larval CNS (A, B) and no staining in the *npd*-mutant larval CNS (C). CNSs were dissected at Zt 2 from the SD-reared larvae when 16-d-old. The representative pictures are displayed. Four treatments were compared: wild-type LD; wild-type SD; *npd*-mutant LD; *npd*-mutant SD. Ten larval CNSs of each treatment were fixed in Bouin-Hollande solution, embeded in Paraplast and sectioned using 820 Spencer Microtome (American Optical Corp.). Sections were treated with primary (rabbit anti-TIM) and secondary (goat-anti-rabbit HRP conjugated) antibodies. Preparations were stained with 3',3'-diaminobenzidine, mounted in DPX medium and viewed under Zeiss Axioplane 2 microscope (magnification 160x). All wild-type brains (both LD and SD) showed similarly positioned two neurons in each hemisphere. None of the *npd*-mutant brains showed staining.

2.3. The long-day *tim* mRNA abundance pattern rapidly changes to the short-day pattern upon transfer of larvae from one photoperiodic regime to the other.

When the wild-type LD-reared larvae were transferred from the LD to SD conditions on day 15, the cell proliferation in their CNS gradually ceased and almost 100 % of them changed their developmental destiny from pupariation to larval diapause. Simultaneously, the cycling of *tim* mRNA abundance rapidly changed (entrained) from the LD to SD pattern (Fig. 4, green line). In fact, the change in the development of *tim* mRNA abundance was apparent already during the first day (day 16) after transfer from LD to SD. On day 20, almost identical cycling-patterns were observed in the SD-reared and LD→SD-transferred wild-type larvae.

2.4. An endogenous component of diurnal cycling of *tim* mRNA abundance is weak.

When the LD- or SD-reared wild-type larvae were transferred to continuous darkness on day 16, the cycling-patterns of *tim* mRNA abundance mostly dissapeared (Fig. 6A, B). Under the SD-conditions, a weakly expressed continuation of the diurnal-like pattern was observed, which suggests that a weak endogenous component may participate in driving the diurnal oscillations of *tim* mRNA abundance. The transfers to continuous darkness did not change developmental destinies of the larvae.

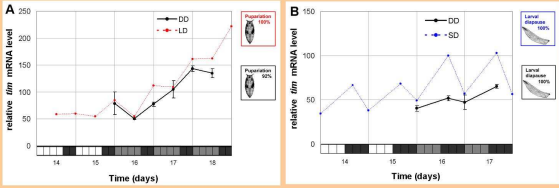

Fig. 6: Changes in the relative abundance of *tim* mRNA transcripts in the CNS dissected from larvae of *C. costata*, which were transferred from photoperiodic regime to continuous darkness when they were 15-d-old. The larvae were initially reared either at long-day (A), or short-day (B), conditions (see legend of Fig. 3 for more details). The dashed lines are redrawn from Fig. 4 for comparison. They show how the *tim* mRNA abundance develops in the larvae when they are maintained under the respective photoperiodic conditions.

ABSTRACT 2

Involvement of circadian clock in photoperiodic calendar function and diapause induction of the fly, *Chymomyza costata*.

Kostal V., Stehlik J., Zavodska R., Sauman I., and Shimada K.

Molecular mechanisms underlying the photoperiodic induction of larval diapause were studied in the drosophilid fly, *Chymomyza costata*. Our previous results revealed that the expression of clock genes *period* and *timeless* is abnormal and the function of central circadian clock is impaired in the *npd* (non-photoperiodic-diapause) mutants. Disrupting the *timeless* transcription in the wild-type strain by injection of *timeless* dsRNA into early embryos resulted in adoption of a *npd*-mutant phenotype (no-diapause in response to short-daylength) in a certain proportion of individuals. Genetic linkage analysis confirmed that a mutant allele of *timeless* gene, *timelss*^{npd}, is strictly co-inherited with the mutant phenotype. Recently, clear daily cycling of *tim* transcripts was observed in larval CNS during the 3rd instar development under short-day conditions. Such cycling was less apparent under the long-day conditions, where a gradual increase of *tim* transcripts abundance appeared as a previling trend. TIM protein was localized in two neurons in each brain hemisphere of wild-type larvae using anti-TIM antibody. In a marked contrast to wild-type strain, no neurons were stained in the CNS of *npd*-mutant larvae and the level of *tim* transcripts was approximately 10-fold lower in them. By transferring sensitive wild-type larvae to continuous darkness, we found that an endogenous component of daily *tim* transcript oscillations was relatively weak (short-day larvae) or unapparent (long-day larvae). When sensitive wild-type larvae were transferred from long-day to short-day conditions, they responded by entering diapause and their *tim* expression changed within less than a single day from a long-day to a short-day pattern. Analysis of genomic structure of *timeless* gene in both strains revealed that *timeless*^{npd} allele carries a large (ca. 1800 bp) deletion in the promoter region. As the deletion covers important regulatory sequences, we hypothesize, that this mutation in *timeless* promoter might cause a disruption of circadian clock function in adult insects and disruption of photoperiodic calendar function in larvae of *C. costata.*

2nd World Congress of Chronobiology
November 4 - 6, 2007, Tokyo, Japan