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CIRCADIAN CLOCK GENES IN INSECTS

Ph.D. Thesis

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České Budějovice 2017

This thesis should be cited as: Bazalová O, 2017. Circadian Clock Genes in Instects. Ph.D. Thesis Series, University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, Czech Republic.

Annotation

This thesis focuses on molecular characterization of circadian clock genes in insects. It explores genetic diversity of circadian clock genes by molecular characterization of several insect species including two dipteran flies (*Musca domestica* and *Drosophila melanogaster*), two cockroach species representing ancestral insects, and the linden bug, *Pyrrhocoris apterus*. Furthermore it considers various roles of circadian clock genes in insect physiology. Application of molecular-biology methods in *Pyrrhocoris apterus*, non-model insect species, enable us to investigate involvement of circadian clock genes in photoperiod induced physiological responses. Application of molecular-biology methods in *Periplaneta americana* and *Blattella germanica* was used to explore involvement of circadian clock genes in magnetoreception.

Declaration [in Czech]

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This thesis originated from a partnership of Faculty of Science, University of South Bohemia, and Institute of Entomology, Biology Centre CAS v.v.i., supporting doctoral studies in the Molecular biology and genetics study program.

Přírodovědecká fakulta
Faculty of Science

Jihočeská univerzita v Českých Budějovicích
University of South Bohemia in České Budějovice

Financial support

This work was financially supported by following grants and supporting institutions:

- Ministry of Education of the Czech Republic LH14029
- Grant Agency of the University of South Bohemia 16/2007/P
- Grant Agency of the University of South Bohemia 130/2012/P
- Grant Agency of the University of South Bohemia 099/2013/P
- Grant Agency of the Czech Republic project 206/07/J041
- Grant Agency of the Czech Republic project 13-11908J
- Grant Agency of the Czech Republic project 14-32654J
- Grant Agency of the Czech Republic project 17-01003S

Acknowledgements (poděkování)

First of all, I would like to thank to my supervisor David Doležel for providing me with excellent conditions to perform all the work. It was him who introduced me into the basic molecular biology techniques (let´s say) a few years ago. I am grateful for his endless patience and empathy, and for his never-ending enthusiasm when proposing more and more work. And for no complaints about the time I spent with my dogs while not working in the lab.

I would also like to thank to all of my colleagues Alena Kobelková, Silva Fexová, Adam Bajgar, Roman Naužil, Katka Švehlová, Honzík Provazník, Lenka Pivarčiová, Martin Pivarči, Hanka Vaněčková, Honzík Martinek, Verča Urbanová, Samarjeet Singh, Bulah Wu, Aška (Joaana Kotwica-Rolinska), Milena Damulewicz, Vlastík Smýkal, Markéta Hejníková, Hanka Sehadová and Radka Závodská who helped me during my studies by their always inspiring advices, collaborations and (huge) mental support. I´m very thankful to prof. Ivo Šauman for his professional advices and help. My special thanks belongs to Lenka and Aška for their critical reading of the thesis.

My great THANKS belongs to my love Jaromír Cihlář for his patience, empathy and love he gives me. And for the help with my two dog-devils in the last days, without that I would never ever finish the thesis.

Můj největší dík patří mé rodině – mamce, taťkovi, Míše, babičkám a dědům – za jejich neutuchající podporu jak psychickou, tak finanční.

List of papers and author's contribution

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

Two of the presented papers are already accepted in respected journals, the last one is in form of manuscript (prepared for final correction and submission in impact journal)

Bazalova O, Kvicalova M, Valkova T, Slaby P, Bartos P, Netusil R, Tomanova K, Braeunig P, Lee HJ, Sauman I, Damulewicz M, Provaznik J, Pokorny R, Dolezel D, Vacha M. (2016) *Cryptochrome 2* mediates directional magnetoreception in cockroaches. Proc Natl Acad Sci U S A. (Feb 2016), 9; 113(6): 1660-5. doi: 10.1073/pnas.1518622113.

O. Bazalová and M. Kvíčalová contributed equally to this work. Olga designed and performed all of the locomotor activity experiments, prepared most of the clones used as the template for double stranded RNA preparation, prepared all of the dsRNAs used in experiments, performed all the RT-qPCR assay verifying the knocking down of the gene expression and most of the imunocytohistochemical experiments followed by the laser-scanning confocal microscopy.

Urbanova V, Bazalova O, Vaneckova H, Dolezel D. (2016) Photoperiod regulates growth of male accessory glands through juvenile hormone signaling in the linden bug, *Pyrrhocoris apterus*. Insect Biochem Mol Biol. (Mar 2016), 70: 184-90. doi: 10.1016/j.ibmb.2016.01.003.

O. Bazalová and V. Urbanová contributed equally to this work. Olga prepared the dsRNA used to knock down particular genes of interest and heterologous controls, designed and performed the behavioral experiments – some locomotor activity and all of the mating experiments, measured mRNA level of certain circadian genes using the RT-qPCR method and analyzed most of the data.

Bazalova O, Dolezel D. Daily activity of the housefly, *Musca domestica*, is influenced by temperature independently on period gene splicing. In preparation for Behavioral Genetics

Olga designed and performed all the behavioral experiment, using RT-qPCR measured the mRNA levels of circadian clock genes and the possible splicing variants in all the group of animals and analyzed all the data. Olga participated on research design, and revision of the article.

List of abbreviations

CLK - clock
CYC - cycle
PER - period
TIM - timeless
CRY1 – cryptochrome 1
CRY2 – cryptochrome 2
PDP 1ε – par domain protein 1 epsilon
CWO – clock work orange
VRI - vrille
PDF – pigment dispersing factor
SD – short day
LD – long day
JH – juvenile hormone
MET – methopren-tolerant
TAI - taiman
CA – corpus allatum
CC – corpora cardiaca
PI – pars intercerebralis
MAG – male accesory gland
GMF – geomagnetic field
MIR – magnetically induced restlessness
RNAi – RNA interference

Abstract

Organisms experience regular changes of environment, such as periodic alternation of day/night and seasonal weather changes. To maximize their fitness, organisms evolved the biological clocks that anticipate these conditions in advance – circadian clock measuring approximately 24h intervals and photoperiodic clock that distinguishes between long (summer) and short (autumn) days. Although circadian clock genes are conserved even between *Drosophila* and human, certain important and interesting differences exist. This thesis focuses on molecular characterization of circadian clock genes in insects. The goal of this work was to explore genetic diversity of circadian clock genes by molecular characterization of several insect species including two dipteran flies, two cockroach species representing ancestral insects, and the linden bug, *Pyrrhocoris apterus*. This selection allowed identifying surprising diversity even between related species. Moreover, species-specific biology of these organisms created opportunity to explore additional circadian clock-related phenomena, such as photoperiodic regulation of diapause and role of circadian clock proteins cryptochromes in magnetoreception. Comparison of *Drosophila* and *Musca* indicates that both species adjust their daily locomotor activity pattern similarly in respect to surrounding temperature. However, molecular mechanisms orchestrating this activity distribution are probably different between these two species. Functional reverse genetic experiments on the linden bug, *P. apterus*, suggest that circadian genes *Clock* and *cryptochrome* 2 are essential for the photoperiod measurement. To shed some light on possible role of cryptochrome proteins in magnetoreception, two cockroach species were studied by combination of reverse genetics, immunocytochemistry and behavioral experiments. Taken together, diversity of insect circadian clocks is not only interesting and inspiring, but also an amazing opportunity to decipher molecular basis of widely distributed adaptation for which molecular mechanisms is unknown.

Abstrakt

Většina organismů žijící na planetě Zemi je vystavena různým denním a sezónním změnám. V průběhu evoluce byly organismy těmto změnám vystaveny a vyvinuly si vlastní systém, jak měřit ubíhající čas a přizpůsobovat svůj vývoj, fyziologické procesy a chování aktuálním podmínkám prostředí. Systém přizpůsobení se není však jen pasivním kopírováním měnících se životních podmínek, ale je to systém vnitřních hodin, které každému živočichovi umožňuje předvídat následující změny a podle toho přizpůsobit svou životní strategii tak, aby mohl organismus úspěšně přežít. Geny cirkadiánních hodin jsou značně konzervované mezidruhově. Funkční homology lze nalézt jak u octomilky, tak u člověka. Na druhou stranu existují rozdíly mezi jednotlivými druhy na hladině fungování celého mechanismu. Tato práce se zaměřuje na molekulární charakteristiku cirkadiánních genů u různých druhů hmyzu, jako jsou mouchy z řádu dvoukřídlých (octomilka a moucha domácí), dále dva zástupci řádu švábi (rus domácí a šváb americký) a jeden zástupce řádu polokřídlých (ruměnice pospolná). Tento výběr nám umožňuje studovat nejen molekulární podstatu biologických hodin u jednotlivých zástupců hmyzu, ale také studium možných dalších úloh genů cirkadiánních hodin, jako je například fotoperiodická regulace, nebo schopnost megnetorecepce. Teplota ovlivňuje chování (lokomoční aktivitu) u mouchy domácí podobně jako u octomilky, ovšem z našich výsledků vyplývá, že molekulární podstata tohoto chování bude u těchto dvou druhů odlišná, navzdory jejich relativně blízké příbuznosti. Studiem fotoperiodických hodin u ruměnice pospolné jsme odhalili důležitou roli cirkadiánních genů *Clk* a *cry2* v tomto mechanismu. U švábů jsme pomocí reverzní gentiky, behaviorálních studií a zobrazovacích technik dokázali odhalit důležitou roli CRY2 v magnetorecepci. Výsledky této práce ukazují, jak důležité je studovat molekulární podstatu biologických hodin u různých zástupců hmyzu. Nejen kvůli odlišnému mechanismu fungování na hladině genů, ale také vzhledem k různé úloze těchto genů v dalších procesech v organismu.

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1 Introduction

Living on Earth is challenging for all organisms. They have to cope with the fact their environment vary during the day and the year, including light/dark cycles, light intensity, temperature, moisture, nutrient availability and many other factors. Many of these changes periodically cycle, although their periods may differ from hours to years. To enhance their fitness, organisms evolved mechanisms how to predict the upcoming conditions in advance. The emergence of biological clocks has turned responders to predictors and become widespread across taxa (Dunlap et al., 2004). This ability serves to synchronize many physiological and behavioral functions which bring to the individual considerable benefits. The science studying biological clock is referred as CHRONOBIOLOGY. The word is derived from three Greek stems: CHRONOS stands for time, BIOS for life and LOGOS for science. In general we can divide the appearance of biological aspects according to the cycle of its duration to **1)** Ultradian clocks with the period of less than 24 hours; **2)** Circadian clock (period close to 24 hours); **3)** Circatidal clock (period of approximately 12,4h); **4)** Circannual clock (period of approximately 1 year); and **5)** Occurrence once in a lifetime with precise timing to the most beneficial phase of the animal's life (Dunlap et al., 2004).

The earliest recorded account of a circadian process dates from the 4th century B.C.E., when Androsthenes, a ship captain serving under Alexander the Great, described diurnal leaf movements of the tamarind tree (Bretzl, 1903). But the very first scientific endogenous circadian oscillation was recorded by the French scientist Jean-Jacques d'Ortous de Mairan in 1729. He noted that 24-hour patterns of the movement of the leaves of the plant *Mimosa pudica* continued even when the plants were kept in constant darkness. At the end of 19th century and at the beginning of the 20th century scientist started to study clocks in humans and animals in more details. For insects *Drosophila melanogaster* became a model organism. Some principles discovered in *Drosophila* helped to identify molecular basis of human disorders (Toh et al., 2001; Xu et al., 2005).

1. 1 Circadian clocks in insect

In the 1971 Konopka and Benzer published their findings on the fruit fly (*Drospohila melanogaster*). Their mutant lines of flies showed abnormal daily locomotor activity and eclosion timing with shortened or prolonged period or with no rhythmicity. Since that time scientists have been studying circadian clock not only in model organisms, *Drosophila melanogaster*, but also in many other insect species (Levine et al. 1995; Sauman et al. 1996; Sauman a Reppert 1996; Rubin et al., 2006; Tomioka et Matsumoto, 2010). Circadian clocks are endogenous, genetically determined mechanisms which evolved as an adaptation to cyclic changes in light, temperature, and other environmental factors during the day and night. One can find conserved homologues of almost all important circadian clock genes in almost all animals, not only insects. Recent studies revealed unexpected variability in the central clock mechanism (Yuan et al., 2007) and this intriguing diversity enables scientist to observe how genes with highly conserved amino-acid sequences can manifest functional polymorphism. The most conserved part of the circadian clock molecular mechanisms consists of clock proteins accumulating in the cytoplasm, which then enter the nucleus where they negatively feed back on their own transcription. When the level of the protein is decreased the transcription is restored again (Dunlap, 1999; Cyran et al., 2003; Peschel et Helfrich-Förster, 2011; Tomioka et Matsumoto, 2010). Typical behavioral circadian phenotypes are sleep/wake cycles, daily locomotor activity changes, egg hatching and eclosion.

1.1.1 Mechanisms of the core oscillator – *Drosophila* model

In flies the central peacemaker is located in the lateral brain close to the optic lobes in a group of 5-6 clusters of neurons. Each cluster is named according to its location and the size of individual neurons (Helfrich-Förster et al., 2007, Fig. 1). It still remains unclear whether all clusters of clock neurons are important for controlling behavioral rhythms, because several studies suggest that they are not all of equal importance (Yoshii et al., 2012).

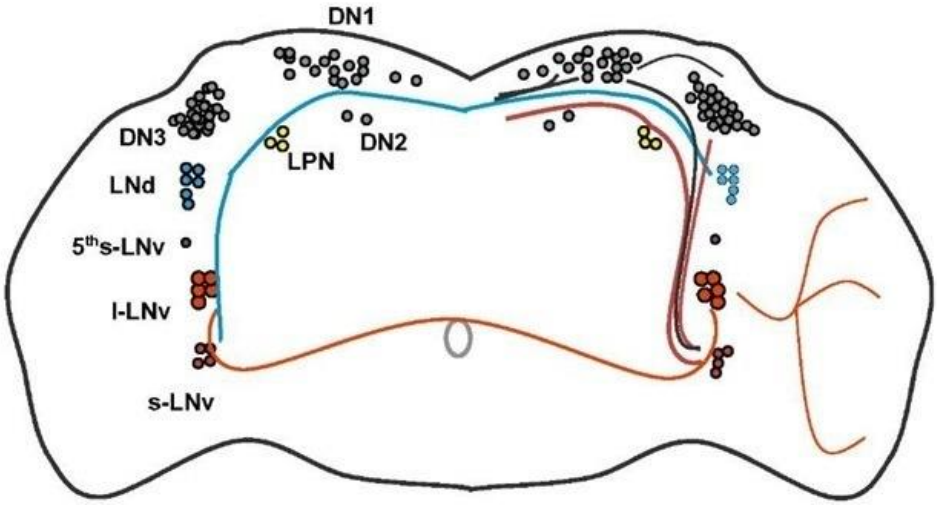

Fig. 1 Simplified model of clock gene expressing neurons in the *Drosophila* brain. For simplicity, only the projections from the neurons of the right hemisphere are shown. The lateral neurons consist of the LN_d cells (blue), the PDF-positive l-LN_v (orange) and s-LN_v cells (dark brown), and the PDF-negative fifth s-LN_v cell (brown). The dorsal neurons comprise the DN_1, DN_2, and DN_3 cells (all in grey). Furthermore, three PER/TIM neurons are located in the posterior lateral brain (lateral posterior neurons; LPN, in yellow).

1.1.1.1. Feedback loops

Breakthrough in studying molecular basis of the core oscillator was reached by successful cloning of the clock gene *period* from *Drosophila melanogaster* (Bargiello et Young, 1984; Reddy et al., 1984; Jackson et al., 1986; Hardin et al., 1990). The following research on fruit fly was quite expansive and led to deepen the knowledge about the molecular mechanisms of *Drosophila* clock (reviewed by Dunlap 1999; Stanewsky 2002; Tomioka et Matsumoto, 2010; Rivas et al., 2016). Nowadays scientists identified many crucial genes playing an important role in the clock machinery and we know a lot about their reciprocal interactions. Figures 2 and 3 schematically show the central loops of *Drosophila* clock mechanisms – the most important genes and their interaction.

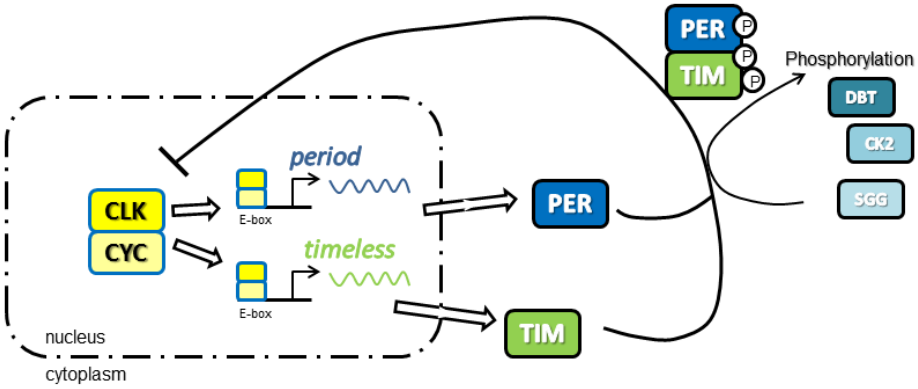

Fig. 2 Diagram of the central clock molecular machinery of *Drosophila melanogaster* – the first feedback loop, which consists of clock genes *Clock*, *cycle*, *period* and *timeless*. Arrows indicate positive/stimulatory regulation, while blunt ends indicate negative/inhibitory regulation. See details in text. Adapted from Tomioka et Matsumoto, 2010.

The first feedback loop (Fig. 2) constitutes of basic helix-loop-helix transcription factors *Clock* (*Clk*) and *cycle* (*cyc*), and circadian clock genes *period* (*per*) and *timeless* (*tim*) (Hardin, 2006; Stanewsky, 2002). Proteins CLK and CYC make heterodimers through their PAS domains in the nucleus (Allada et al., 1998; Rutila et al., 1998), where they act as a transcription activators of *per* and *tim* transcription by binding to their promoters (E-boxes) during the afternoon or early night (Curtin et al.,1995; Darlington et al., 1998). As the concentration of PER and TIM proteins rise in the cytoplasm, they dimerize and enter the nucleus where they inhibit their own transcription by preventing the CLK/CYC complex from binding *per* and *tim* promoters (Lee et al., 1999, Bae et al., 2000; Yu et al., 2006). Since *per* and *tim* transcription decline, PER and TIM level decrease so the CLK-CYC dimer can bind to the E-boxes and the loop can be restored again.

The stability of TIM-PER complex is affected by many factors such as **1) Kinases** - C*asein kinase 2* (CK2 (Akten et al., 2003; Lin et al., 2002)), *doubletime* (DBT – ortholog of human *casein kinase 1 epsilon;* (Price et al., 1998)) and *shaggy* (SGG (Martinek et al., 2001)); **2) phosphatases** - *Protein phosphatase 2A* (PP2A (Sathyanarayanan et al., 2004)), F-box/WD40-repeat protein SLIMB (Ko et. al, 2002; Grimma et al., 2002) and

Leukocyte-antigen-related-like gene (LAR, (Agrawal et Hardin, 2016)) ; and **3) E3 ligase.** All these factors tune the timing of the TIM-PER complex entry to the nucleus or - in case of DBT and PP2A - regulate also CLK activity (Kim et Edery, 2006; Yu et al., 2006).

The second loop (Fig. 3) assesses oscillations of *Clk*, *vrille* (*vri*) and *Par domain protein 1 epsilon* (*Pdp 1ε*) transcripts. CLK-CYC heterodimer initiates not only transcription of *per* and *tim*, but also binds to E-box of *vri* and P*dp 1ε* and stimulates their transcription during late day to early night. *Vri* mRNA is quickly translated to VRI protein, which enters the nucleus where it binds to V/P – box of C*lk* and inhibits its transcription, thus the *Clk* mRNA is reduced during the night. Translation of P*dp 1ε* mRNA is delayed, therefore the protein increases during late night to early day. Then it enters nucleus, where it competitively to VRI binds V/P- box of *Clk* and activates its transcription. Thus, the *Clk* transcripts increase during the day, also leading to an increase of CLK protein during the day. But there are some suggestions that the relevance of PDP 1ε participation in the loop is debatable (Benito et. al, 2007).

The third negative feedback loop (Fig. 3) is formed by the clock gene *clock work orange* (*cwo*), a basic helix-loop-helix ORANGE family protein, which acts as a transcriptional repressor that synergizes with PER and inhibits its own transcription as well as expression of other clock genes through E-box elements (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007; Richier et al., 2008; Zhou et al., 2016).

Recently it was shown that nuclear receptors *unfulfilled* (*unf*) and *E75* play an important role in circadian pacemaker. The precise molecular basis of how they contribute to the mechanisms is still not well known. Moreover they are both expressed in the circadian neurons and collaborate to enhance CLK-CYC mediated transcription of *per* gene (Beuchle et al., 2012; Jaumouillé et al., 2015). E75 protein also works together with VRI as a repressor of C*lk* transcription, and its activity is lowered by PER, revealing new possible role for PER as a de-repressor of *Clk* transcription (Kumar et al., 2014).

Expression of some of circadian clock genes (*Clk*, *cwo* and *vri* in *Drosophila*) is post transcriptionally regulated by micro RNA machinery (miRNA) which occurs through binding to the regulatory sequences, or by degradation of messenger RNA of circadian clock genes. The best studied miRNA bantam is present in circadian clock neurons and

binds to the regulatory sequence of *Clk* gene, while its overexpression prolongs period of the clock (Yang et al., 2008).

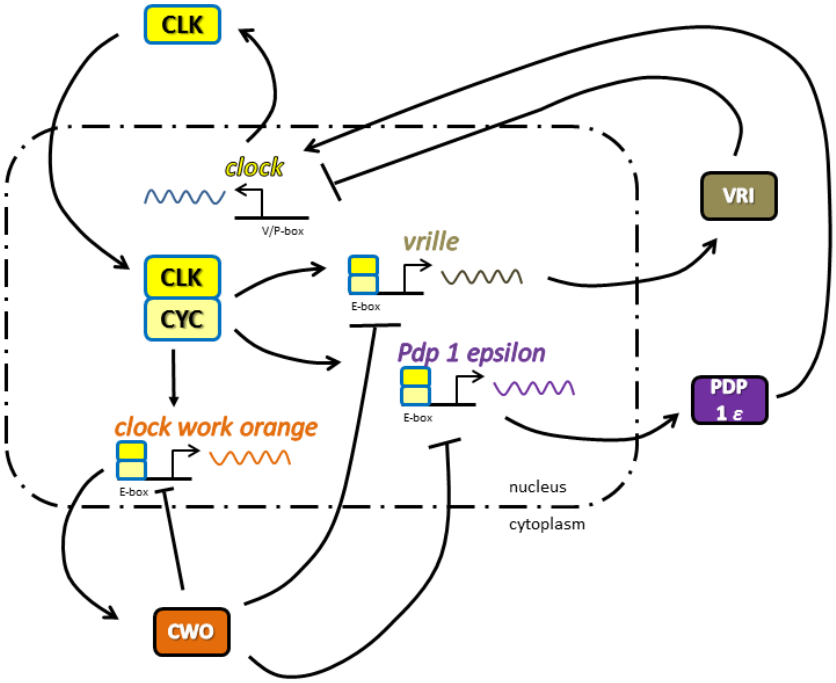

Fig. 3 Diagram of the central circadian clock molecular machinery of *Drosophila melanogaster* – the second and third feedback loop. Arrows indicate positive/stimulatory regulation, while blunt ends indicate negative/inhibitory regulation. See details in text. Adapted from Tomioka et Matsumoto, 2010.

1.1.1.2 External entrainment of core oscillator

Even though the clocks are endogenous, they can be entrained by external cues to adjust to a proper time in synchrony with the surrounding environment. Light is denoted as the major external cue. Insects have traditionally been considered as poikilotherms, so not only light but also temperature is the major external cue synchronizing their inside clocks. Furthermore, it has been shown that *Drosophila melanogaster* flies during cold days are more active during the day, and during hot days they prolong their midday "siesta" and the morning and evening activity peaks are shifted into the cooler nighttime hours (Majercak et al., 1999). On the other hand in case of *Drosophila yacuba* flies, which have more ancestral distribution indigenous to Afro-equatorial regions wherein day length

and temperature exhibit little fluctuation throughout the year, the little effect of temperature on the daily distribution of activity is observed (Low et al., 2008).

Insects use several structures and molecules to receive light and temperature input signals and forward them to the main oscillator. *Drosophila* genome-wide expression analysis showed hundreds of genes responsive to the light and temperature. Small number of transcripts is directly affected by light, whereas temperature can drive the expression of large number of genes (Boothroyd et al., 2007), which indicate importance of this environmental cue for the entrainment. It was shown that low temperatures enhance the splicing efficiency of a 3´UTR intron in *Drosophila melanogaster per* gene, which leads to advanced phase of accumulation of its mRNA and protein level, respectively (Majercak et al., 1999). Similarly, the gene *timeless* is affected by temperature, since in the lower temperatures the isoform of *tim*, called tim^{cold} is dominant and contributes in the increase of overall *tim* transcripts (Boothroyd et al., 2007).

Temperature

The role of temperature in circadian clock is interesting for several reasons. If the clock has to measure time precisely, it has to be temperature-independent. Indeed, circadian clock runs with comparable pace over physiologically relevant range of temperatures. This phenomenon is called temperature compensation. On the other hand, cyclical changes in temperature might serve as an excellent synchronization signal. Indeed, even a few degree steps can phase shift locomotor activity of flies in otherwise constant conditions. And lastly, organisms prefer to dwell in certain ambient temperatures. Indeed, animals can shift their activities to the early morning or the late evening to avoid mid-day high temperatures. Conversely, low temperatures influence the shift of the behavior in opposite direction. Obviously, all these aspect related to temperature sensing and temperature compensation of circadian clocks might be regulated at different levels, either anatomic (cells, organs) or genetic.

The input signals about temperature cycles are delivered to the *Drosophila* brain via chordotonal organ (Sehadova et al., 2009; Simoni et al., 2014) where genes *nocte* and *pyrexia* are expressed. Both of them are important for synchronization at low

temperatures, while *Ionotropic Receptor 25a*, plays an important role in perception of small temperature cycles (Wolfgang et al., 2013; Chen et al., 2015). It was suggested that *Transient Potential A1* gene (*TrpA1,* expressed both in clock and non-clock neurons in *Drosophila* brain) might play an important role in temperature dependent regulation of afternoon siesta in *Drosophila melanogaster* (Lee, 2013; Lee et Montell, 2013, Das et al., 2015, Green et al., 2015). The molecular mechanism of temperature entrainment is not fully understood, but the ability to entrain to temperature cycles depends on functional clocks, since per^0 mutants merely respond to temperature cycles (Wheeler et al., 1993; Yoshii et al., 2005). Temperature increases intracellular Ca^{2+} levels, which then triggers CALMODULLIN degradation of TIM by the SOL (Small Optic Lobe) protease (Tataroglu et al., 2015). The gene *norpA* (no receptor potential A) might also play a role in synchronization to temperature cycles (Sehadová et al., 2009; Glaser et al., 2005; Collins et al., 2004). Since n*orpA* gene works also in photo transduction, it is suggested that light and temperature act not only independently, but also together to compensate and reinforce the adaptation to the specific time of a day (Fig. 4).

Light

The most important photoreceptor organs are the compound eyes (Tomioka et Chiba, 1984; Loher, 1972), ocelli (Rence et al., 1988; Helfrich-Förster et al., 2002), and in *Drosophila* H-B eyelets, which are remnants of the larval eye (Helfrich-Förster et al., 2002). In addition, CRY-expressing circadian clock neurons in *Drosophila* have ability to be entrained by the blue light (Emery et al., 2000). The molecular mechanisms of light/dark cycles entrainment is relatively well defined. In *Drosophila melanogaster* this is mainly achieved through the flavoprotein Cryptochrome1 (CRY1). Light induces the conformational change that activates CRY1, which then can bind to TIM and conduct it to the proteasome degradation through the E3 ligase protein JETLAG (JET, Fig. 4) (Suri et al., 1998; Yang et al.,1998; Ceriani et al.,1999; Naidoo et al.,1999; Lin et al.,2001; Busza et al.,2004; Koh et al.,2006; Peschel et al., 2006) and WD40 protein – Bromodomain and WD repeat domain containing 3 protein (BRWD3) – a substrate for cullin 4 ring finger E3 ligase (Ozturk et al., 2013). As the TIM degradation occurs only during the day, TIM

presence is restricted to the night only. As the PER is unstable without TIM, PER levels decrease during morning and the CLK-CYC inhibition is released. Besides CRY1, rhodopsins (Rhs) are important in photic entrainment. In *Drosophila* Rh1 and Rh6 are important in photic entrainment to the red light (Hanai et al., 2008) and Rh1, Rh5 and Rh6 in entrainment to green and yellow light (Hanai et Ishida, 2009). In *Drosophila* rhodopsins acting through G proteins, target the protein NorpA encoded by a phosphoinositide-specific phospholipase C (PLC). NorpA in turn catalyzes the breakdown of phospholipids and generates inositol triphosphate and diacylglycerol.

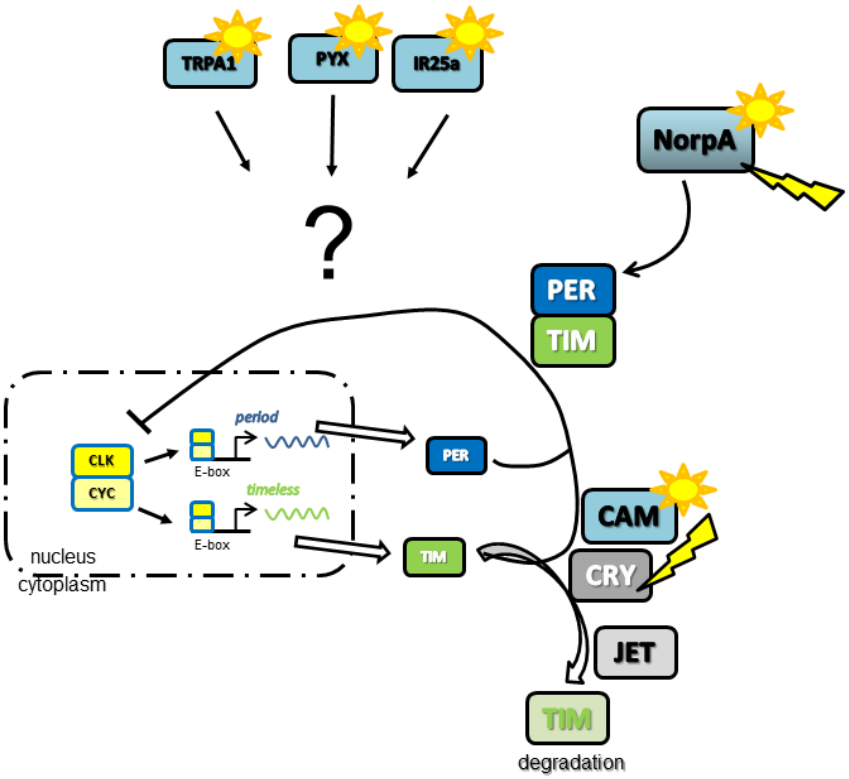

Fig 4 Light (lightning) and temperature (sun) entrainment pathways in *Drosophila melanogaster*. CRY activated by light binds to TIM, which is then led to proteasome through JET. CAM activated by released Ca^{2+} levels conduct the TIM to proteasome. While the role of IR25a TRPA1 and PYX channels in temperature sensing remain to be elucidated. Temperature and light entrainment is also modulated by PLC encoded by *norpA*. Adapted from Rivaz et al., 2016.

Diacylglycerol is a potential precursor for several polyunsaturated fatty acids, which activate *Drosophila* light-sensitive channels (Chyb et al., 1999). Histamine, dopamine and serotonine are suggested to be important neurotransmitters in the compound eyes (Rieger et al., 2003), histamine and acetylcholine are neurotransmitters that convey light inputs from the HB eyelets and directly affects the clock neurons (Duvall et al., 2012; Guo et al., 2014; Li et al., 2014).

1.1.2 Output pathway

The central clock is necessary for the circadian physiology and behavior regulation and also for entrainment with external conditions, on the other hand there are many tissue specific peripheral circadian clocks in the organism (Tomioka et al., 2012). These peripheral oscillators are responsible for tissue specific circadian organization of organ function and act in more or less autonomous manner. The synchronization of these peripheral oscillators with the master clock seems to be crucial, since many physiological processes, such as metabolic settings, reproduction, and homeostasis maintenance require interconnection and proper timing of physiological processes in different tissues (Xu et al., 2008). Unfortunately, very little is known about the circadian output pathways directing the signal to the other clocks in periphery. It has been suggested that neurohormones can play crucial role in transmitting signals to downstream neurons (Maywood et al., 2006; Nässel et Homberg, 2006). The only well described neurohormone in insects is Pigment dispersing factor (PDF; Homberg et al., 1991; Renn et al., 1999; Peng et al., 2003; Lin et al., 2004; Schneider et Stengl, 2005; Nitabach et al., 2006). PDF works via closely related G-protein-coupled receptors of the B1 subfamily (classical hormone receptor) that are present on the clock neurons themselves (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005; Shafer et al., 2008). PDF is periodically released from circadian clock neurons, *Drosophila Pdf* null mutants show weak rhythmicity with the short period of the locomotor activity behavior compared to wild type flies, or the flies are arrhythmic (Renn et al., 1999). Further studies show multiple effect of PDF on clock neurons and under natural condition PDF may be required for adapting

*Drosophila'*s clock to varying photoperiods, since the *Pdf*^{01} mutants are not able to adapt their activity to long photoperiods (Yoshii et al., 2009).

Another non-hormonal transduction pathway through which peripheral oscillators are synchronized with the main oscillator has been shown in mammals. Although liver clock system can be shifted by glucose and insulin signalling, (Yamajuku et al., 2012), oscillations of circadian clock genes in liver, heart and kidney of rat were influenced by the restricted feeding (Wu et al., 2012). It has been also shown that the central clock system regulates peripheral clocks through body temperature in mammals (Dibner et al., 2010). Although *Drosophila* peripheral clocks seem to be synchronized directly by light, the other insect species might differ and direct investigation is needed.

1.1.3 Circadian clock in non-*Drosophila* insect species

Outside the *Drosophila* model, the best studied insects are lepidopteran – monarch butterfly (*Danaus plexippus*), silkmoth (*Antheraea pernyi*), the hawkmoth (*Manduca sexta*) and silkworm (*Bombyx mori*); then hymenopteran – honeybee (*Apis mellifera*) and bumblebee (*Bombus*); blattodean – cockroaches (*Blatella germanica*, *Periplaneta americana, Leucophaea maderae*); orthopteran – crickets (e.g. from the genus *Laupala*); coleopteran – red flour beetle (*Tribolium castaneum*); and dipteran – mosquitoes in the genus *Anopheles*, fleshflies (*Sarcophaga sp*.), housefly (*Musca domestica*), and the drosophilid fly *Chymomyza costata*. Studies performed on these insect species showed that although the main clock mechanisms is more or less conserved, not all the gene homologs play the same role in the machinery of circadian clocks.

In the **housefly *Musca domestica*** it was shown that cycling of PER protein is not abundant during the night in the head (Codd et al., 2007). Furthermore, the spatial organization of clock cells differ in *Musca* brain in comparison to the one observed in *Drosophila*, as well as the expression pattern of PER and TIM is different. On the other hand the *Md*PER rescued the circadian locomotor activity rhythms in *Drosophila per*^{01} mutant flies (Piccin et al., 2000).

In the **silkmoth *Antheraea pernyi*** the *per* mRNA and PER protein levels oscillate in the central brain and eyes (Reppert et al., 1994; Šauman et Reppert, 1996a,b), however the number of more than 100 cells in the *Drosophila* brain expressing PER and TIM shrank in silkmoth to 8 large neurosecretory cells (four in each hemisphere). Furthermore PER and TIM proteins seem not to enter the nucleus, which is a critical feature of the *Drosophila* negative feedback oscillator model (Šauman et Reppert, 1996b). A peculiar finding was the antisense *per* RNA oscillating with the circadian period, but with the opposite phase in relation to *per* (sense) mRNA cycles, so the oscillating level of PER could be achieved via RNA interference (RNAi) machinery (Šauman et Hashimi, 1999). Unfortunately, further studies show that the locus for *per* sense and antisense RNA is settled to different sex chromosomes – sense to Z and antisense to W – meaning oscillating pattern of *per* RNA could be reached via RNAi machinery in females (ZW) only (Gotter et al., 1999). So the role of this phenomenon in circadian clock machinery was elucidated forasmuch as in males (ZZ) the antisense *per* RNA is not present. But it is still possible, that the RNAi machinery somehow participates in sex specific female´s circadian clock machinery. However, since the sense *per* RNA is oscillating during the day in both males and females, Gotter and colleagues assumed presence of posttrascriptional mechanisms in the silkmoth (Gotter et al., 1999), similar to those found in *Drosophila melanogaster* (So et Rosbasch, 1997; Suri et al., 1998). They suggest that the auto-repression function of PER-TIM dimer might be substituted by other clock components. Possible repressor in this case could be a cryptochrome (CRY), whose variants were found in many organisms from bacteria, to high vertebrates such as humans.

Cryptochromes

Cryptochromes (CRY´s) are flavin containing blue light photoreceptors related to photolyases. The phylogenetic analysis of the photolyase–cryptochrome family strongly suggests that cryptochrome blue-light photoreceptors have evolved from photolyases, lost enzymatic activity, and evolved a mechanism for signaling to the circadian clock (Cashmore et al., 1999; Sancar, 2003). CRYs have two main domains an N-terminal

conserved domain, and a carboxy-terminal “tail” that is intrinsically unstructured and varies considerably in length and primary amino acid sequence and results in functional diversity within the cryptochrome family (Sancar et al., 2003; Green et al., 2004; Partch et al., 2005; Chaves et al., 2006).

As it was previously described (chapter 1.1.1.2), the *Drosophila melanogaster* CRY (dCRY) acts as a major circadian photoreceptor with flavin adenin dinucleotide and methenyltetrahydrofolate as chromophores (Emery et al., 1998; Stanewsky et al., 1998). dCRY mediates light input to circadian oscillators in both brain and peripheral tissues and influence clocks by binding TIM protein when activated by light. TIM is then degraded in the proteasome which leads to destabilization of PER, and de-repression of the CLK-CYC mediated transcription. It was shown dCRY also plays a photoreceptor-independent role in the periphery (Stanewsky et al., 1998; Krishnan et al., 2001).

In contrast to *Drosophila*, mouse (*Mus muscullus*) cryptochromes (mCRY´s) are essential components of the central pacemaker (van der Horst et al. 1999; Vitaterna et al. 1999), since they interact with PERs (PER 1 and PER2), translocate them to the nucleus and thereby inhibit the CLK–BMAL1 transcriptional activity (BMAL1 is the mammalian orthologue of *Drosophila’s* CYC). Hereby mammalian CRY´s function in the negative feedback loop of the clock, with a similar function to TIM in *Drosophila* model. Mammals do not have a true orthologue of *Drosophila* TIM but rather have an orthologue of *Drosophila* gene *Timeout*, a gene with no known function in the fly’s clock. Mammalian TIM is expressed in the suprachiasmatic nucleus (SCN), the site of the mammalian pacemaker, but its role, if any, in central rhythm generation is very debatable. In addition to the number of cryptochromes, activity of vertebrate mCRYs does not appear to be depend on light (Griffin Jr. et al. 1999; Kume et al. 1999; Froy et al. 2002), but it is still for the circadian light response (Selby et al., 2000; Cashmore 2003; Sancar 2003). The repressive function of mCRY proteins on CLK-BMAL1 – activated transcription has been extended to homologous CRY proteins from other vertebrates, including those from zebrafish *Danio rerio* (zCRY1A, 1B, 2A and 2B) (Kobayashi et al., 2000), the African clawed frog *Xenopus laevis* (xCRY1 and xCRY2b) (Zhu et Green, 2001), and the domestic chicken *Gallus gallus* (cCRY1 and cCRY2) (Yamamoto et al., 2001).

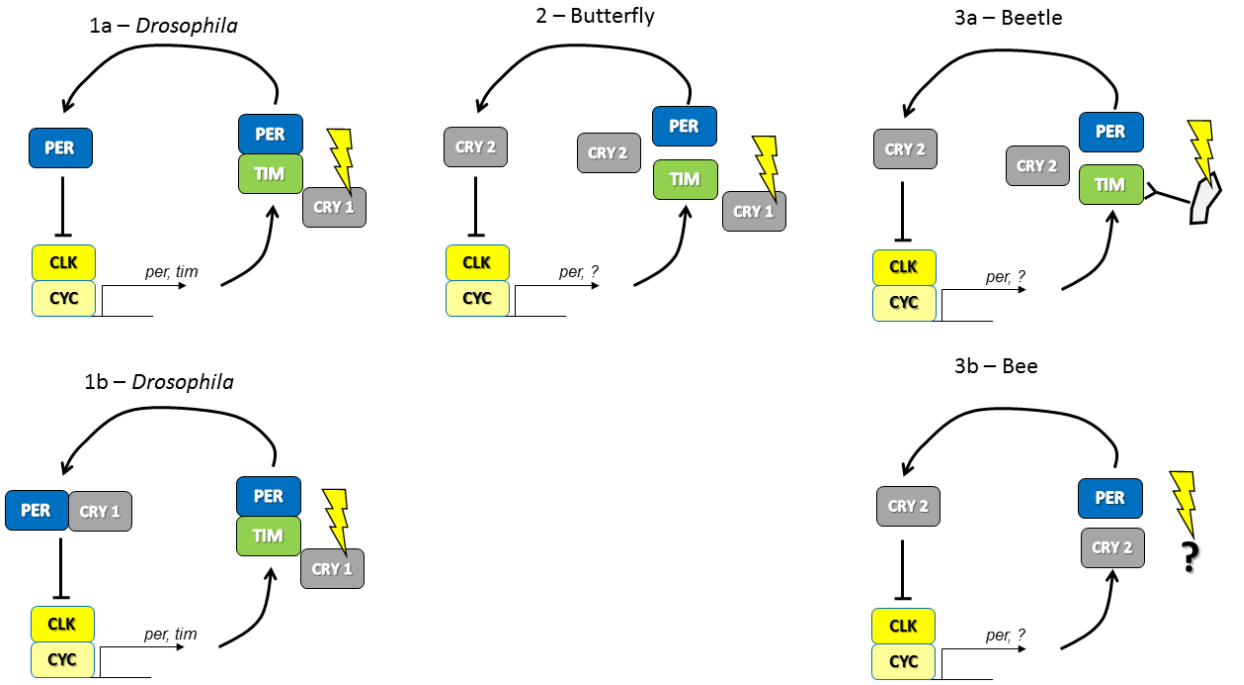

Fig. 5 Insects clockwork models. Two functionally distinct CRYs are present in insects, three major types of clockwork models can be proposed: Type 1 (the *Drosophila* form) – CRY1 only functions in the central brain clock as a circadian photoreceptor (panel 1a) or in peripheral clocks as both a photoreceptor and central clock component (panel 1b). Type 2 – both CRY1 and CRY2 exist and function differentially within the clockwork (panel 2). Type 3 – only CRY2 exists and functions within the clockwork. In beetles, CRY2 acts as a transcriptional repressor of the clockwork and light input may be mediated through the degradation of TIM (panel 3a). In bees (which lack TIM) CRY2 acts as a transcriptional repressor and novel light input pathways (?) are used to entrain the clock (panel 3b). Adapted from Yuan et al., 2007.

A phylogenetic analyses show at least two rounds of gene duplication at the base of the metazoan radiation, as well as several losses, which gave rise to 2 *cryptochrome* gene families in insects, a *Drosophila*-like CRY1 gene family and a vertebrate-like CRY2 family. *Drosophila* express CRY1 only, while several other insect species such as mosquitos and monarch butterflies express both CRY1 and CRY2 (Zhu et al., 2005) and surprisingly some insect species such as honey bee and the beetle express the CRY2 solely (Zhu et al., 2005; Rubin et al., 2006). This entails two remarkable facts. First, the core oscillator in insects has itself evolved in the way that at least three kinds of clocks exist, those containing only CRY1 (as in *Drosophila*), those containing both CRY1 and CRY2 (as in

monarch butterfly and mosquito), and those containing CRY2 alone (as in beetle and honey bee). Secondly, in insects containing CRY2 exclusively, the cryptochrome may serve dual functions, as both a transcriptional repressor and a photoreceptor (summary - Fig.5). What is even more interesting the absence of the *timeless* gene in the honey bee genome (Rubin et al., 2006), which suggests a completely novel light input mechanism to the bee central clock. Also this multiplicity of circadian clock shows how important is to study core clock mechanisms not only in model organisms, but also in other organisms, more or less related to the model-ones.

1.2 Photoperiodic clock in insect

A photoperiod is the interval in a 24-hour period during which an organism is exposed to light. It is the natural ratio between light (photophase) and dark (scotophase) phase during the day. While the Earth orbits Sun, this light/dark ratio is changing during the year and is corresponding to certain seasons of the year. The ability to measure photoperiod is advantageous and is widely used to predict upcoming seasonal change and enable to prepare on changing environmental conditions in advance (Tauber et al., 1986). One of the mostly used mechanisms how to cope with the upcoming stark season is diapause (Kostal, 2011), state which is characterized by reduced metabolic and energy-demanding processes in the body such as reproduction (Hahn and Denlinger, 2011). Usually insects in temperate regions enter the diapause to survive the upcoming winter, but there are some insect species entering the diapause during the summer to protect the body from the desiccation.

Light/dark ratio in which half of the experimental individuals enter the diapause is called critical photoperiod (CPP) and is species and population specific and changes with latitude (Lankinen et Lumme, 1984). Various insects enter diapause in different, species-specific developmental stages (Meuti et Denlinger, 2013; Dolezel, 2015). For example *Chymomyza costata* can live in a diapause larval stage up to one year and even more, compared to the lifespan of days or weeks of non-diapausing siblings. The diapause at

pupal stage in *Antheraea pernyi* can be reverted by photoperiod, which can be measured due to the transparent cuticular window over the pupal brain. However, once the diapause is terminated by the low temperature, the individual is not photoperiodic anymore and after exposure to suitable conditions, the development is resumed (in both larval and pupal stage of diapause). Additionally diapause and reproductive stage in *Pyrhocorris apterus* adult females can be switched repeatedly just by photoperiod.

To induce the diapause state, there has to be some certain number of days with the respective photoperiod. The number of these days is counted by the photoperiodic counter (Kostal, 2006). As a so called “required day number” (RDN) scientists describe the number of days needed for reaching specific threshold, when the photoreceptive neurons translate the information by modulating output pathway signalization. RDN is species specific (Saunders, 1971). The initiation of diapause is influenced also by other environmental factors, such as temperature, diet, social interaction etc. (Saunders, 2002). The molecular mechanisms of photoperiodic counter remain undetermined, but it was suggested that it somehow cooperate with photoperiodic clock system. It was supposed that the counter summarize the incoming information, direct them to the downstream mechanisms and send them to the clock, where the yes/no decision is made (Vaz Nunes, 1990).

1.2.1. Architecture of the photoperiodic clock

As far as we know from the observations about the photoperiodic phenotypes, we can assume the photoperiodic clock mechanisms consist of 3 subunits: **1)** light receptors as important external light transducers; **2)** the photoperiod measuring device to measure the length of the day or night, and **3)** an endocrine effector - output pathways connecting the clock with the rest of the organisms to direct function of subordinated physiological processes, such as diapause (Saunders, 1981).

1.2.1.1. Light receptors and organs

The light receptors and the photoreceptive organ are crucial for the photoperiod clockwork. Early observations, mainly with larvae and pupae of Lepidoptera, suggest that the photoreceptive organ and cells are situated to the brain area (Williams et Adkisson, 1964; Bowen et al., 1984; Hasegawa et Shimizu, 1987). Some experiments were also done in adult insects with functional compound eyes. Working with blow fly (*Calliphora vicina*), the brain was effectively disconnected from optic lobe by surgical removal of both optic lobes. These flies were found to be blind, but able to distinguish short from long photoperiods in the production of diapausing or nondiapausing larval progeny (Saunders et Cymborowski, 1996). First proof that not only brain, but also the compound eyes are the photoreceptive organs comes from studies with the beetle *Pterostichus nigrita*, in which bilateral ablation of the eyes in male beetles leads to suppressed maturation of the sperm, similar to observed in continuous darkness (Ferenz, 1975). Other studies on other Heteroptera and Orthoptera insect species showed the compound eye as the principal, if not the only, photoperiodic photoreceptors (Numata et Hidaka, 1983; Goto et al., 2010). However, in heteropteran *Plautia stali* the brain may play the crucial role as a photoreceptive organ (Morita et Numata, 1999). In addition in the beetle *Leptocarabus kumagaii*, compound eyes were shown to be the receptors in the adults, although stemmata were found to be important in the larvae (Shintani et Numata, 2009). And to make the story even more complicated, in the black blow fly (*Protophormia terraenovae*), ovarian diapause is regulated by receiving light information through the compound eyes, on the other hand, in the related blow fly (*Calliphora vicina*) it is the brain, which appears to be the main photoreceptor (Saunders et Cymborowski, 1996). From these findings we can assume that the light signal can be detected by more than just one type of cells and photoreceptive organ and these input pathways can cooperate (Morita et Numata, 1999). The input pathways seem to be multiple, redundant and cooperative. The reverse genetic experiments on the cricket (*Modicogryllus siamnesis*) revealed an important role of opsin in the obtaining photoperiodical signal (Tamaki et al., 2013).

1.2.1.2. Molecular mechanisms

The molecular mechanisms of photoperiodic clock remains elusive. There are several hypothesis, but none of it was proved, yet, especially because there is no insect model organisms designated for studying photoperiodic clock. *Drosophila melanogaster*, which is very well known model organisms for studying circadian clock, is not very suitable model for studying photoperiodism for its weak photoperiodic response. Some experiments were held on non-model insect species and combined together with weak results obtained from *Drosophila* pointed some genes playing an important role in photoperiodic clock mechanism. Not surprisingly, these were genes acting as the key players in the circadian clock machinery – *per*, *tim* and *Cry* (reviews Košťál, 2011; Meuti et Denlinger, 2013; Goto, 2013). Several genes promotes different expression patterns under long day and short day conditions. Further experiments show the modulation of cyclic expression pattern of clock genes *Clk*, *per*, *tim*, *Cry* and *Pdp1* by the photoperiod (Goto et Denlinger 2002; Stehlik et al., 2008; Kobelkova et al., 2010; Dolezel et al., 2008), but the results are species-specific and inconsistent. Some RNAi experiments showed possible role of *per*, *Cry2* and *cyc* in photoperiodic clock of *Riptortus pedestris* (Ikeno et al., 2010, 2011a,b). Unfortunately, experiments with mutant flies or photoperiodic modulation of expression cannot bring convincing evidence about involvement of these genes in photoperiodic clock mechanism. It is very complicated to separate if the photoperiodic phenotype is altered by the abolished circadian clock system, or if the circadian clocks only influence the photoperiodic clock. Even though there are some indications showing possible interconnection of circadian and photoperiodic clock, the direct involvement of the circadian clockwork in photoperiodic clock mechanism still remains unclear.

1.2.1.3. Endocrine effector

For further investigation it was crucial to identify photoperiodic clock residing neurons, quantify the expression pattern of clock genes in single cell and find neuronal clusters responsible for processing of photoperiodic output signal. Williams (1964) performed microsurgical interventions in *Antherea pernyi* and revealed the *dorsolateral*

protocerebrum to be essential for photoperiodic clock function. Added experiments identified these cells to be the site of circadian clock system connected to *corpora allata* and *prothoracicotropic hormone* (PTTH) (Sauman et Reppert, 1996). Additionally these neurons express PER and regulate the hormonal release from the neurosecretory glands. Ablation of these PER expressing neurons prevents induction of diapause in *Manduca sexta* (Shiga et al., 2003). In flies, five groups of *per* and *tim* expressing cells were found. Since the expression level of both genes peak during the dusk, it has been suggested their involvement in photoperiodic clock mechanism (Muguruma et al., 2010). What more all of the five groups of cells co-express the *Pdf*, which is important output player in the circadian clock machinery (Shiga et Numata, 2009). Ablations of these lateral neurons abolished both, the circadian and the photoperiodic responses. Such results indicate that these five neuronal groups act as a multi-oscillatory cooperative system and all of them participate in diapause induction (Muguruma et al., 2010).

Transplantation experiments in two insect species *Antherea pernyi* and *Pyrrhocoris apterus* determined cells of *pars intercerebralis* (PI) and *pars lateralis* (PL) to be essential in photoperiodic phenotype regulation (Truman 1971, 1972; Hodkova, 1976). These two neuronal groups directly innervate neurosecretory glands *corpora cardiaca* (CC) and *corpus allatum* (CA), which act as hormone-releasing glands in insects. Ablation of cells of PI and neurosecretory gland CA significantly influence expression level of circadian clock genes in the fat body (Dolezel et al., 2008) as well as in the gut (Bajgar et al., 2013a). These results showed the gene expression response to hormonal signals and can thus suggest a possible photoperiodic clock output signalling pathway in insect. One of hormones released from *corpora* glands and playing an important role in induction of adult diapause in insects is juvenile hormone (JH) (Hodkova, 1976; Hodková et al., 2001; Shimokawa et al., 2008; Denlinger et al., 2012). JH is an insects' sesquiterpenoid that controls reproduction (Raikhel et al., 2005) and entry into metamorphosis (Jindra et al., 2013). JH and its analog methoprene acts through the *Methoprene-tolerant* (*Met)*, which has been described as JH receptor (Miura et al., 2005; Charles et al., 2011). MET is a transcription factor of the basic helix–loop–helix Per–ARNT–Sim (bHLH–PAS) family (Ashok et al., 1998) preventing premature metamorphosis in *P. apterus* juveniles

(Konopova et al., 2011). Capability of MET to dimerize with other transcription factors is JH dependent (Charles et al., 2011). It has been shown that heterodimer MET-CYC binds to E-box promoter region and acts like a transcription regulator, similarly to the CLK-CYC heterodimer in circadian clock machinery (Miura et al., 2005). Recent work revealed that diapause/reproductive regulation of gut physiology in *Pyrrhocoris apterus* requires JH, its receptor MET and two circadian transcription factors CLK and CYC, thus linking the insect photoperiodic clock with circadian and hormonal signaling (Bajgar et al., 2013a). To make the story even more interesting and complicated, it turned out that the JH response in different organ, the fat body, requires JH, its receptor MET and Taiman, while CLK and CYC are not involved in JH signaling in this tissue (Bajgar et al., 2013b).

To conclude, the photoperiodic output pathway is based on hormonal signalling. Hormones released from the neurosecretory cells in brain activate receptors and transcription factors in tissues. Circadian clock genes seem to be essential for maintaining the photoperiod induced changes. JH is not the exclusive neurosecretory hormone, the other hormones possibly involved in photoperiodic responses are ecdyson, prothoracicotropic hormone, and insulins (Denlinger et al., 2005; Shiga et al., 2003; Tatar et al., 2001).

1.2.2 Circadian clock, circadian clock genes and their possible role in the photoperiodic time measurement

The possible role of circadian clock in photoperiodic responses is intensively discussed question. First theory suggesting the possible crosstalk between circadian and photoperiodic clock was postulated by Bünning in 1936. He hypothesized that photoperiodic timing is mediated by the interaction, either directly or indirectly, with the circadian pacemaker. This was an alternative to the “hour-glass” model that postulates that the photoperiodic timer is entirely driven by the external light–dark cycle, and is reseted every day. Bünning´s suggestions were then elaborated by Pittendrigh to three

main models: **1)** external coincidence; **2)** internal coincidence; and **3)** resonance model (Schiesari et al., 2011).

The external coincidence model is based on occurrence of short photoperiodic light sensitive periods which occur in a circadian manner, and that light have a dual role in the system. By exposing organisms to artificial light regimes (Nanda-Hamner behavioral test) it was shown that insect exhibit regularly timed multiple peaks of their seasonal response and these peaks are spaced at 24h intervals. Behavioral experiments with light pulses during the night phase revealed that there are two sensitive time points during the night. According to external coincidence model, through these two time points organism measures length of the night more than length of the day (Saunders, 1975). But this model is relevant only for some insect species, such as *Sarcophaga argyrostoma*, *Megoura bicie*, *Aphis phabae*, and some *Lepidoptera* species (Saunders, 2011).

The other model – internal coincidence – is based on the necessity of two or more circadian oscillating mechanisms, which influence each other. The light signal has a single role of entrainment in this model. One oscillator is the dawn (morning) measuring device and the other is dusk (evening) oscillator. The changing external light conditions change the relation of these two oscillators and results in different regulation of downstream gene expression. In fact *Drosophila* studies supports this model, where two groups of neurons respond to morning or evening light conditions and regulate morning or evening activity cycles (Grima et al., 2004; Stoleru et al., 2004).

Last but not least the resonance model is based on multi-oscillatory system, too. It implies that the circadian clock system itself is not involved in the measurement of night length, but it is needed for proper function of photoperiodic clock.

The role of circadian clock genes in the photoperiodic measurement still remains intensely discussed. Working with *Drosophila melanogaster* per^{01} mutants, Saunders showed that despite the flies had absolutely arrhythmic behavior they were still able to recognize long and short day conditions. But the mutants had shortened critical photoperiod, what suggests that the *per* gene itself is not involved in photoperiodic mechanism, but circadian clock cycling is essential for correct photoperiod measurement (Saunders et al., 1989). In another drosophilid fly *Chymomyza costata* it was shown that

mutant lines with deletion in *tim* promotor display disrupted eclosion rhythms and also abolished diapause induction. This implies that the gene *timeless* function in both circadian and photoperiodic clocks (Pavelka et al., 2003; Kobelková et al., 2010). In both males and females of heteropteran insect bean bug *Riptortus pedestris* knocking-down expression of circadian clock genes *per* and *Cry2*, by systemic RNAi, prevents the bugs of entry into diapause in short day (diapause-inducing) photoperiod conditions (Ikeno et al., 2010, 2011a,b). What more, knocking-down circadian clock genes *cyc* (in both males and females) and *Clk* (in females) resulted in blocking development of reproductive organs in long day (diapause-averting) photoperiod conditions (Ikeno et al., 2010, 2011b, 2013). These results provide strong evidence, that circadian clock genes play important role in photoperiodic responses, such as diapause. Whether the circadian clock genes have a pleiotropic role in biological clocks and are crucial players of both circadian and photoperiodic clock machinery stays disputable (Bradshaw et Holzapfel, 2010). In females of other heteropteran insect species – linden bug *Pyrrhocoris apterus*, the tissue-specific role of circadian clock genes *Clk*, *cyc*, *Pdp1* and *Cry2* on photoperiodic responses has been postulated, since their interaction with MET is needed for proper function of several downstream genes involved in gut physiology (Bajgar et al., 2013a,b; Smykal et al., 2014). Further RNAi experiments also showed that the expression pattern of circadian clock genes in the periphery (in the gut (Bajgar et al., 2013a,b); and in the fat body – unpublished data) reflects the physiological state of the linden bug (diapause or reproductive) females rather than the photic conditions. Nevertheless, these results only illustrate the complexity of the mechanisms leading to the response – diapause/reproduction, with no explanation of the convenient role of circadian clock genes in photoperiodic clockwork.

1.3 Possible role of circadian clock genes in other processes

The role of circadian clock genes in photoperiodism was discussed in the previous chapter, however beside this generally respected function, clock genes has been shown to be important for many other processes in organisms.

It has been demonstrated that circadian clock genes act as the key player in regulation of **sleep** in mammals. Mice deficient for one or more circadian clock genes show altered response to sleep deprivation. What more, the high levels of PER2 negatively regulate sleep deprivation recovery (Franken et al., 2007). In *Drosophila melanogaster* it has been shown that neurons in the brain responsible for the transfer of the sleep/wake signal are downstream targets of the PDF positive neurons (Chen et al., 2016).

Circadian clock genes are connected also with **metabolism** and its regulation. Many genes involved in metabolic processes (such as glucose transport, gluconeogenesis, lipolysis, etc.) display cyclic expression pattern (Kohsaka et Bass, 2007). Mice lacking the circadian clock gene BMAL1 exhibit beta cells dysfunction and diabetes due to a loss of glucose-stimulated insulin secretion, suggesting the working clock is required for the rhythmic secretion of insulin (Lee et al., 2013). Other interconnection of circadian clock genes and metabolism is the gene *Nocturnin* (*Noc*). Mice lacking the *Noc* gene, which is expressed in robustly rhythmic pattern, display resistance to diet-induced obesity and hepatic steatosis. *Noc* might play an important role in other tissues and has been implicated in lipid metabolism, adipogenesis, glucose homeostasis, inflammation and osteogenesis (Stubblefield et al., 2012). It has been shown in mice and also in humans that non-traditional working hours has been linked to several metabolic and immune related disorders, including obesity (Karlsson et al., 2001), diabetes (Morikawa et al., 2005, Karlsson et al., 2005), stroke (Karlsson et al., 2005), atherosclerosis (Haupt et al., 2008) and coronary heart disease (Tenkanen et al., 1998). Various cancers have been linked to disrupted circadian cycles, such as lymphatic (Lahti

et al., 2008), prostate (Kubo et al., 2006; Conlon et al., 2007) and breast cancer (Schernhammer et al., 2006). The link of circadian clocks–sleep–metabolism has been suggested. The orphan nuclear receptor REV-ERBα might be the possible linking partner of all these cascades (Mang et al., 2016).

Circadian clock genes are important participants in **immune responses** and diseases in mammals. It has been well documented that many parameters (such as circulating hematopoietic cells and the levels of cytokines) in immune response exhibit circadian rhythms (Nakao, 2014). It has been shown that central clock in SCN drives the expression of adhesive molecules (ICAM-1 and VCAM-1) in endothelial cells or chemokines/chemokine receptors in tissue or leukocytes (Scheiermann et al., 2012). CLK-BMAL1 heterodimer has been established as the initiator of adaptive immune response (Silver et al., 2012) and regulator of immune response of monocytes (Nguyen et al., 2013). What more, another circadian clock gene – *Cryptochrome* – plays an important role in regulation of cytokine expression. Narasimamurthy and colagues reported that CRY-deficient macrophages exhibited a marked increase in TNF-α (tumor necrosis factor alpha) and IL-6 (interleukin-6) protein secretions compared with wild-type macrophages. Authors also showed that CRY works through Nf-κB signalling (Narasimamurthy et al., 2012).

The highest multi-functionality was shown for the circadian clock gene *Cryptochrome*. *Drosophila Cry* is established as the major transducer of light signals to the circadian core oscillator and was recently described to be involved in light perception and vision. The C–terminal region harbors several protein–protein interaction motifs, likely relevant for signal transduction regulation and has been shown to participate in interaction with proteins that belong to a multiprotein complex (the Signalplex) that includes visual signaling molecules (Mazzotta et al., 2013). Gene duplication and several loses gave rise to many *Cry* variants across the taxa and even within the insect class (see chapter 1.1.3.). Resent works implied its role not only in circadian clock mechanisms but also in magnetoreception. Last paragraphs will focus especially on that phenomenon.

Magnetoreception

The ability of perception the information of the magnetic field on Earth and utilization of this information for navigation or simple passive lateralization was studied in many animals across different taxa (reviews Wiltschko et Wiltschko 2002, 2005). Scientists postulated some hypothesis of how the animals “see and feel” the magnetic field, which were based on the results obtained mostly from vertebrates (mainly birds): **1)** electromagnetic induction model, **2)** pheromagnetic particles – magnetite based model and **3)** magnetic field dependent chemical reactions model (radical pair model).

The first model seems to work only in sea-living organisms, since only the surrounding sea water functions as the motionless conducting medium, and additionally animals have to possess some special organ, which can act as a conduction bar. Structures known as ampullae of Lorenzini, which function as the conducting bar, have been found on the fishes (Lohmann et Johnsen, 2000). However, the electric fields induced by ocean currents complicate this simple model considerably.

The magnetite based model requires presence of ferromagnetic particles in the organisms. These particles can be made from magnetite (Fe_3O_4) or greigite (Fe_3S_4) as strong magnetic particles producing crystals (Kirschvink et al., 2001). This model has been studied in many taxa (from bacteria to vertebrates). Important organs are the trigeminal nerves in birds and fishes (Semm et Beason, 1990; Walker et al., 1997) and upper part of the beak in birds (Fleissner et al., 2003). Well studied are the eusocial species of insects. Ants and bees use the geomagnetic field to orientate and navigate in areas around their nests and along their migratory paths. In *Apis malifera* the suspected magnetoreceptors are the iron granules in the abdomens of the bees (Liang et al., 2016).

In the radical pair based magnetoreception model the role of photo-excitable particles is postulated. The light-induced electron transfer results in the generation of a radical pair intermediate that can either exist in a singlet or a triplet excited state and can be modified by the external magnetic field. The first proposal that the ***Cryptochromes*** could act as the likely organic reactants came from Ritz (Ritz et al., 2000). There are many reasons for CRY to be a promising radical-pair photoreceptor. For example CRYs occur in many organisms across the taxa. It has been shown in birds that CRYs are

present in the cytosol of the ganglion cells, large displaced ganglion cells and in photoreceptor cells (Mouritsen et al., 2004). These cells structures provide the cylindrical membrane, which seems to serve as an ideal substrate for highly oriented ensemble of molecules needed for radical pair based magnetoreception model. Another key prerequisite of cryptochrome based magnetorception is that the protein has to be able to orient in the Earth´s magnetic field, which was documented for the *E. coli* photolyase (Henbest et al., 2008). Furthermore, the *Cry* expression levels differ between migratory and non-migratory birds at night (Fu et al., 2002; Haque et al., 2002; Mouritsen et al., 2004). *Cryptochromes* are well studied in vertebrates, alternative splicing, gene duplication or loss, resulted in variety of types of cryptochromes in different vertebrates (4 in migratory birds, up to 6 in zebrafish (Kobayashi et al., 2000)). However the radical pair model is not well supported model of magnetic sense in insects. The most convincing evidence was implemented in *Drosophila melanogaster* (Gegear et al., 2008, 2010). The ability to recognize the magnetic field relies on the functional CRY1. The *Cry1* deficient mutants can be rescued by using the mammalian type of *Cry* – *Cry2* (Foley et al., 2011). The flies were trained to recognize the local magnetic anomaly up to 10 times stronger than the natural GMF in T-shape maze experiments (Ritz et al., 2010). Unfortunately, there is no study showing the effect of natural geomagnetic field on insect and no research supporting the CRY-dependent sensitivity to the direction of geomagnetic field, yet.

2 Aims of the study

The main aim of the study was to shed some light on the role of circadian clock genes in the circadian clocks machinery and also their impact on physiological processes in model and non-model insect organisms.

First, we focused on the influence of the temperature on the function of the circadian clock machinery in the model organisms *Drosopihla melanogaster*. Our previous results obtained from closely related dipteran species – housefly *Musca domestica* – showed that surrounding temperature acts as a modulatory factor influencing expression pattern of core circadian clock genes. In this goal of the study we wanted to test the hypothesis that temperature affects expression of circadian clock genes also in the model organism *Drosophila melanogaster,* and whether the mechanisms of modulation is complex, or unique. Using the RT-qPCR method we aimed to characterize expression patterns of circadian clock genes in flies kept in different temperatures and to compare obtained results with closely related species.

Next goal of this study was to examine the role of the circadian clock genes and JH signaling in the photoperiodic clock of the linden bug *Pyrrhocoris apterus* MALES. The role of JH signaling in female reproduction of this species is well established; however, its function in male reproductive development and behavior is completely unclear. To achieve this goal we used reverse genetic approach (RNA interference) to down-regulate expression of genes of interest and hormone analogue application to study their effect on male reproductive organs development and mating behavior.

The last goal of this study was to test the hypothesis, that the *cryptorchome* is involved in magnetic sense in two non-model, closely related cockroach species *Periplaneta americana* and *Blatella germanica*.

3 Publications

Publications included in the thesis

1. **Bazalova O**, Dolezel D. *Daily activity of the housefly, Musca domestica, is influenced by temperature independently on period gene splicing,* in preparation for Genetics
2. Urbanova V, **Bazalova O**, Vaneckova H, Dolezel D. (2016) *Photoperiod regulates growth of male accessory glands through juvenile hormone signaling in the linden bug, Pyrrhocoris apterus.* Insect Biochem Mol Biol. 2016 Mar; 70: 184-90. doi: 10.1016/j.ibmb.2016.01.003.
3. **Bazalova O**, Kvicalova M, Valkova T, Slaby P, Bartos P, Netusil R, Tomanova K, Braeunig P, Lee HJ, Sauman I, Damulewicz M, Provaznik J, Pokorny R, Dolezel D, Vacha M. (2016) *Cryptochrome 2 mediates directional magnetoreception in cockroaches.* Proc Natl Acad Sci U S A. 2016 Feb 9; 113(6): 1660-5. doi: 10.1073/pnas.1518622113.

Publications not included in the thesis

1. Kobelková A, Závodská R, Sauman I, **Bazalová O**, Dolezel D. (2015) *Expression of clock genes period and timeless in the central nervous system of the Mediterranean flour moth, Ephestia kuehniella*. J Biol Rhythms. Apr;30(2): 104-16. doi: 10.1177/0748730414568430.
2. Pivarciova L, Vaneckova H, Provaznik J, Wu BC, Pivarci M, Peckova O, **Bazalova O**, Cada S, Kment P, Kotwica-Rolinska J, Dolezel D. (2016) *Unexpected Geographic Variability of the Free Running Period in the Linden Bug Pyrrhocoris apterus.* J Biol Rhythms. Oct 5. pii: 0748730416671213.
3. Jirošová A, Jančařík A, Menezes RC, **Bazalová O**, Dolejšová K, Vogel H, Jedlička P, Buček A, Brabcová J, Majer P, Hanus R, Svatoš A (2017) *Co-option of the sphingolipid metabolism for the production of nitroalkene defensive chemicals in termite soldiers* Insect Biochem Molec Biol in press (available online from 23 January 2017) http://dx.doi.org/10.1016/j.ibmb.2017.01.008

3.1 Publication 1: *Daily activity of the housefly, Musca domestica, is influenced by temperature independently on period gene splicing*

Background

The circadian clock is well studied and its molecular model is quite well established in lots of species across the tree of life from bacteria to humans. Although the circadian clock machinery is well-understood in insects, there are still some “white spots” in the machinery. One of these spots is the influence of the temperature on the circadian clock system. Previously it was shown that the temperature affects the behavior of the *Drosophila melanogaster* flies. During hot days flies prolong the midday siesta and restrict their activity to the early morning (morning activity peak is before the dawn) and to the evening (evening activity peak is in the early night) displaying almost no activity during the day. During cold days flies exhibit increased activity during the day. This behavior is due to the intron alternative splicing thermal efficiency of two core clock genes – *period* and *timeless*. On the other hand in *Drosophila yacuba*, which is closely related to *D. melanogaster*, but with a more ancestral distribution indigenous to Afro-equatorial regions, wherein day length and temperature exhibit little fluctuation throughout the year, this behavioral response to the surrounding temperature was not shown. Also the splicing efficiency of *per* intron was not temperature dependent in this species. The impact of the temperature on the rhythmic daily pattern of other circadian clock genes has never been reported in any insect species (including *Drosophila melanogaster*). Therefore, we compared the impact of temperature on the behavior and the clock genes expression patterns in two dipteran species – *Drosophila melanogaster* and *Musca domestica*.

Summary

In this study we compared how the surrounding temperature affects the behavior and circadian clock core machinery in two dipteran species, *Drosophila melanogaster* and *Musca domestica*. It was already known that *Drosophila melanogaster* exhibit different locomotor activity behavior in different temperatures (Low et al., 2009). We have shown that housefly *Musca domestica* display similar behavior. In the cold temperature male flies restrict their activity to the day (with maximal activity around the noon) with almost

no activity during the night, whereas when placed into the hot temperature they tend to be active in the early morning (morning activity peak), then the activity decreases and after the midday siesta it rises again reaching the maximum around the dusk. In addition the housefly´s females placed to cold temperature are also active particularly during the day. On the other hand when placed to high temperature females are active mostly before the dusk and at the early night, with no significant morning activity peak and lower activity during the day when compared to males.

Having the more or less similar phenotype in both fruit fly and housefly we were wondering if the molecular mechanism regulating this behavior is similar in these two species. It was shown that in *Drosophila* the expression pattern of *per* gene changes according to the temperature. In the cold temperature the relative mRNA level of *per* rises quickly when the light turns on and reaches its maximum sooner in comparison to that one in high temperature. This difference is maintained through the alternative splicing efficiency of 3´UTR intron (Majercak et al.,1999). The expression pattern of other core circadian clock genes was never reviewed in any study. The only other gene known to be thermosensitive in *Drosophila* is *tim*. Its version called tim^{cold} is abundant in the low temperatures (Boothroyd et al., 2007). We have explored the expression pattern of all core clock genes in *Drosophila* finding the 3 possible groups of genes with 3 more or less similar expression pattern: 1) *per*, *vri* and *cwo* mRNA levels rise quickly when the light turns on in flies placed in cold temperature (18°C), and what more, the overall level of transcripts of these genes appears to be increased at low temperatures compared to the higher temperature; 2) The relative mRNA levels of *tim* and *Pdp 1ε* equals in the cold and normal temperature (25°C), but differ in flies placed in the high temperature (29°C) - the increase of the transcripts is delayed when compared to the colder temperatures; 3) Expression levels of *Clk* and *cry1* are not thermosensitive.

In the housefly we explored the expression pattern of the same circadian clock genes as in *Drosophila* plus other cryptochrome-related gene *photolyase*. We observed these similarities with *Drosophila*: 1) *per*, *vri*, *cwo* (and possibly *Clk*) mRNA levels rise quickly when the light turns on in flies surrounded by cold temperature (15°C), and what more the overall transcripts of these genes appear to be increased in lower compared to the higher temperature; 2) the expression pattern of *tim* and *Pdp 1ε* equals in the cold

and normal temperature (25°C), but in high temperature (35°C) the increase of the level of transcripts is delayed compared to the colder temperatures; 3) expression patterns of *cry1* and *photolyase* are not significantly thermosensitive (summary - Fig. 6).

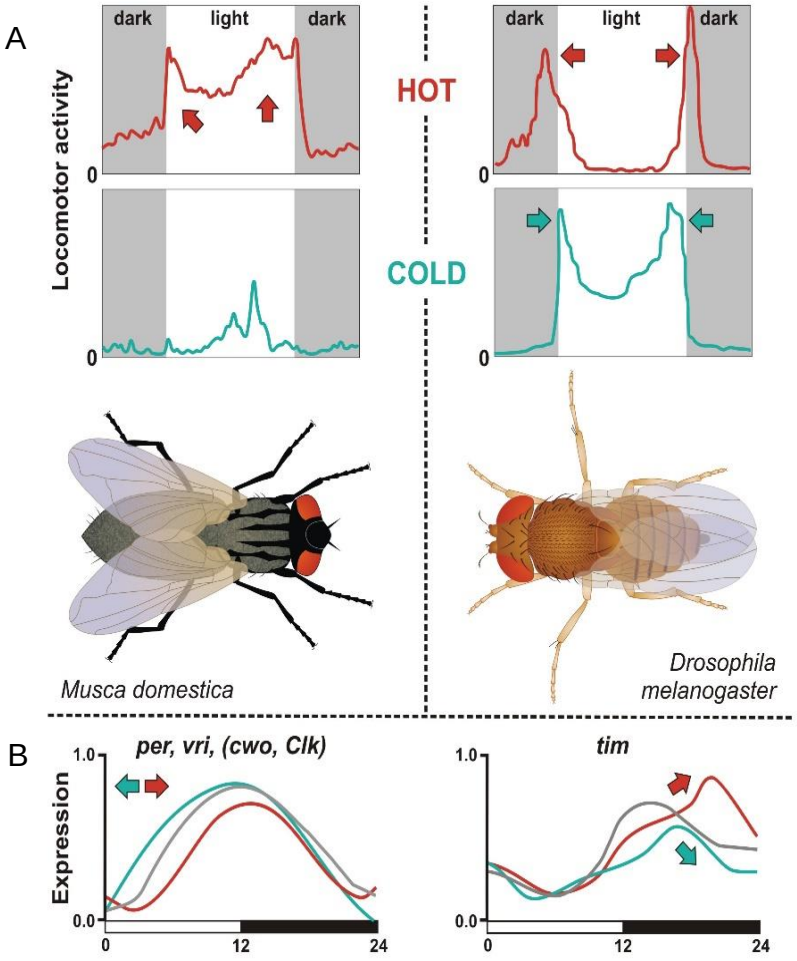

Fig. 6 The impact of temperature on the behavior and molecular basis of the clock. (A) Locomotor activity is influenced by the temperature in both flies - housefly (*Musca domestica* – left) and fruit fly (*Drosophila melanogaster*, right). During hot days (red line) flies prolong the midday siesta and are more active during the early morning and late afternoon, or night. In the cold temperatures (blue line) flies are more active during the day displaying almost no activity during the night. (B) Interesting similarities in expression patterns of selected clock genes in different temperatures were found in both flies (cold temperature – blue line, normal temperature – grey line, and high temperature – red line, for details see the text).

We have also found the possible intron candidates in the *Musca´*s *per* 3´UTR region and plausible *tim*^{cold} variant of *tim* gene and tested the hypothesis, that the molecular mechanism of how temperature influence the inner clock would be general. However, none of the tested introns displayed the thermosensitive splicing, although their positions in the gene correspond to the ones found in fruit fly. We also tested two additional introns located near the 3´UTR in the *Md pe*r gene, and we obtained the same result showing no temperature dependent splicing. One explanation of these results could be that intron with temperature-specific splicing is located in different region of the gene in *Musca domestica*. We favor another explanation that there is some additional, unknown mechanisms in the housefly, causing the same expression pattern of *per* and *tim* genes.

My contribution

I designed all the behavioral experiment. Using RT-qPCR I measured the mRNA levels of circadian clock genes and the possible splicing variants in all the group of animals. I analyzed all the data.

3. 2 Publication 2: *Photoperiod regulates growth of male accessory glands through juvenile hormone signaling in the linden bug, Pyrrhocoris apterus.*

Background

To cope with the adverse effect of upcoming stark season insects evolved the diapause as the surviving state in which they reduce metabolic and energy-demanding processes in the body, such as development or reproduction. From the previous research we know that the reproductive arrest (reproductive diapause) in insects is characterized by the absence of JH. After application of JH, or its mimic – methoprene – the animals terminate the diapause and induce reproduction even in diapause-promoting photoperiodic conditions. The JH works through its receptor MET, which belongs to the bHLH-PAS protein family and acts as a transcription factor. Interaction of MET with another bHLH-PAS transcription factor – Tai was previously shown in mosquito females to induce the oogenesis in the JH-dependent manner. In the bean bug *Riptortus pedestris* it was shown that circadian clock genes *Clk*, *cyc*, *per* and *Cry* play an important role in choosing reproduction, or entering the diapause. In our lab the linden bug, *Pyrhocorris apterus,* is well established model for studying seasonality. Adults can be switched from the diapause to the reproductive state repeatedly only by changing the photoperiodic conditions. The crosstalk of JH signaling and the photoperiodism was established in females and the possible role of circadian clock genes in the photoperiodic clock mechanisms has been suggested. Nevertheless nothing is known about the photoperiodic responses in males. Therefore we aimed to explore which factors regulate reproductive/diapause physiology in *P. apterus* MALES.

Summary

This study evaluates factors involved in regulation of reproduction and diapause in the linden bug *Pyrrhocoris apterus* males. It was known from previous research that in the short day (SD) photoperiodic conditions the male accessory gland (MAG) remains shrunken irrespective on the animal age. In contrast, in the long day (LD) photoperiodic conditions MAGs gradually grow and their size in the presence of JH is positively correlated with the successful reproduction (Socha, 2006; Socha and Hodkova, 2006).

The similar impact of the photoperiod and JH on the oogenesis was observed in females (Jedlicka et al., 2009).

We have shown that the suppressive role of short photoperiodic conditions is overridden by the JH mimicking compound methoprene, which requires functional MET and its binding partner Tai, since knocking -down of *Met* or *tai* prevents MAG's grow even in the reproduction-promoting photoperiodic conditions. These findings are supported by the research done previously in *P. apterus* females (Smykal et al., 2014) and some other insects (Guo et al., 2014; Marchal et al., 2014). We have shown that *Met* and *tai* are highly expressed in the MAG, therefore the local role of these two factors is plausible, although the mechanisms of how they act in the gland is not established, yet. Using Yeast Two Hybrid essay we also found out that *P. apterus* MET and Tai proteins interact in the methoprene dependent manner (data not shown), supporting the previous observations in *Aedes aegypti* (Li et al., 2011).

We found that the circadian clock genes *Clk* and *Cry2* are important in mediating photoperiodic information to the bug, since depletion of these two genes significantly reduced the growth of MAGs when bugs were transferred from SD into the reproduction-promoting conditions. Since circadian factors CLK, CYC, and protein CRY2 are not involved in MAG growth upon JH mimic administration to males in SD, we suggest, that these circadian homologs are involved in the regulation of JH hormone synthesis or activity of the c*orpus allatum* (Fig. 7).

We also tested the possible role of JH on the bug´s locomotor activity and mating capability. From our results it was clear that males kept in SD are less active compared to males placed in LD, which supported the previous findings in females (Hodková et al., 2003). Curiously the high activity in reproductive males and low activity of diapause males seems to be independent on JH presence or absence, respectively. Importantly, activity of *Clk* knockdown males remained low after the transfer from SD to LD conditions. Similarly, depletion of *Clk* did not affect reproduction of LD males, but destroyed the ability of diapause male to switch to reproductive mode after the transfer to LD regime, supporting the possible crucial role of circadian clock genes in photoperiodic time measurement. To clarify whether all of them are involved, or if only some of the certain

circadian clock genes were recruited independent of their circadian function (gene pleiotropy) need more investigation in the future.

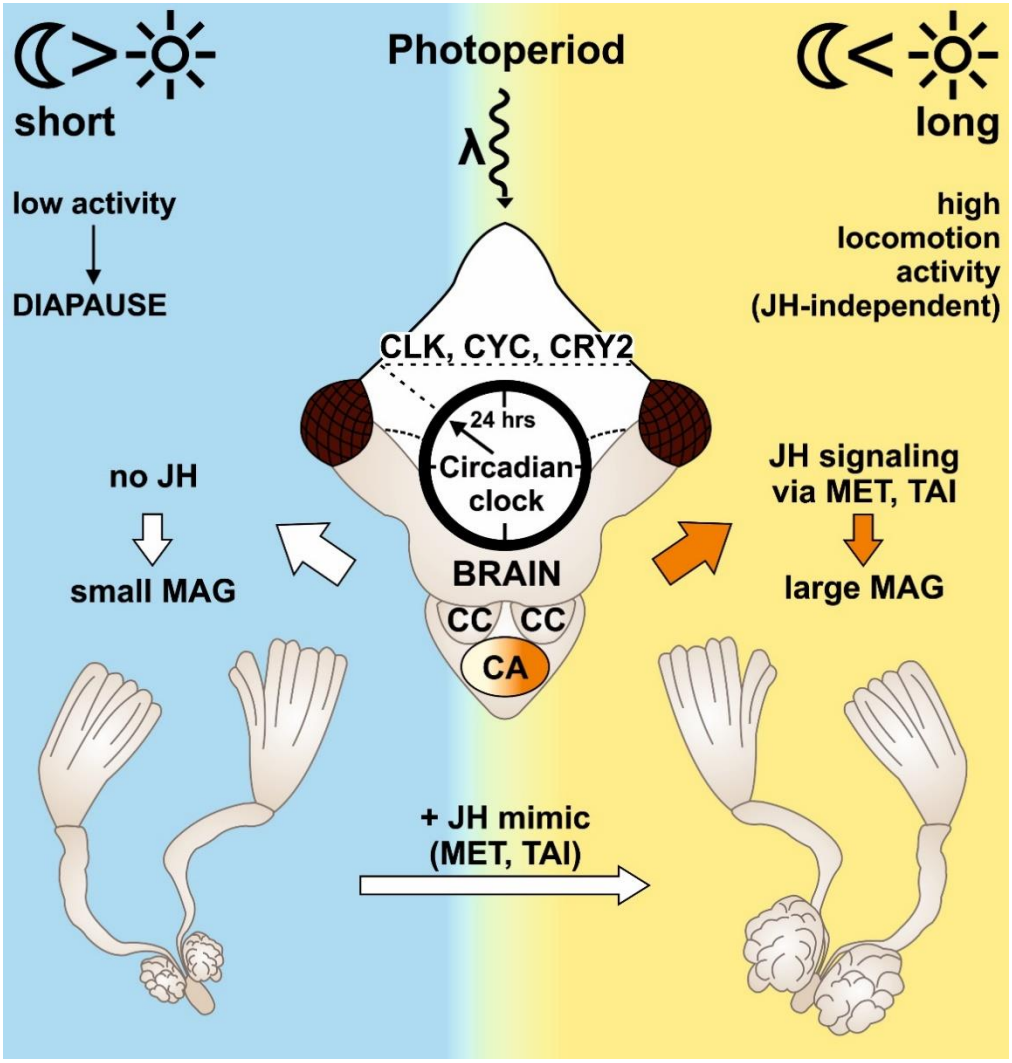

Fig. 7 Model summarizing our results on MAG regulation in *Pyrrhocoris apterus*. Under short days (left, blue background), males enter diapause characterized by JH absence resulting in small MAGs. Administration of JH mimic induces growth of MAGs to sizes observed under a long photoperiod (right, yellow background). In both situations, the growth requires expression of *Met* and *tai.* When males are transferred from short to long days, expression of circadian genes *Clk* and *Cry2* is needed to initiate MAG growth. The locomotor activity differs between males in LD and SD conditions and is not influenced by either JH mimic administration or *Met* knockdown.

Surprisingly, knocking down of *Met* by RNAi did not affect the locomotor activity, or the mating behavior of depleted bugs. They still increased the locomotor activity after the SD to LD transfer and similarly their mating behavior was not reduced. It is possible that *Met* was knocked down insufficiently, allowing for residual protein expression. However it is also plausible that MET is not necessary for male reproduction. Microsurgery ablation of the CA, JH-producing organ, might further address the role of the JH in male's reproduction and behavior.

My contribution

I have prepared the dsRNA used to knock down particular genes of interest and heterologous controls. I have performed the behavioral experiments – some locomotor activity and all of the mating experiments. I measured mRNA level of certain circadian genes using the RT-qPCR method and analyzed most of the data.

3.3 Publication 3: *Cryptochrome2 mediates directional magnetoreception in cockroaches*

Background

The ability of perception the information of the magnetic field on Earth and utilization of this information for navigation or simple passive lateralization was studied in many animal across different taxa, including insects. Studies on transgenic *Drosophila melanogaster* flies imply that the working circadian clock machinery is needed for the ability to sense the geomagnetic field. It has been shown in fruit flies that the photosensitive *Drosophila*-like *Cryptochrome* is involved in the responses to the magnetic field. However there is no evidence showing if the CRY-dependent magnetosensitivity is coupled to the sole magnetic field presence or to the direction of magnetic field vector. Furthermore there are no data providing the evidence of the location of the magnetosensitive organ in insects. In our study we focused on two non-model species, both cockroaches – *Periplaneta americana* and *Blattella germanica*. Although closely related, these two cockroaches still sustain some differences in their genome – such as missing photosensitive *Drosophila*-like *Cry1* in *Periplaneta americana*, while both *Cryptochromes* are present in the *Blattella germanica* genome (the *Drosophila*-like *Cry1* and mammalian-like *Cry2*). Using the novel assay we explored the involvement of the circadian clock genes in the insect magnetosensitivity under intensities comparable to intensity of natural geomagnetic field.

Summary

In this study we used previously developed assay for studying directional magneto-sensitivity. Although this assay does not monitor the directional locomotion from point A to point B, it still gives us the evidence of the direction of the magnetic vector. Cockroaches are nocturnal animals, around the noon they display minimal locomotor activity. When the direction of horizontal magnetic vector of geomagnetic field (GMF) is rotated periodically, the animals change their resting positions more frequently. This behavior is termed as magnetically induced restlessness (MIR) (Vácha, 2006).

Since we did not observed MIR in cockroaches placed in constant darkness, we assumed that the involvement of the light is crucial for magnetic sensing. To test the hypothesis that *Cryptochromes* might be involved in directional magnetic sensing as it was previously proved in birds (Du et al., 2014), we used previously described MIR assay combined with reverse genetic approach – the RNAi. *Periplaneta americana* contains only one *Cryptochrome* – mammalian-like *Cry2*, whereas both cryptochromes (*Drosophila*-like *Cry1* and mammalian-like *Cry2*) are present in *Blattella germanica*. Whereas the *Cry2* depleted animals showed no MIR phenotype together with abolished circadian rhythmicity in both cockroaches, knocking down of *Cry1* in the German cockroach affected only the circadian clocks, with no impact on MIR. Additionally, knocking down both *Crys* in the German cockroach we were able to abrogate both circadian rhythmicity as well as magnetic sensing.

To address the question if there is an overlap of the circadian clock and magnetic sensing, we performed the MIR assay in the constant light. These photic conditions abolished the circadian clocks, but did not affect the MIR (cockroaches still showed the MIR phenotype). The same result was obtained by knocking down the core clock gene *tim*. All these outcomes clearly support the separation of GMF sensing from circadian clock, leaving only the CRY2 as a prerequisite for magnetic sensing in both cockroach species MIR (Fig. 8).

To further characterized magnetoreception, we tested several wavelengths of light under different intensities. We found out that the minimal light wavelength needed for the working MIR was at the UVA (365mn). We observed steep decline of sensitivity in the cyan range (505-528nm), which matches the decline of the light absorption of three redox forms of flavin.

Because the presence of light is one of the crucial conditions for magnetic sensing, we attempted to localize the magneto-sensitive organ by shielding/covering the compound eyes. Cockroaches with black painted eyes were not able to respond to the rotating magnetic field, while the ones with eyes covered by transparent enamel retained MIR behavior, suggesting eyes as necessary organ to perceive GMF information. Laser-scanning confocal microscopy localized CRY2 in the hemispheral multicellular structure

beneath the pigment layer of the retina. High magnification analysis identified CRY2 staining strongest in the close vicinity of the plasma membrane.

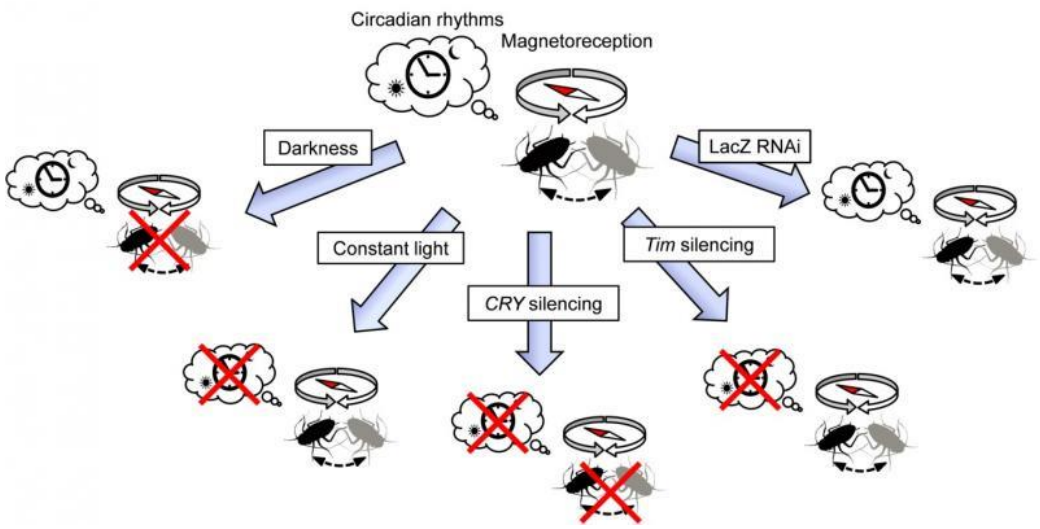

Fig. 8 The graphical summary of experiments performed on the American cockroach. In the constant darkness conditions cockroaches do not respond to the rotating magnetic field, but their locomotor activity is unaffected. Contrary in the constant light only the circadian clock is interrupted. Knocking down of *Cry2* aborts both MIR and locomotor activity, while silencing of circadian clock gene *tim* affect only the locomotor activity. Injecting heterologous double stranded RNA of *LacZ* leaves both MIR and circadian clocks unaffected.

Furthermore the microinjection of a fluorescent neuronal tracer into the cockroach ocular photoreceptor cells revealed an intimate contact between the neuronal output from the retina and the CRY2-positive cells proposing their possible interplay. Taken together our results established the role of CRY2 in light-dependent magnetoreception in two cockroach species and delivered the original evidence that CRY2 protein is the important player in the detection of GMF vector direction in the natural magnetic field intensities.

My contribution

I performed all the locomotor activity experiments. I prepared most of the clones used as the template for double stranded RNA preparation, prepared all of the dsRNAs used in experiments. I performed all the RT-qPCR experiments verifying the knocking down of the gene expression. I did all the imunocytohistochemical experiments followed by the laser-scanning confocal microscopy (except for high-magnification analysis). I performed the injection of a neuronal tracer into the cockroach ocular photoreceptor cells.

4 Discussion and final conclusion

In the first chapter of this thesis I focused on the effect of temperature on the circadian clock network and circadian behavior of two dipteran insect species – fruit fly (*Drosophila melanogaster)* and housefly *(Musca domestica)*. It was previously shown that *D. melanogaster* flies exhibit bimodal activity pattern in their behavior in normal temperature (25°C). In these conditions flies are active mainly around the dawn (the morning activity peak) and around the dusk (the evening activity peak) displaying almost no activity during the day, or night. When exposed to the cold temperature (18°C) flies increase their activity during the day so the morning and evening activity peaks are shifted closer together. On the contrary, the high temperature (29°C) is associated with the slight advance of the morning activity peak, more robust and longer siesta time and significant delay of the evening activity peak (Majercak et al., 1999; Low et al., 2009). The mechanism regulating temperature-dependent changes of the behavior was studied on the molecular level with particular interest paid to genes *period* and *timeless*. It was suggested that the temperature-regulated splicing efficiency of 3´UTR intron of *Dm per* and the last intron in the coding region of *Dm tim* gene (so called tim^{cold}) could be the molecular basis of this behavior, since these splicing variants predominate in cold temperatures (Majercak et al., 1999; Boothroyd et al., 2007). We have shown the impact of temperature on expression pattern of three additional clock genes (*Dm cwo*, *Dm vri*, *Dm Pdp 1ε*) and also *Dm tim*. In contrast, the expression pattern of two circadian clock genes (*Dm Cry1* and *Dm Clk*) was temperature-independent. The important question was, if such a behavior is unique for *D. melanogaster*, or whether it's conserved across taxa. Therefore we chose housefly, a dipteran insect species with a wide distribution pattern from tropical to temperate regions. The free running period of houseflies kept in different temperatures stays unaffected, confirming that the circadian clock is temperature compensated. Moreover, temperature influenced daily locomotor activity of houseflies similarly (but not identical) to *Drosophila*. Even though the expression pattern of the circadian genes of the housefly is modulated by temperature in the analogous way as in the fruit fly, diverse molecular mechanisms might be used to advance the rise of *Md per* levels in cold temperatures, since no temperature-dependent alternative splicing of *per*

intron was observed. Moreover, no alternative splicing was observed for *Md tim* gene either. Another difference was observed for *Md Clk*, which was affected by the temperature, contrary to fruit fly. These data indicate connection between temperature-dependent circadian clock genes expression and locomotor activity distribution. Further studies should explore the principal mechanism of temperature-modulation of circadian clocks.

In the second chapter I focused on possible role of circadian genes in the photoperiodic clock. The ability to measure photoperiod is widely used by many organisms to predict upcoming seasonal change and enables to prepare for changing environmental conditions in advance (Tauber et al., 1986). To cope with the adverse effect of upcoming season insect evolved the diapause as the surviving state in which they reduce metabolic and energy-demanding processes such as development or reproduction. In our laboratory the linden bug, *Pyrrhocoris apterus*, is used as model organisms for studying chronobiology for its strong photoperiodic and circadian phenotype and very simple manipulation and rearing. RNA interference works effectively in this species and thus experimental manipulation of gene expression is possible (Bajgar et al.2013a,b; Smykal et al., 2014). It was previously shown that the reproductive arrest is characterized by the absence of JH. The diapause can be overridden to reproductive mode even in diapause-promoting photoperiodic conditions, simply by adding the JH mimicking methopren, in both males and females (Socha, 2006; Socha and Hodkova, 2006, Jedlicka et al., 2009). The role of JH in females´ reproduction was further confirmed by genetic depletion of its receptor MET and its partner Tai resulting in non-reproductive females (Smykal et al., 2014). In this study we explored the role of JH signaling in males. We showed that JH is essential for growth of MAGs, indicating that JH is important for development of both males and females. However, our next experiments suggest that reproduction of *P. apterus* males continues even in *Met* RNAi knockdown animals. Since RNAi might result in only partial knock down, alternative explanation is that some residual amounts of MET were sufficient for male's reproduction. Alternatively, JH is not essential for reproduction in males. Indeed, microsurgery of CA, a gland producing JH, does not prevent male reproduction (Hejnikova et al., 2016), further supporting the outcome of RNAi experiments. Our study further explored the role of circadian genes in regulation of

reproduction/diapause switch. It was shown that in the bean bug, *Riptortus pedestris*, circadian clock genes *Clk*, *cyc*, *per* and *Cry* play an important role in the photoperiod measurement (Ikeno et al., 2010, 2011). First we have confirmed that circadian genes are not directly mediating JH reception. However, depletion of circadian clock gene *Clk* or *Cry2* prevented measurement of the photoperiod, since depletion of these two genes significantly reduced the growth of MAGs when bugs were transferred from SD into the reproduction-promoting conditions. We further noticed differences in the locomotor activity of the diapausing and reproductively active males. Males in SD conditions displayed low locomotor activity, whereas males exposed to LD photoperiodic conditions were more active. Interestingly, the high activity in reproductive males and low activity of diapause males seems to be independent on JH presence or absence, respectively. The activity of *Clk* knockdown males remained low after the transfer from SD to LD conditions, and also the ability of diapausing males to switch to reproduction mode after the transfer to LD regime was blocked, supporting the possible crucial role of circadian clock genes in photoperiodic measurement. There is a long debate on connection of circadian and photoperiodic clocks (photoperiodic timers) in insects. Numerous evidence from various insect species suggests some involvement of circadian clock genes either in diapause regulation, or up-stream, in the photoperiodic clock mechanism. However, it is not clear if all circadian genes are involved (complete genetic overlap), or if only some of them are necessary (partial overlap). There is even suggestion that circadian genes were recruited independently of their circadian function (so called gene pleiotropy) and hence the connection of circadian and photoperiodic clocks is misleading (Bradshaw et Holzapfel, 2010). Clearly, detailed mechanism of photoperiodic clock is missing and this work illustrates experimental potential of *P. apterus* for this research.

In the third chapter I focused on possible role of circadian clock genes in magnetoreception. The data obtained from vertebrates suggest that circadian clock genes might play an important role even in magnetoreception and navigation, yet this never been confirmed in natural geomagnetic field condition in insects. In birds it was shown that the light have strong effect on the orientation, suggesting that reactions of radical pairs (RPs) formed by photosensitive biological processes may be susceptive to the external magnetic fields, and thus provide the basis for *in vivo* chemical

magnetoreception (Schulten et al., 1978). Proteins from the Cryptochrome – Photolyase family have been widely discussed as being relevant to the light-dependent biological compass relying on the RP mechanism (Ritz et al., 2000; Solov'yov et al., 2010, 2012). In *Drosophila melanogaster* both circadian rhythmicity and geotaxis turned out to be Cry-dependent and were also affected by a magnetic field in intensities exceeding the natural geomagnetic field conditions (Gegear et al., 2008; Yoshii et al., 2009; Gegear et al., 2010; Fedele et al., 2014a,b). In our work we used MIR assay combined with reverse genetic approach to address the question if the magnetoreception in the natural GMF conditions in two cockroach species is cryptochrome-dependent. We used two cockroach species in our study, one with two *Cryptochromes* – *Cry1* and *Cry2* (*Blattella germanica*) and the other one with only one cryptochrome – *Cry2* (*Periplaneta americana*) present in the genome. We showed that *Cry2* knockdown abolished both circadian rhythms and magnetoreception in both cockroach species. When knocking down *Cry1* in the German cockroach we were able to abolish the circadian rhythmicity, but no significant effect was observed in MIR. Using eye-covering experiments we localized the GMF receptive organ to the compound eye. Laser-scanning confocal microscopy localized CRY2 to the hemispherical multicellular structure beneath the pigment layer of the retina with the strongest staining signal in the close vicinity of the plasma membrane. By microinjections of the fluorescent dye we revealed an intimate contact between the neuronal output from the retina and the CRY2-positive cells proposing their possible interplay. All these results indicate that CRY2 mediates directional magnetoreception in these two insects, phylogenetically distant to *Drosophila*.

Despite the diverse topics of presented chapters, we can conclude that the investigation of the role of circadian clock genes in different insect species is very important because: **1)** Circadian genes might participate in different biological phenomena, but some of them are robustly pronounced (i.e., diapause) only in certain species, and **2)** we confirmed important diversity in genetic composition of even closely related species, although most of the key functional homologs of the clock genes are conserved and can be found even in vertebrates. In our studies we have used 3 hemimetabola insect species – German and American cockroaches and linden bug; and 2 holometabolan insect species – fruit fly and housefly (Fig. 9). Although both flies are closely related (they diverged approximately 100 Mya (Henning, 1981)), we have found some important differences in their circadian clock mechanisms. Similarly cockroaches although closely related (they diverged approximately 80Mya (Che et al., 2017)) we observed the strong genetic variability in these two ancient insect species.

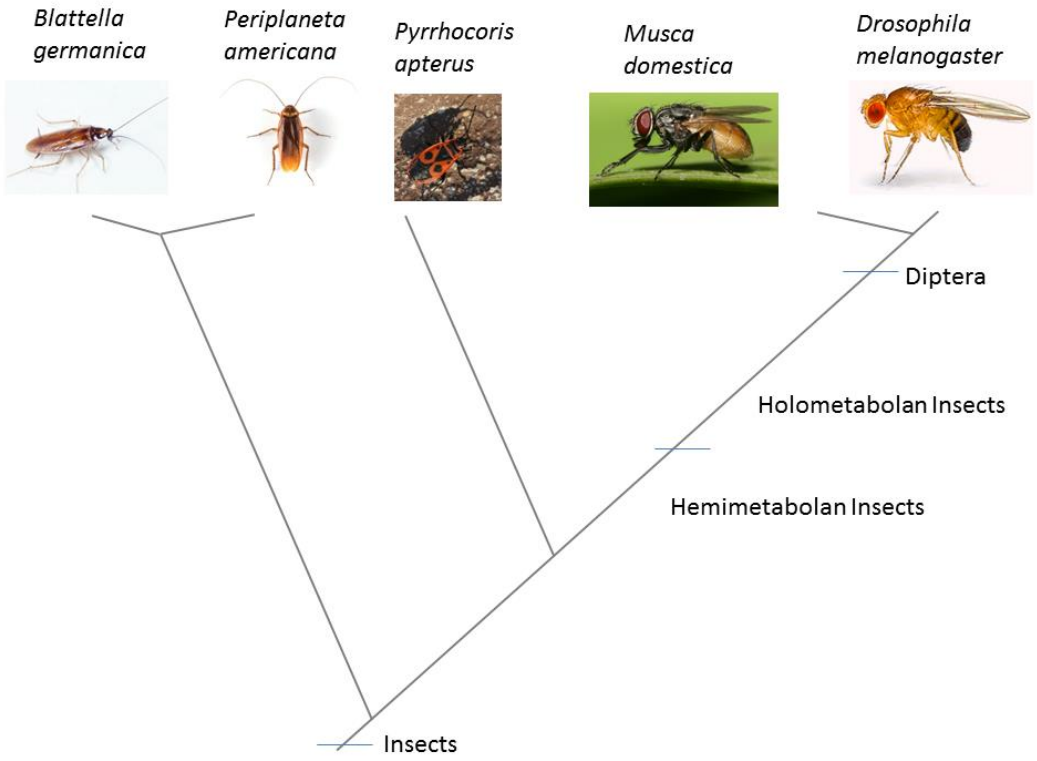

Fig. 9 Simplified phylogenetic tree showing model organisms we used in our studies.

For the further investigation the functional experiments seems to be crucial. While all of these species have strong circadian phenotype, the functional homologs of clock genes were identified and the animal can be reared in the lab conditions easily, there are some important limiting factors complicating the functional-genetics research. Specifically, the recently emerging genome editing offers amazing experimental opportunities. However, long generation time of organism (such as several months in cockroach species) makes experiments unrealistic. In case of cockroaches, the real obstacle is the formation of eggcases (oothecas), which practically prevents injection into early embryos. Therefore, experimental tools are limited to RNAi in cockroaches, which was effective strategy for some experiments (Bazalova et al., 2016), but it might be limiting when complete null mutant is necessary.

In general, we can expect that recent boom of technology, such as genome editing and massively parallel sequencing, allows working even on non-model species effectively. Hopefully, this remarkable biological diversity of insect will contribute to our understanding of function and evolution of biological rhythms.

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Suplement

Suplement 1 – Daily activity of the housefly, *Musca domestica*, is influenced by temperature independently on *period* gene splicing (paper in preparation for Genetics)

Suplement 2 – Photoperiod regulates growth of male accessory glands through juvenile hormone signaling in the linden bug, *Pyrrhocoris apterus*. (Insect Biochem Mol Biol. 2016 Mar; 70: 184-90. doi: 10.1016/j.ibmb.2016.01.003)

Suplement 3 – Cryptochrome2 mediates directional magnetoreception in cockroaches. (Proc Natl Acad Sci U S A. 2016 Feb 9; 113(6): 1660-5. doi: 10.1073/pnas.1518622113.)

Suplement 4 – *curriculum vitae*

Daily activity of the housefly, *Musca domestica*, is influenced by temperature independently on *period* gene splicing

running head: Impact of temperature on daily activity of *Musca domestica*

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Abstract, (preferably no longer than 150 words)

Circadian clocks orchestrate daily activity pattern and free running period of locomotion in constant conditions. While the first often depends on the temperature, the latter is temperature compensated over physiologically relevant range. Here, we have explored locomotor activity of a temperate housefly, *Musca domestica*. Under low temperatures, activity is centered to a major and broad afternoon peak, while high temperatures result in overall activity throughout the photophase with a mild mid-day depression pronounced especially in males exposed to long photoperiods. While *period* mRNA is peaking earlier under low temperatures, no temperature-dependent splicing of the last *per* 3'end intron was identified. Expression of *timeless*, *vrille* and *Par domain protein 1* was also influenced by temperature, yet each of them differently. Our data indicate that comparable behavioral trends in daily activity distribution have evolved in *Drosophila melanogaster* and *Musca domestica*, yet behavior of these two species is orchestrated by different molecular mechanisms.

Keywords: temperature compensation of circadian rhythms; locomotor activity; *period* gene; *timeless*; mRNA splicing; transcription; *vrille; Par domain protein I epsilon; cryptochrome;* mRNA splicing;

INTRODUCTION

Circadian clocks orchestrate daily activity pattern and free running period of locomotor activities. While the first often depends on the temperature, the latter is temperature compensated over physiologically relevant range. For instance, *Drosophila melanogaster* displays bimodal activity with morning and evening peaks separated by siesta under constant temperature (Rosato & Kyriacou, 2006) or by afternoon activity burst under natural environmental conditions (Green et al, 2015; Vanin et al, 2012). Under elevated temperatures, morning peak is advanced and evening peak delayed. In contrast, overall free running period in constant conditions is temperature compensated over physiologically relevant range. Importantly, both of these activities are driven by circadian genes.

Temperature strongly affected weak splice site of the last *period* (*per*) gene intron of *D. melanogaster* (dmpi8) and locomotor activity pattern in this fly (Collins et al, 2004; Majercak et al, 2004; Majercak et al, 1999). Comparison of two tropical *Drosophila* species, *D. yakuba* and *D. santomea*, revealed that temperature has neither effect on splicing efficiency of this last *per* intron, nor on the daily activity of flies (Low et al, 2008). Transgenic constructs specifically addressed structure of this intron and established its splicing efficiency as an important molecular mechanism adjusting behavior to temperature. The impact of temperature on transcription of *per* and *timeless* (*tim*) was explored both in *D. melanogaster* exposed to a laboratory-controlled environment (Boothroyd et al, 2007) and recently also under natural conditions (Montelli et al, 2015).

Notably, locomotor activity of northern *Drosophilid* species, including *D. littoralis* and *D. montana* is tightly linked to weather and their behavior becomes arrhythmic under constant dark conditions (Kauranen et al, 2012; Lankinen, 1986; Dolezel and Hall, unpublished). Given these interspecific differences within the *Drosophila* genus, we decided

to explore impact of temperature on locomotor activity of the housefly*, Musca domestica*, a dipteran species with characterized circadian toolkit (Codd et al, 2007), including rescue experiments of *D. melanogaster per*^{0} mutant (Piccin et al, 2000) and numerous anatomical data (Miskiewicz et al, 2008; Pyza & Meinertzhagen, 1997).

METHODS

Fly maintenance

M. domestica larvae were raised on a medium made of wheat bran (55 g), heat-inactivated yeast (3 g), milk (150 ml), and the antimycotic nipagin (0.35 g, Sigma) until pupariation. After eclosion, adult flies were fed on water, sugar, and dried milk. Fly stocks were maintained at 25°C either under 12hr light/12hr dark cycles (LD 12:12) or under 16 hr light/8 hr dark cycles (LD 16:8). In our study, we have mainly used strain homozygous for mutations *white eye* and *apterous* (gift from Daniel Bopp, University of Zurich, (Codd et al, 2007; Hediger et al, 2001). For behavioral experiments we have also collected wild type strain in Ceske Budejovice (Czech Republic) which was amplified for 3-5 generations and used for locomotor activity recording. The species identity was verified by sequencing intron of *per* gene.

Locomotor activity

3 to 5 days old male or female flies were individually housed in glass test tubes (24 mm x 150 mm, PYREX, Sigma) with a sugar lump wrapped in a mesh bag on one side and water reservoir on the other side. Movement of the fly was automatically detected by an infrared photosensor (Large Activity Monitors, LAM, TriKinetics, Waltham, MA, U.S.A.) and recorded in 5 min intervals. Monitors were placed in controlled regime of MIR 153 or 253 incubators

(SANYO/Panasonic) with 15W cool white lamp (ZS15, Top Light) generating light of approximately 400-800 lux.

To determine the free running period, flies were entrained for four days at either LD16:8, LD12:12 or LD 8:16 (15°C, 25°C or 35°C) and released into constant dark (DD) of the same temperature for another 10 days. The activity data were evaluated using ActogramJ software (Schmid et al, 2011).

Daily pattern of locomotor activity was recorded at three different constant temperatures (15°C, 25°C or 35°C) under either 16h light/8h dark (LD 16:8) or 12h light/12h dark (LD 12:12) regimes. Data were collected for at least 14 days, while the first four days were omitted from the analysis. To compare daily activity distribution, data were plotted as Mean +/- SEM in Excel (Microsoft). To compare absolute activity levels at different conditions, total average activity per 24 h interval was calculated from day 5-14 and presented as one point for each individual fly (Graphpad Prism).

Gene cloning

To collect samples for cDNA cloning, flies were snap frozen and their heads were either stored at -80°C, or immediately used for total RNA isolation with TRIzol-reagent® (Ambion). 5µg of total RNA was reverse transcribed using the oligo(dT) primer (24mer) and Superscript™ III reverse transcriptase (Invitrogen). Sequences for *M. domestica* circadian clock genes *casein kinase 2beta* (*Md_ck 2beta*), *clockwork orange* (*Md_cwo*), and *Par domain protein 1* (*Md_Pdp1*) were obtained using degenerate PCR approach with primers designed according to conserved protein regions from insects (*D. melanogaster*, *Apis melifera*, *Culex pipiens*,

Aedes aegypti, *Dannaus plexipus*, *Tribolium castaneum*), amphibians (*Xenopus laevis*) and mammals (*Rattus norvegicus*, *Homo sapiens sapiens*) clock gene orthologs. Short fragments (200-700bp) were amplified, cloned and sequenced. Gene sequences were further extended by primer walking, 3` - RACE (rapid amplification of 3` ends) and 5` - RACE (Ambion). The sequence of *Musca pigment dispersing hormone* (*Md_pdf*)(Matsushima et al, 2004), *period* (Piccin et al, 2000), *vrille* (*Md_vri*), *Clock* (*Md_Clk*), *cycle* (*Md_cyc*) and *timeless* (*Md_tim*) (Codd et al, 2007) were described previously. The intron sequences close to the coding sequences (CDS) 3` end of *cwo*, *ck 2beta* and *pdf* genes and close to the start codon of *Pdp 1 epsilon* gene were obtained from genomic DNA using specific primers. No intron sequences were identified in the *double time* gene. All primers used are listed in Table S2.

Phylogenetic analysis

To clarify which *cryptochromes* are encoded in *Musca* genome, CRY proteins were aligned using MAFFT program (Geneious, Biometters), trimmed and substitution models were tested in ProTest 2.4 program (Abascal et al, 2005). Phylogenetic trees were constructed under WAG and LG models (RaXML) and in Fasttree algorithm (Geneious 8.1, Biometters). Since all trees shared the same topology, we present only phylogenetic analysis performed in RaXML 7.2.8 under LG+Gamma model with 500 bootstrap replications.

As a reference, we used insects containing both mammalian-type and Drosophila-type CRYs: *Anopheles gambiae, Danaus plexippus* (Yuan et al, 2007), *Blatella germanica* (Bazalova et al, 2016), *Pogonus chalcetus*; insect with only mammalian CRY: *Apis mellifera* (Rubin et al, 2006) and *Pyrrhocoris apterus* (Bajgar et al, 2013b), and *Drosophila melanogaster* representing insects with only the photosensitive CRY (Stanewsky et al, 1998).

Expression analysis

Three independent animal groups were reared and sacrificed for each experiment. For each experiment, 5-10-day-old males and females were collected separately, and the heads stored at -80°C until RNA extraction. Total RNA was isolated from 25 heads per timepoint using TRIzol-reagent® (Ambion) according to the manufacturer's instructions. 1µg of total RNA was reverse transcribed using oligo(dT) primer and Superscript™ III reverse transcriptase (Invitrogen). To safeguard against amplification of possible contaminant genomic DNA, the primers were designed either to anneal only to a template corresponding to the spliced transcript or (in case of *dbt*) the genomic DNA was removed from the sample using TURBO DNase (TURBO DNA-free™ Kit, Ambion) according to the manufactures instructions or (in case of *tim*) the primers were designed to include a large (2.5 kb) intron in the genomic DNA template (Codd et al, 2007).

A total 3µl of 50 times diluted cDNA were used for 12µl PCR reaction (IQ™ SYBR®Green Supermix, 1x conc. – Bio-Rad, primers 0.4 µM each, milliQ H_2O). Amplifications were carried out on C1000™ Thermal Cycler (Bio-Rad) in 96-well micro plates (Bio-Rad Hard-Shell®) according following protocol: initial denaturation (94°C, 2 min) followed 35 cycles of denaturation (94°C, 10s), annealing primers (60°C, 20s), elongation (72°C, 20s). Product size was always confirmed by the melting analysis. Each cDNA sample was amplified in triplicates for each primer combination, where *rp49* served as a normalization reference. List and specification of used primers is in Table 1. Data were analyzed and quantified using CFX Manager™ Software. Relative values were standardized to *rp49* and normalized to the sample with the highest expression (Kobelkova et al, 2010). Values represent the mean of three independent biological replicates +/- standard deviation.

Promoter comparison

Promoters of *D. melanogaster* and *M. domestica* were explored by automatic prediction server to identify transcription start and manually in Genious 7.1 (Biometers) to identify conserved cis-regulatory motifs. Identified motifs were highlighted as sequence annotations in Genious 7.1 (Biometers). Corresponding figures were redrawn in Adobe Illustrator CS5 manually. The expected frequencies of particular cis-motifs were compared to observed frequency in Microsoft Excel 2010 spreadsheet, obtained values were color coded (Conditional formatting in Excel) and the figure was finalized in Adobe Illustrator.

RESULTS

Temperature compensation of the free running period (FRP)

Our first goal was to verify, if circadian clock of *M. domestica* is functional at all temperatures. Flies were entrained to three different photoperiods at three different temperatures (9 different combinations) and afterwards released to DD. While free running period was observed in all flies exposed to 25°C, both lower (15°C) and higher (35°C) temperatures resulted in a lower percentage of rhythmic individuals, particularly males, and in higher variability of the FRP in rhythmic individuals (Fig. 1A-C). Moreover, short photoperiod entrainment (LD 8:16) slightly reduced percentage of rhythmic individuals even at 25°C (Fig. 1A). Examples of double plotted actograms are shown in Supplementary Fig S1.

Locomotor activity distribution in LD

Then we explored impact of the temperature and the photoperiod on the daily activity pattern. In general, low temperature of 15°C results in locomotion centred to the middle of

the photophase (Fig. 2A, D, G, J) in both photoperiods, whereas flies are active at 25°C and 35°C during the whole photophase (Fig. 2B, C, E, F, H, I, K, L). There is also mild difference between male and female activity at 25°C and 35°C, when males show slightly bi-modal pattern with the light on peak followed by a mild trough in the first half of the photophase followed by a gradual activity increase during the afternoon reaching maximum approximately 10 hrs after light on signal (Fig. 2B, C, H, I). The afternoon activity peak is delayed with the temperature increase (compare Fig. 2A-C for the short day and Fig 2G-I for the long day). The activity is gradually decreasing during the second half of photophase under the long photoperiod of LD 16:8 (Fig. 2H, I). The activity of females rises progressively during the photophase at 25°C and 35°C peaking either after light off signal (Fig. 2E, F, L) or just at the end of photophase (Fig. 2K). The overall activity at 15°C is markedly lower than activity detected at 25°C or 35°C in both sexes (Fig. 3, Tab S2). At 25°C and 35°C, males are slightly more active than females (Fig. 3, Tab S2).

Cryptochrome genes in *Musca* – phylogenetic analysis

Initial search identified two *cry*-like genes in *M. domestica* genome. Phylogenetic analysis revealed three clearly distinguished clusters: (*i*) 6_4 photolyase, (*ii*) insect cryptochrome 1 (*Drosophila*-type CRYs), and (*iii*) group containing both human CRYs and insects CRY2 (Figure 4). This analysis, unambiguously supported by bootstrap values, completely agrees with previously published phylogeny of CRYs (Yuan et al, 2007). Hence, *M. domestica* contains one CRY protein of *Drosophila*-type (Md_CRY1) and the second gene clearly belongs to 6-4 photolyases (Figure 4).

Our phylogenetic analysis revealed an additional interesting aspect. In an attempt to add CRY proteins from relevant holometabolan insect species, we have prospected transcriptomes of beetles (Coleoptera). Whereas in *Tribolium castaneum* only one gene

belonging to CRY2 group was identified, in another beetle, *Pogonus chalcetus*, both CRY1 (*Drosophila*-type) and CRY2 -coding genes were found (Figure 4). This finding supports the remarkable diversity of circadian clock design, particularly the role of various CRY combinations reported in holometabola (Yuan et al, 2007) and recently also in hemimetabola (Bazalova et al, 2016).

Given the non-cyclical expression of *Md_cry1* mRNA (Codd et al, 2007), which is in contrast with expression of *Drosophila cry* (Emery et al, 1998), and possible role of *Dm_cry* in temperature-dependent rhythmicity (Dolezelova et al, 2007), we sought to analyze expression profile of both *Md_cry1* and *Md_phr6-4*.

mRNA expression at different temperatures – *Musca domestica*

qRT PCR was performed on samples isolated from male *Musca domestica* heads collected every 2 hours. Since the expression of *Md_casein kinase2 beta*, *Md_pdf* and *Md_dbt* did not show any cyclical expression either in LD or in DD condition at 25°C (Fig Sx A-C; see expression of *Md_per*, *Md_Pdp1 epsilon* and *Md_cwo* for comparison in Fig Sx D-F), further measurements addressed the influence of temperature on cyclically expressed transcripts.

Previous study identified peak of *per* abundance at ZT 16 in houseflies kept at 25°C (Codd et al, 2007). Our data relying on more detailed 2hr resolution indicate the mRNA maximum at ZT14 (Fig. 5A). Both the expression rise and its peak are advanced by approximately 2 hrs at 15°C. Consistently, *Md_per* accumulation is phase delayed by approximately 2 hrs at 35°C (Fig. 5A).

Similar trend was observed in *Md_vri*, where exposure to 15°C results in 5-6 hr advance in mRNA expression profile (Fig. 5B). In contrast to *Md_per*, no difference was observed in *Md_vri* expression at 25°C versus 35°C (Fig. 5B grey vs magenta). Comparable trend to *Md_vri*

expression, phase advance at 15°C and no difference between expression at 25°C and 35°C, was found for *Md_cwo* (Fig. 5E) and *Md_Clk* (Fig. 5F). The main difference is in the phase of expression: *Md_Clk* starts to accumulate during the scotophase and peaks early in the photophase, particularly at 15°C (Fig. 5F), whereas *Md_cwo* peak lies at the end of the photophase (Fig. 5E) similar to *Md_per, Md_vri,* and *Md_Pdp1*$_{epsilon}$ (Fig. 5A,B,D).

Expression of *Md_tim* was influenced by the temperature differently. At 25°C, mRNA peaks at ZT 14 (Fig. 5C), identically to *Md_per* maximum. Low temperature of 15°C results in a phase delay (peak at ZT 16-18) and a slightly lower expression level. High temperature of 35°C delays the phase of *Md_tim* maximum even more (peak at ZT 20) and the expression is higher than levels observed at 15°C or 25°C (Fig. 5C).

Temperature did not affect the phase of *Md_Pdp1*$_{epsilon}$, which peaks during the early photophase in all three temperatures (Fig. 5D). Level of *Md_cry* was temperature-independent and non-cyclical (Fig. 5G), confirming result published previously (Codd et al, 2007). Similarly, expression was non-cyclical for *Md_photolyase* (Fig. 5H).

mRNA expression at different temperatures – *Drosophila melanogaster*

To compare if similar trends are conserved in *D. melanogaster*, expression profiles at low (18°C), ambient (25°C) and high (29°C) temperatures were defined with 2hrs resolution. *Dm_per* peak was phase advanced to ZT10 at low temperature (Fig. 6A), whereas mRNA reached maximum at ZT14-16 in both ambient and high temperature. Similarly, *Dm_vri* peak was advanced at 18°C and delayed in 29°C (Fig. 6B). Similarly to *Musca*, accumulation of *Dm_tim* started identically at all three temperatures (Fig. 6C). The only differences, bordered in a statistical significant value (see supplementary Table Sxx), were observed later in the

middle of the scotophase, where higher temperature is associated with the higher *Dm_tim* levels in contrast to low temperature samples (Fig. 6C). *Dm_ Pdp1*$_{epsilon}$, was influenced by temperature differently from *Md_ Pdp1*$_{epsilon}$ (Fig. 5D). The high temperature resulted in a higher *Dm_ Pdp1*$_{epsilon}$ levels that peaked at ZT18, approximately 8 hrs later than the low temperature maximum (Fig. 6C).

Mdpi11 splicing

Two PER-immunoreactive bands were identified in head extracts, whereas only single band was recognised in the thorax previously (Figure 4 in Codd et al., 2007). We failed to identify any intron in the 3'UTR of Musca *per* gene corresponding to *dmpi8* (Majercak et al, 1999) either by *in silico* search in the published *Musca* genome (Scott et al, 2014), or by PCR-based approach. Nevertheless, alternative retention of *mdpi11*, the last intron within the coding sequence, would result in PER protein shorter by 18 amino acids (Fig. 7A,B). Therefore we have measured expression of both transcript variants using splice isoform-specific primer combination. Clearly, the expression of *mdpi11* is not affected by the temperature (Fig. 7C).

***timeless* splicing in Musca**

Interspecific comparison of TIM proteins across all insect species identified conserved 14-15 amino acid motif near the C terminus (Fig. 8), whereas its preceding sequences are highly variable. This motif, characteristic by 3-4 acidic residues at the C end, is found even in ancestral insect such *Thermobia domestica*.

Low temperature triggers alternative splicing in *D. melanogaster tim* resulting in an earlier stop codon completely removing this conserved TIM motif (Montelli et al, 2015). Comparison of genomic sequence identified existence of identical intronic premature stop

codons in *C. costata* and *M. domestica* (Fig. 8). Therefore, we have explored splicing frequency of the last intron by measuring level of both isoform; the spliced transcript, resulting in full protein with conserved motif, and non-spliced isoform, resulting in shorter protein. Low temperature results in ~1% lower splicing efficiency of *Md_tim*. Although this difference is statistically significant, the low temperature has minimal effect when compared to approximately 10-fold splicing difference in homologous *tim* intron of *D. melanogaster* (Montelli et al, 2015).

Promoter comparison

Having *M. domestica* genome in hands (Scott 2014) we have explored the promoter regions for presence of putative cis-regulatory sequences. No experimental data define promoter sequences in *M. domestica*, and only some *D. melanogaster* circadian genes were rigorously explored using systematic promoter truncation experiments. Therefore we analysed DNA regions upstream of the start codon ("ups" in Fig. 9) and also separately analysed upstream regions including the first intron after the start codon ("ups+" in Fig. 9).

In general, *M. domestica* genes are longer than *D. melanogaster* homologs, often with larger intronic sequences. Frequency of E-box sequences (CACGTG, CACGTH, AACGTG) was lower in *M. domestica* than in *D. melanogaster per*, *tim*, *vri* and *cwo* (Fig. 9). The frequency of canonical E-box (CACGTG) in *M. domestica* was even lower than expected random occurrence of this motif. Remarkable occurrence of D-box (more than 25fold upregulated) was found especially in *D. melanogaster tim* and *Clk.*

DISCUSSION

Expression of circadian genes was explored in many insect species. In general, expression pattern often differs between tissues (Beaver et al, 2003; Damulewicz et al, 2015; Iwai et al, 2006; Tomioka et al, 2012), is affected by reproductive/diapause status of the animal (Bajgar et al, 2013a; Bajgar et al, 2013b; Dolezel et al, 2008; Kostal et al, 2008), and depends on animal age and nutrition access (Dolezel et al, 2007; Rakshit et al, 2012).

Temporal expression of circadian genes also differs between species remarkably. The strong cyclical expression characteristic for several circadian genes in *Drosophila* is not the rule for all insect species. For instance, expression of circadian gene homologs *per* and *Clk* shows no cyclical changes either in heads of the linden bug, *P. apterus* (Syrova et al, 2003), or

even in dissected brains (Kotwica-Rolinska and Dolezel, unpublished data). Importantly, expression changes differ between different brain regions of *D. melanogaster*, including prominent amplitude of many transcripts in PDF cells (Kula-Eversole et al, 2010). Notably, expression in compound eyes contributes remarkably to whole head mRNA levels of several circadian genes. Indeed, this strong expression in the eye allowed for identifying *cryptochrome*^{b} mutation in a luciferase-reporter screen (Stanewsky et al, 1998). Therefore, correlating expression pattern in the whole heads with locomotor activity profile is only the approximation overlooking cell-specific expression. However, the lack of suitable antibodies recognizing *Musca* circadian genes prevents rigorous analysis with anatomical resolution. At the same time, expression profile on whole *D. melanogaster* heads was used extensively and therefore interspecific comparisons with solid data are feasible.

In this study we have addressed the impact of temperature on expression of circadian genes in *Musca* and comparison with expression of *D. melanogaster* homologs. Despite certain differences between these two species, a few general patterns were observed (Fig. 12). Low temperature resulted in phase advance of *period*, *vrille, cwo* and *Clk*. However, *tim* expression is influenced by the temperature differently. Expression of *Pdp1*$_{epsilon}$ was not influenced in *Musca*, whereas expression of *Dm_Pdp1*$_{epsilon}$ was regulated similarly to *Dm_tim*. It is expected that the transcription of *per*, *tim*, *Pdp1*$_{epsilon}$ and *vri* requires CLK-CYC heterodimer (Cyran et al, 2003), therefore it is surprising to see different expression patterns in these genes.

One possible explanation is (*i*) a different temperature-dependent transcription. Indeed, CLK and CYC tissue-specific expression of *Drosophila* is enhanced by transcriptions factors ODD PAIRED and SERPENT (Meireles-Filho et al, 2014). Similar adjustments and redirections might contribute to the temperature-adjustment of circadian gene expression.

Alternatively, (*ii*) posttranscriptional regulation might contribute to altered mRNA stability and hence affect its accumulation. Indeed, three circadian genes, *vri*, *cwo* and *Clk* are regulated by microRNAs (Kadener et al, 2009). Most likely, combination of both regulations will contribute to the temperature compensation mechanism.

Our locomotor activity experiments identified clear impact of the temperature on the activity distribution pattern in *M. domestica*. While houseflies at low temperature show single activity peak located to the middle of the photophase, their activity is distributed throughout the entire photophase under ambient and high temperatures. The activity profile of males at ambient and high temperatures contains mild trough between light on and light off peaks, evoking similarity with morning and evening activity peaks of *Drosophila melanogaster*. It is believed, that lower mid-day activity, the "siesta", is an adaptation protecting *D. melanogaster* from dry environment experienced during the noon. However, this behavior was observed under constant temperatures in laboratory experiments. Locomotor activity obtained in *D. melanogaster* recorded under natural conditions, where temperature cycles during the day (together with the light intensity), reveled more complex pattern with additional noon peak (Vanin et al, 2012).

Temperature-dependent splicing of the last *per* intron (*Dmpi8*) was established as the key factor influencing timing of the morning and evening activity peaks (Majercak et al, 2004; Majercak et al, 1999). Therefore we searched for a homolog of this 3'terminal UTR intron in *Musca*. However, no intron was found in 3' UTR in *Musca per* gene, suggesting different molecular mechanism should be involved in this dipteran insect.

Functional experiments addressing role of particular genes in *Musca* is needed to fully define underlying biological mechanisms. Alternatively, further comparative studies might point to conserved features across taxa. Interestingly, bi-modal activity was found even in

distantly related insects, in Lepidoptera, where flight activity of *Ephestia kuehniella* is restricted to an early night and late night peaks (Kobelkova et al, 2015; Zavodska et al, 2012). It would be interesting to see, how this night-active insect changes its activity pattern at different temperatures, if the expression of circadian genes is affected by the temperature, and how. Similar comparative experiments might identify general patterns, which need to be verified functionally. These reverse genetics experiments are technically demanding in non-model insects, yet current gene editing tools are reaching chronobiology already (Markert et al, 2016).

Acknowledgement

We thank Roman Neuzil for excellent house fly husbandry, Dr. Daniel Bopp (University of Zurich) for *M. domestica* strains and Martin Pivarci for the phylogenetic analysis. We thank Joanna Kotwica-Rolinska and Milena Damulewicz for critical reading of the manuscript. This work was supported from National Science Foundation (Grant No. 14-32654J to D.D.) and locomotor activity monitors were purchased from European Union program FP7/2007–2013, grant No. 316304.

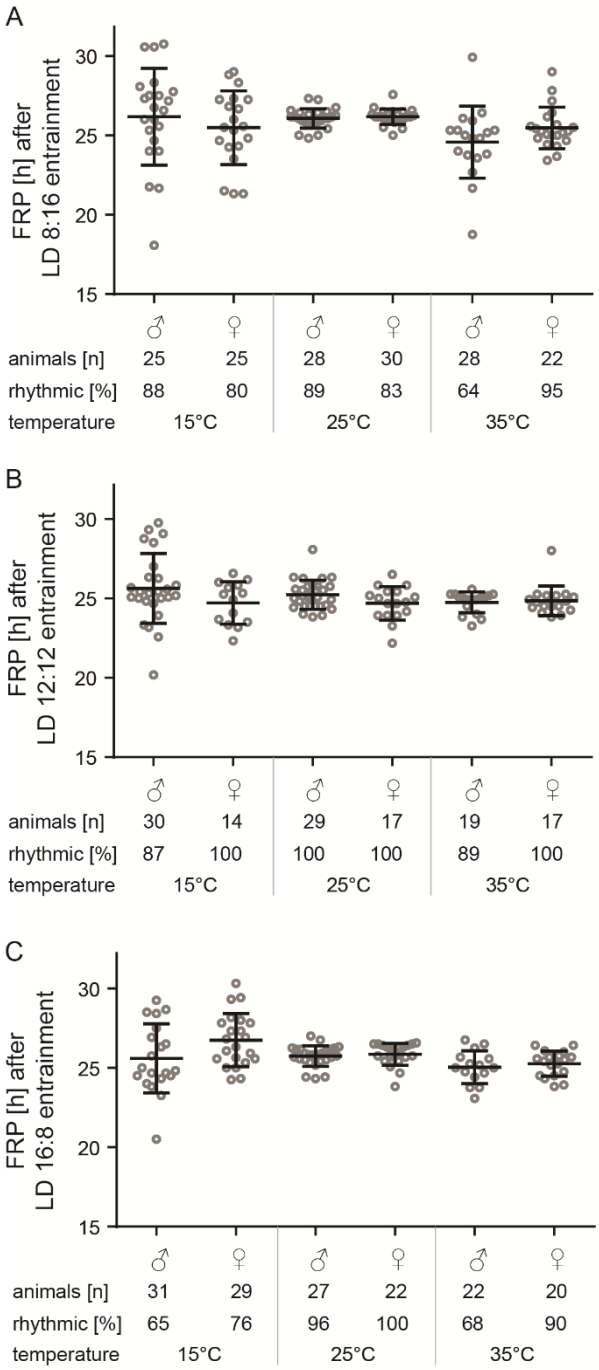

Fig. 1 Free running period of males and females (+/- SD) at 15°C, 25°C and 35°C after entrainment at three different photoperiods: (A) short day LD 8:16, (B) equinox LD 12:12, and (C) long day LD 16:8. Each dot corresponds to individual fly. Number of all animals measured and % of rhythmic individuals is shown under each chart.

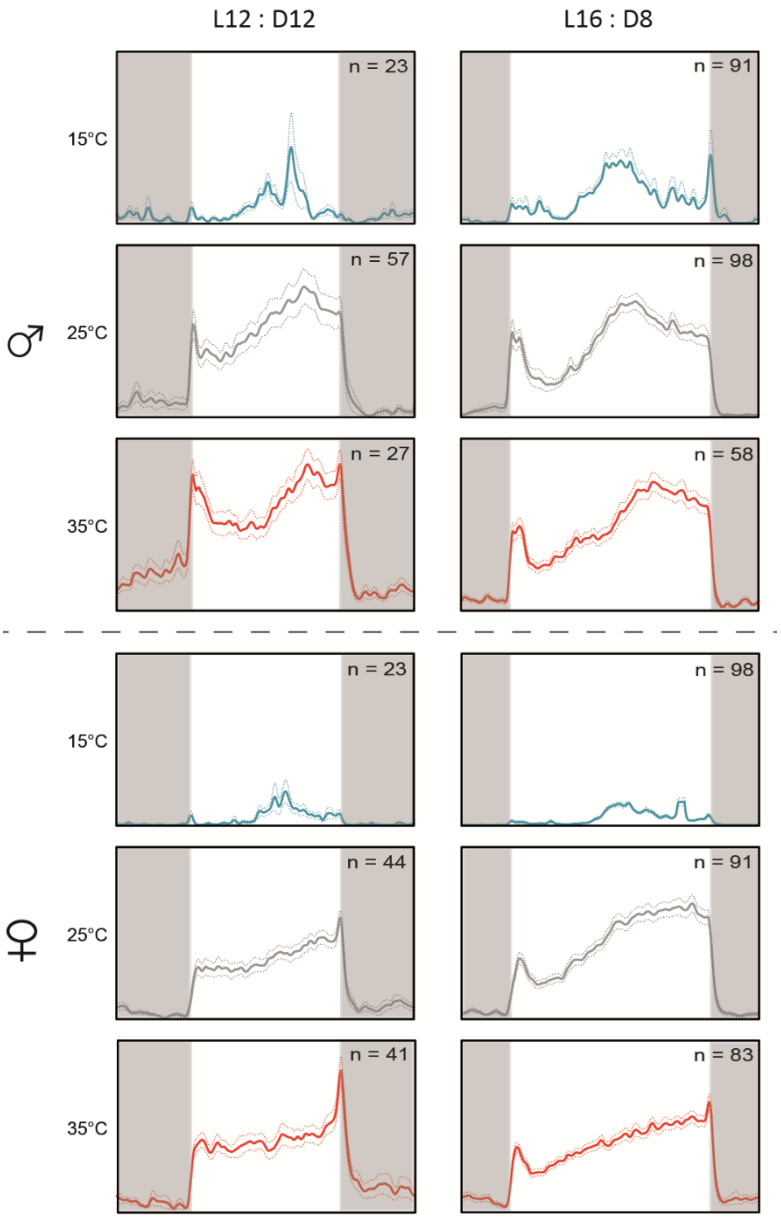

Fig. 2 Locomotor activity pattern of *Musca domestica* kept in LD 12:12 (left column) and LD 16:8 (right column). Blue, grey and red line represents 15°C (low), 25°C (normal) and 35°C (high) temperatures respectively. Flies were measured individually. The average +/- SD shown as dashed colour lines, n = numbers of animals used in the experiment. Vertical dashed grey line corresponds to ZT 12 in L16:D8 regime.

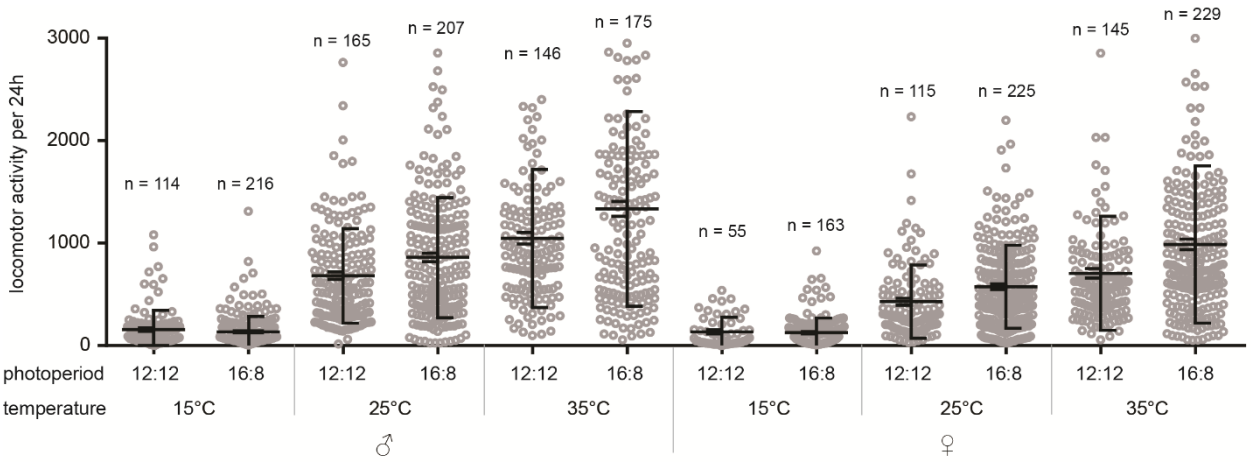

Fig. 3

Locomotor activity levels of *Musca domestica* plotted as number of laser beam crosses per 24h. The left side depicts the mean +/- SEM, the right side depicts the average +/- SD. Each circle corresponds to individual fly, and number of analysed days is shown above each category. See Supplementary table S2 for statistics.

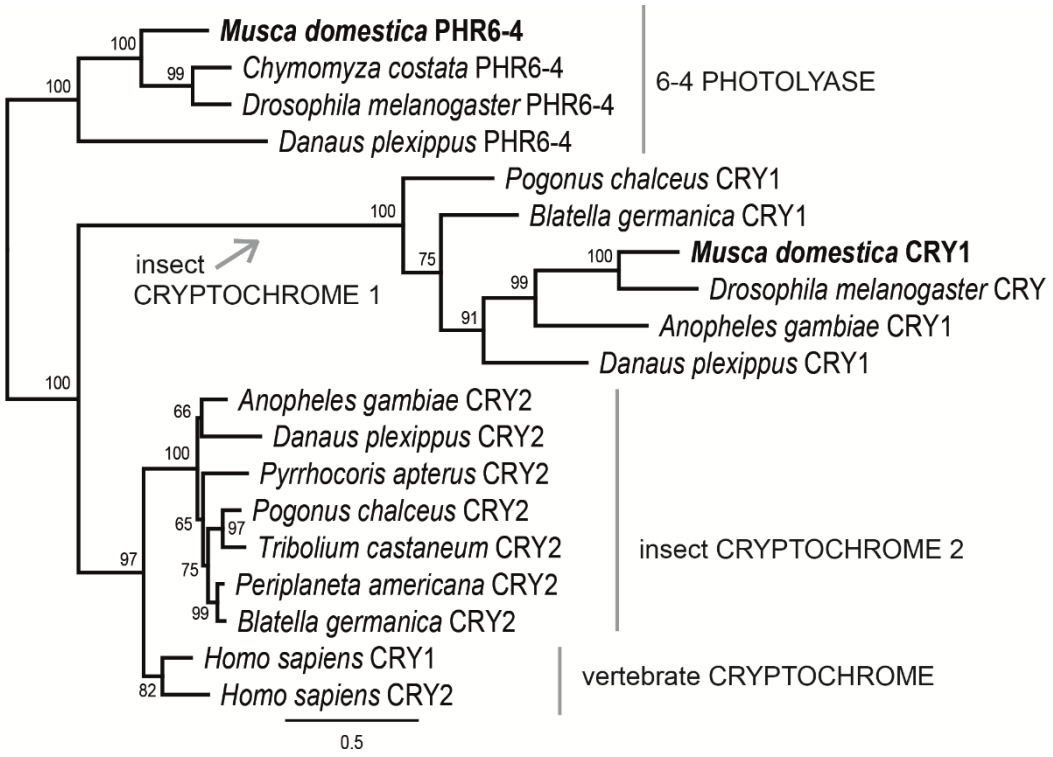

Fig. 4 Phylogeny of *M. domestica* CRYs in context of insect CRYs. The tree was obtained from RAxML 7.2.8 analysis of protein sequence alignment under LG+Gamma substitutional model. Bootstrap support from 500 replicates is shown in % under each node. 6-4 photolyases served as an outgroup.

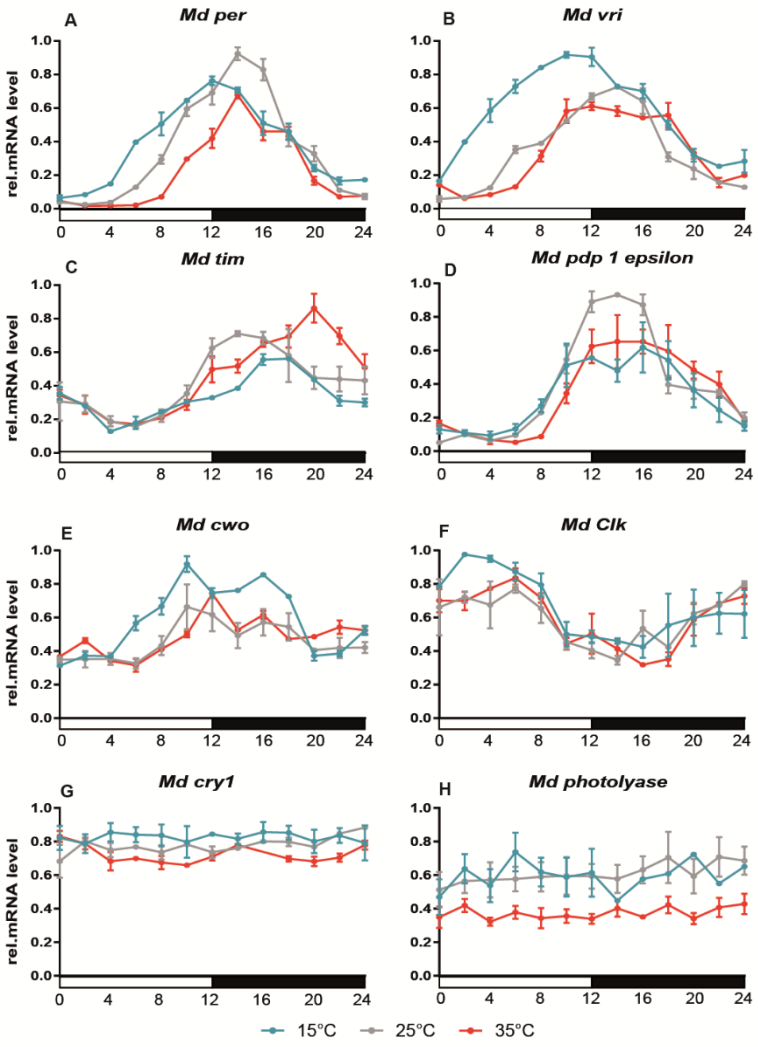

Fig. 5 Relative mRNA level of clock gene expression in *Musca domestica* heads in three different constant temperatures (15°C – blue line, 25°C – grey line and 35°C – red line) under the short photoperiod (LD 12:12): (A) *Md_per*, (B) *Md_vri*, (C) *Md_tim*, (D) *Md_Pdp 1epsilon*, (E) *Md_cwo,* (F) *Md_Clk,* (G) *Md_cry1* and (H) *Md_photolyase.* The average of three independent biological replicates is shown for each temperature, +/- SD. See result section and table S2 for statistical analysis.

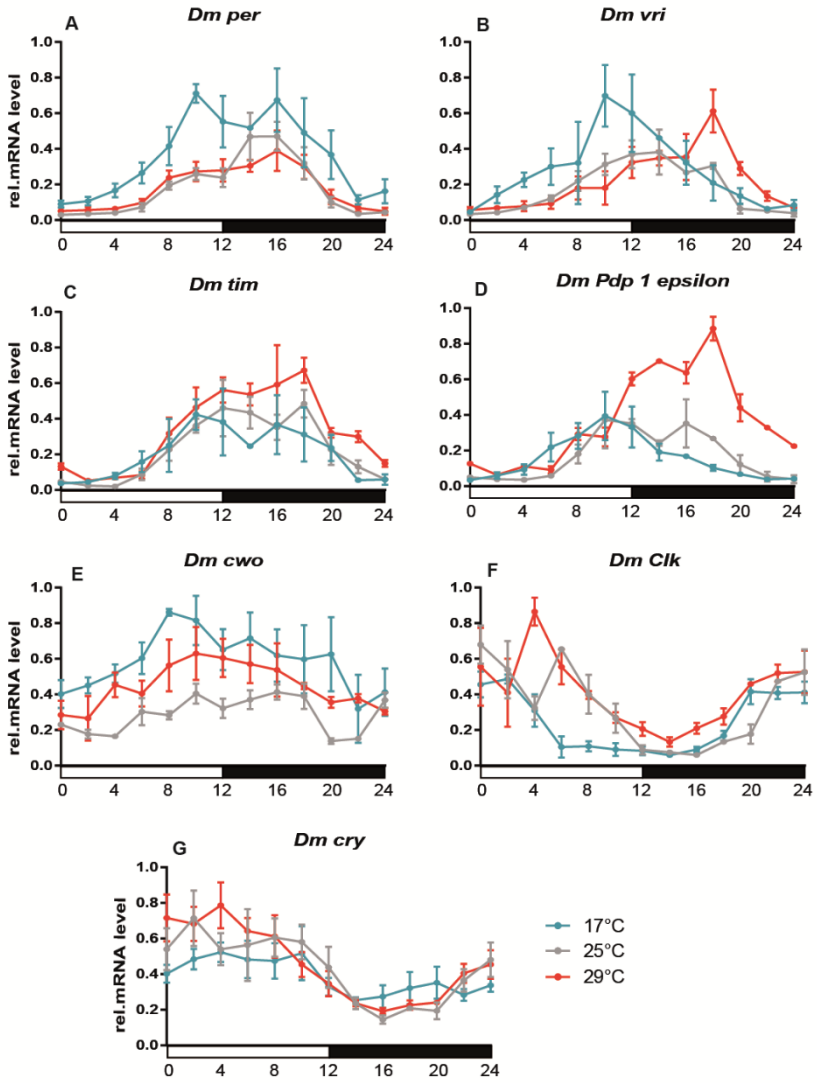

Fig. 6 Relative mRNA level of clock gene expression in *Drosophila melanogaster* heads in three different constant temperatures (15°C – blue line, 25°C – grey line and 35°C – red line) under the short photoperiod (LD 12:12): (A) *Dm_per*, (B) *Dm_vri*, (C) *Dm_tim*, (D) *Dm_Pdp 1epsilon*, (E) *Dm_cwo,* (F) *Dm_Clk,* (G) *Dm_cry.* The average of three independent biological replicates is shown for each temperature, +/- SD. See result section and table S2 for statistical analysis.

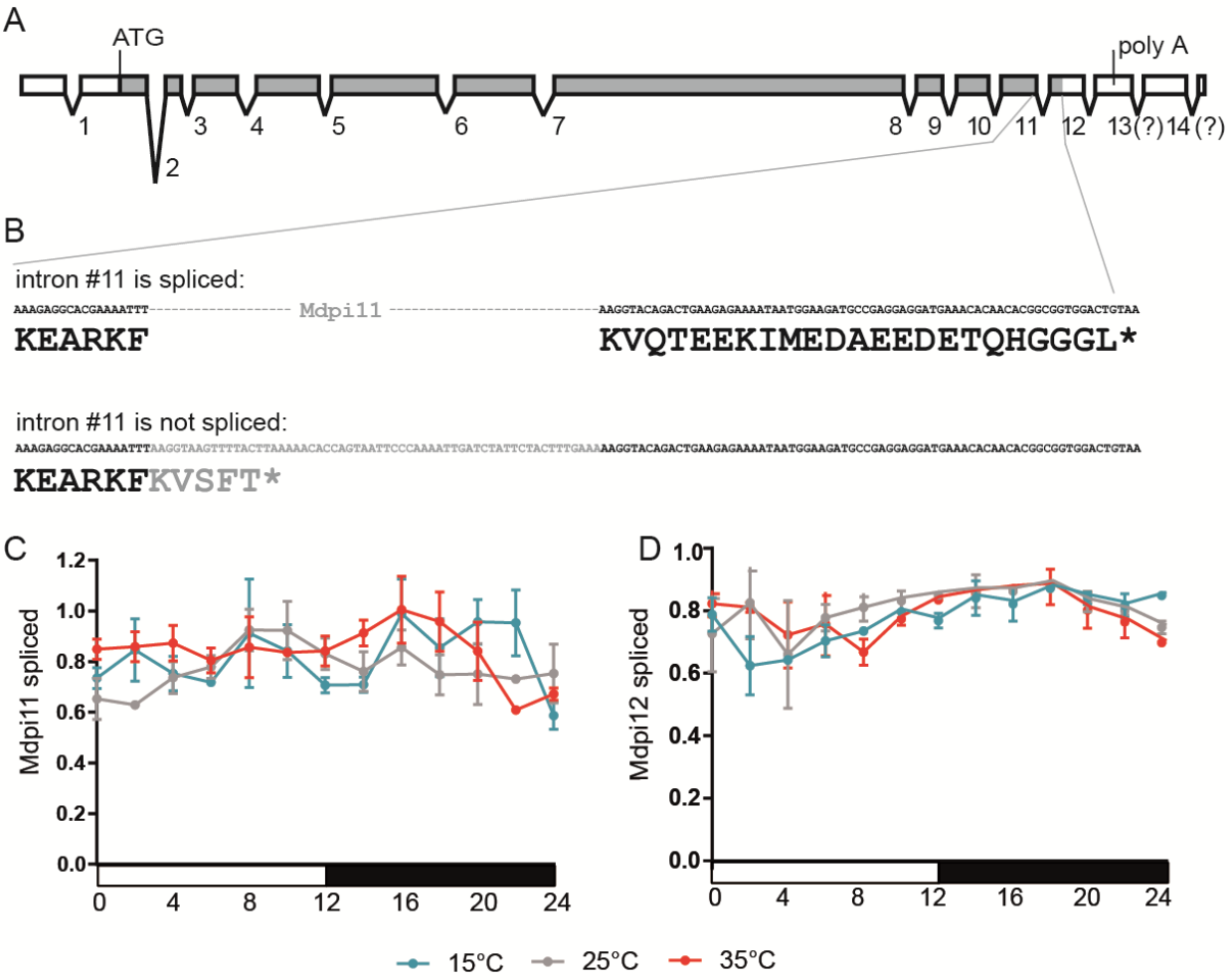

Fig. 7

(A) Structure of *Musca domestica per* gene with exons (rectangles) and introns (lines). Grey fill corresponds to coding sequence, position of the start codon highlighted (ATG) and stop codon (*… stop codon) are shown. Although the genome annotation predicts 14 introns within *per* gene (numbers), our 3'RACE identified poly A tail (poly A) in mRNAs corresponding to the 13th exon. (B) Detail of the intron 11 (in grey color) and surrounding exons with corresponding protein sequence shown below DNA sequence. Both intron Mdpi11 (C) and Mdpi12 (D) are spliced effectively in all three tested temperatures.

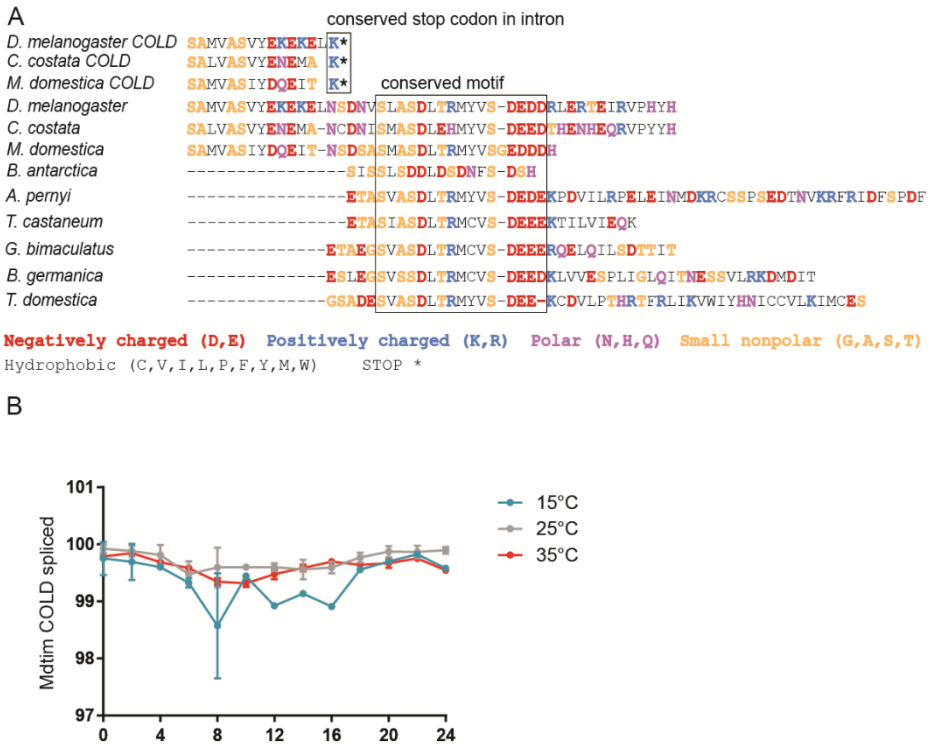

Fig. 8 (A) detail of C terminal region alignment of TIM proteins with highlighted conserved motif in all insect species and conserved stop codon resulting from alternative splicing in *D. melanogaster*, *C. costata* and *M. domestica*. (B) This last *tim* intron is spliced effectively in *M. domestica* in all three tested temperatures.

	per				*tim*				*vri*				*cwo*				*Clk*				*cry*			
	D.m.		M.d.		D.m.		M.d.		D.m.		M.d.		D.m.		M.d.		D.m.		M.d.		D.m.		M.d.	
	ups	ups+	ups	ups+	ups	ups+	ups	ups+	ups	ups+	ups	ups+	ups	ups+	ups	ups+	ups	ups+	ups	ups+	ups	ups+	ups	ups+
E-box: CACGTG	3.7	3.7	0.7	0.5	1.6	1.6	0.4	0.3	3.3	2.5	0.7	0.6	5	4.1	0.4	0.7	0	0.5	0	0	0.8	0.6	0.4	0.5
E-box partial: CACGTH	2.1	2.1	1.7	1.7	3	2.9	1.4	1.4	3.3	2.9	1.2	1	4.8	3.5	1.9	1.8	0.2	0.3	0.8	0.8	1.1	0.9	1.8	1.7
E-box: AACGTG	3.7	3.7	2	1.9	6.6	6.4	2.6	2.6	9	7.5	2.9	2.5	8.1	6.8	2.4	2.6	0.5	0.5	0.8	0.8	0.8	0.6	2	1.2
E-box: CACATG	0.5	0.5	2.4	2.2	3.3	3.2	1.3	1.3	3.3	2.5	2.2	2	2.5	2.7	1.1	2	1.1	0.9	1.2	1.2	2.4	1.9	2	1.2
D-box: TTATGYAA	8.5	8.4	3.5	2.8	26.2	25.4	1.9	1.9	13	10	2.9	2.2	5	2.4	2.3	2.9	26.4	22.7	6.6	6.5	13	10	3.3	4
RRE: WAWNTRGGTCA	17	17	0	0	0	0	15	15	0	0	0	0	0	9.5	4.5	2	0	0	13	13	0	0	0	0
SRP: GATAA	2	2	2.9	2.9	1.4	1.4	1.8	1.8	2.3	2.3	3	3.4	3.4	3.3	2.1	2.4	2.3	2.1	2.8	3	1.6	2.4	2.1	2.1
ME50: TRACRYGCA	0	0	3.5	2.8	0	0	0.9	0.9	0	0	0	2.2	10	7.1	1.1	1	0	3.8	0	0	0	0	3.3	4
ME134/odd: CAGnnGCA	8.5	9.4	1.5	1.4	3.3	3.2	0.7	0.7	1.6	2.5	0.7	0.8	5.6	3	1.6	1.3	1.1	0.9	0.4	0.4	12	9.7	2.4	1.7
Adf1: RCRGMRGSAGC	8.5	8.4	3.5	2.8	0	0	0	0	13	10	0	0	0	0	2.3	1	0	0	0	0	75.8	62.1	13	8
TATA-box: TATAAA	1.6	1.6	9.4	10	7.4	7.1	9.6	9.6	3.3	3.1	9.5	8.9	8.8	8	10	10	10	9	9.1	9	6.3	7.8	4.9	6
CRE: TGACGC	2.1	2.1	0	0.3	2.5	2.4	0.4	0.3	2.5	1.9	0.7	1.1	0.6	1.2	0.7	0.5	0.5	0.9	0	0	1.6	1.3	1.6	1.2
CRE-related: GTGACG	1.1	1	1.1	0.9	0.8	0.8	0.4	0.3	1.6	1.3	1.5	1.1	1.9	2.1	0.6	0.6	0.5	0.9	0.4	0.4	2.4	1.9	0.4	0.2

0 1 2 3 5 8 13 25 75

ups...upstream from ATG

ups+...upstream of ATG + 1th intron

Fig. 9 Relative abundance of *cis*-regulatory motifs in promoters of *D. melanogaster* and *M. domestica*. The values indicate actual occurrence of particular *cis* motif presented as a fold change when 1 is the expected frequency of particular motif in random DNA sequence. Heat map was used for easier orientation in the table. Two regions of gene were analysed: the region upstream of ATG (ups) and the upstream region with the first intron (ups+). Actual position and distribution of selected *cis* motifs on DNA is shown in Figs 10 and 11 and full maps are presented in Figs S3-6.

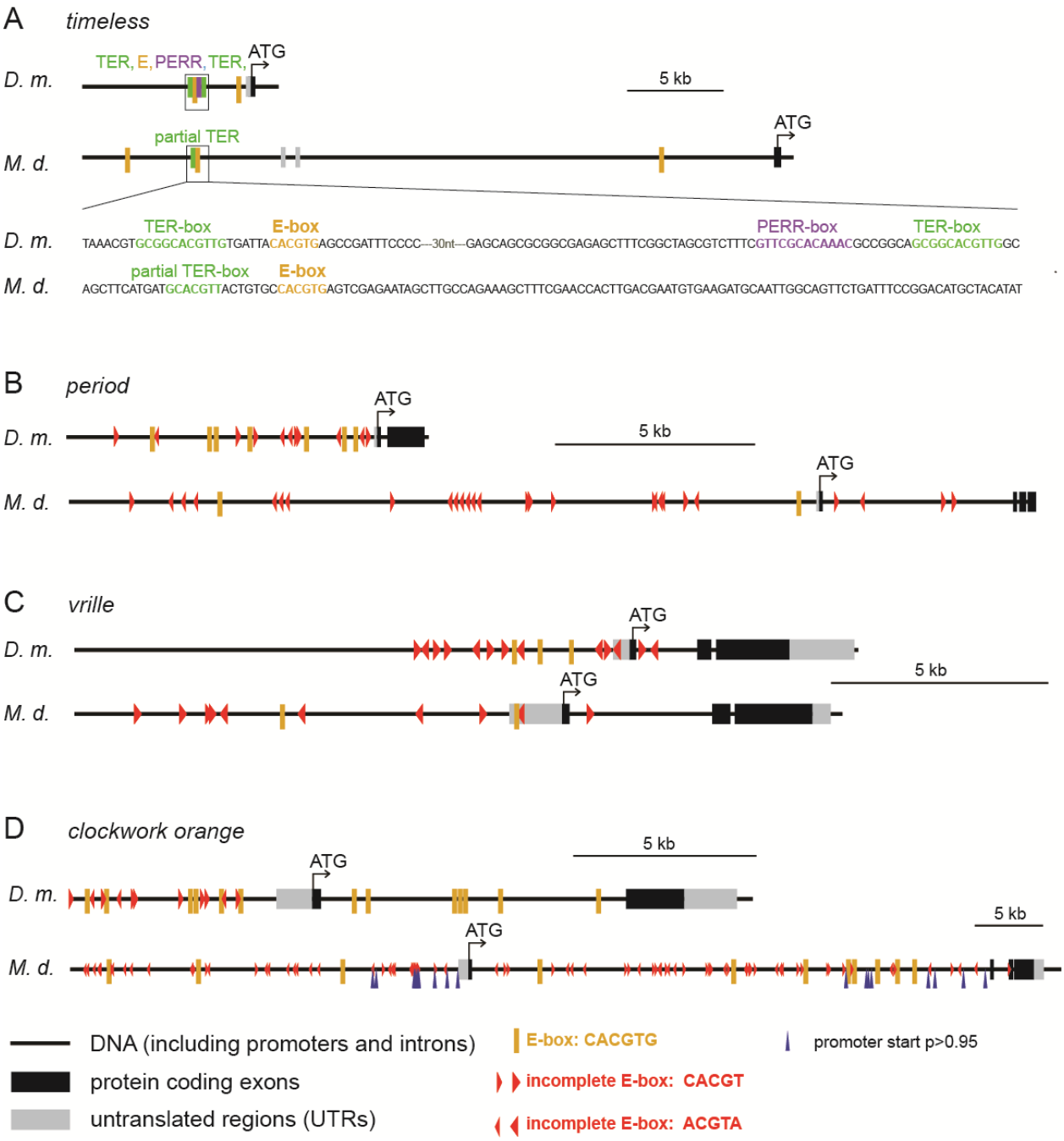

Fig. 10 Graphical comparison of upstream regions in circadian genes of *D. melanogaster* and *M. domestica*. Selected putative *cis*-regulatory motifs are shown.

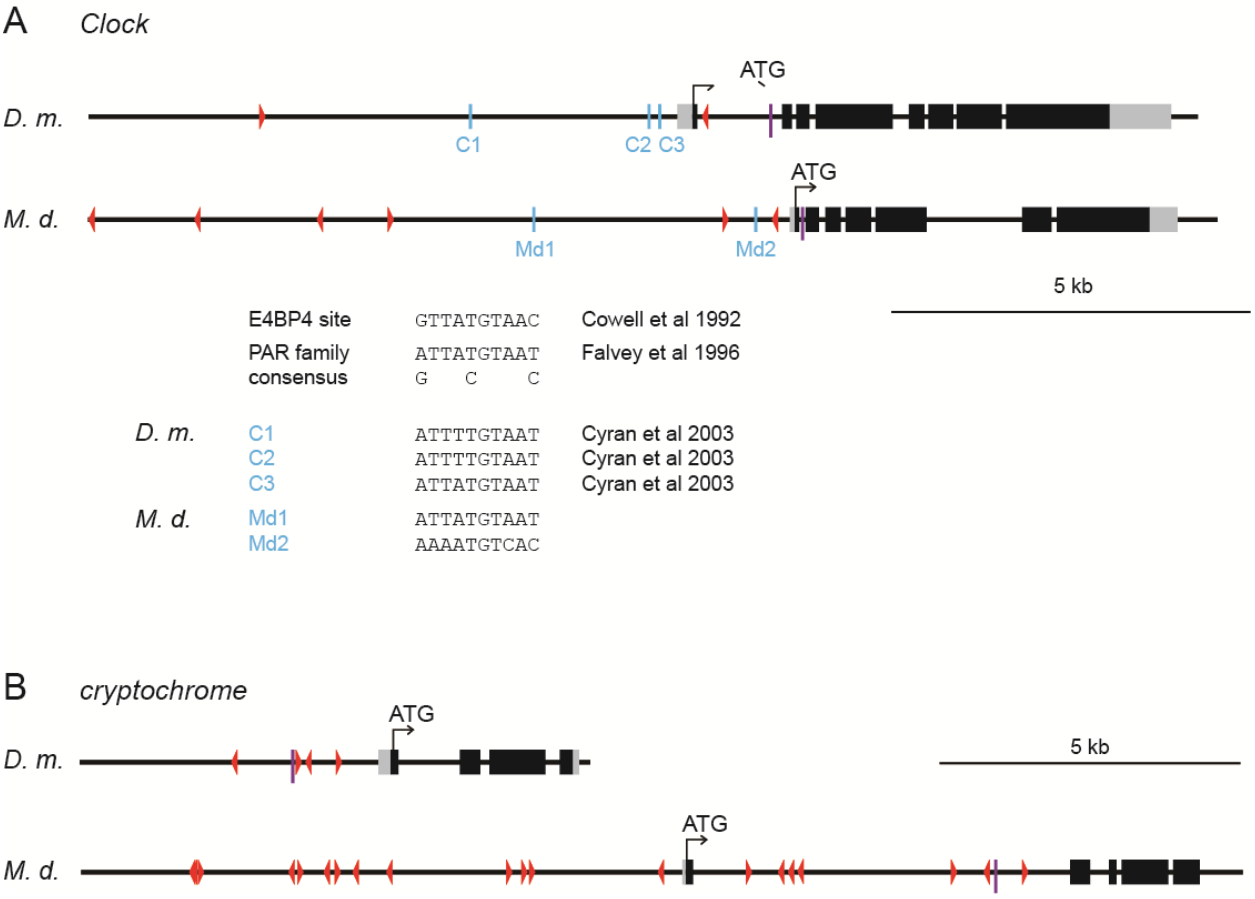

Fig. 11 Schematic depiction of putative cis-regulatory motifs in *Drosophila* and *Musca* promoters. Schematic depiction of *D. melanogaster* (*D.m.*) and *M. domestica* (*M.d.*) promoters with highlighted position of putative cis-regulatory motifs.

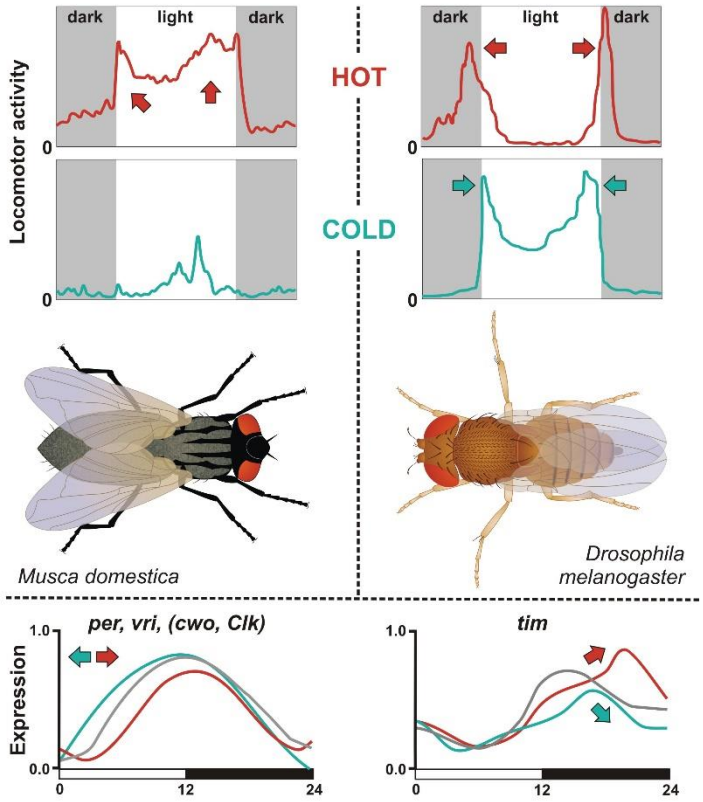

Fig. 12 Graphical summary of response to temperature in *M. domest*ica and *D. melanogaster*. Although the locomotor activity pattern is different, both species display bimodal activity at hot temperatures. Under cold temperatures, M. domestica reduces its activity to a single peak in the center of photophase, whereas D. melanogaster is still bimodal, however, position of both pekas is moved towards the photophase. Cold temperature results in phase advance of *per*, *vri*, *cwo* and *Clk* expression in both species. Both hot and cold temperature results in phase delay of *tim* expression, but the levels are higher under hot temperatures.

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Insect Biochemistry and Molecular Biology 70 (2016) 184–190

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Photoperiod regulates growth of male accessory glands through juvenile hormone signaling in the linden bug, *Pyrrhocoris apterus*

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ARTICLE INFO

Article history:
Received 30 October 2015
Received in revised form
22 January 2016
Accepted 25 January 2016
Available online 28 January 2016

Keywords:
Diapause
Methoprene-tolerant
bHLH-PAS
Circadian genes
Locomotor activity
Mating behavior

ABSTRACT

Adult reproductive diapause is characterized by lower behavioral activity, ceased reproduction and absence of juvenile hormone (JH). The role of JH receptor Methoprene-tolerant (Met) in female reproduction is well established; however, its function in male reproductive development and behavior is unclear. In the bean bug, *Riptortus pedestris*, circadian genes are essential for mediating photoperiodically-dependent growth of the male accessory glands (MAGs). The present study explores the role of circadian genes and JH receptor in male diapause in the linden bug, *Pyrrhocoris apterus*. These data indicate that circadian factors Clock, Cycle and Cry2 are responsible for photoperiod measurement, whereas Met and its partner protein Taiman participate in JH reception. Surprisingly, knockdown of the JH receptor neither lowered locomotor activity nor reduced mating behavior of males. These data suggest existence of a parallel, JH-independent or JH-upstream photoperiodic regulation of reproductive behavior.

1. Introduction

To cope with adverse seasonal conditions, organisms in temperate regions enter diapause, a state characterized by minimized metabolism and reduced energy-demanding processes (Hahn and Denlinger, 2011). Various insects enter diapause at different, species-specific developmental stages (Dolezel, 2015; Meuti and Denlinger, 2013). Diapause in adult stage is characterized by a stopped reproduction and changes in the physiology (Hahn and Denlinger, 2011; Kostal, 2006; Kostal et al., 2008). Some insects reduce locomotor activity (Hodkova et al., 2003; Socha and Zemek, 2000) while others, such as the monarch butterfly, undergo a long migratory flight (Zhan et al., 2014).

A hallmark of adult reproductive diapause is the absence of juvenile hormone (JH), a sesquiterpenoid molecule that controls reproduction (Raikhel et al., 2005), and was first identified for its anti-metamorphic role (Jindra et al., 2013). The connection between JH and reproductive diapause is well documented in various species (Denlinger et al., 2012). Application of the JH-mimicking analogue methoprene to diapausing females of *Pyrrhocoris apterus* or *Riptortus pedestris* bugs is sufficient to terminate diapause and induce ovarian growth (Ikeno et al., 2010; Smykal et al., 2014). Topical application of JH-III to *Plautia stahli* bugs induced ovarian development in females and male accessory gland (MAG) growth in males (Kotaki and Yagi, 1989). Endogenous JH or added methoprene act through the Methoprene-tolerant (Met) protein to mediate reproduction of various insect species including *P. apterus* (Smykal et al., 2014), cockroach (Marchal et al., 2014), locust (Guo et al., 2014) and mosquito (Li et al., 2011).

Met is a transcription factor of the basic helix–loop–helix Per-ARNT-Sim (bHLH-PAS) family (Ashok et al., 1998), and it has been characterized as a JH receptor (Charles et al., 2011; Jindra et al., 2015; Miura et al., 2005). JH-dependent interaction between Met and another bHLH-PAS protein Taiman (Tai; also known as FISC or SRC), have been implicated in oogenesis of *Aedes aegypti* mosquitoes (Li et al., 2011). *A. aegypti* Met and the bHLH-PAS circadian clock protein Cycle (Cyc) have been shown to dimerize and activate circadian rhythm-dependent gene expression in response to JH (Shin et al., 2012). Genetic interaction between Cyc, Met and another circadian bHLH-PAS protein, Clock (Clk), was suggested for gut autonomous JH-dependent response in *P. apterus* (Bajgar et al., 2013b). This loop further involves negative feedback between two circadian factors, Cryptochrome 2 (Cry2) and Par domain protein1

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http://dx.doi.org/10.1016/j.ibmb.2016.01.003
0965-1748/

(Pdp1). However, expression of these two genes does not show any daily changes in abundance suggesting their non-circadian role (Bajgar et al., 2013a, 2013b). Notably, in the fat body of *P. apterus* females, Met function requires Tai for Vg expression independently of either Clk or Cyc (Smykal et al., 2014).

Series of studies from Ikeno et al. explored the role of several circadian genes in regulating diapause in *R. pedestris*. Reproductive development requires long days and presence of *Clk* and *cyc*. If one of these transcription factors is depleted by RNA interference, *R. pedestris* bugs enter diapause mode. In contrary, RNAi knockdown of *per* or *cry* reverse diapause to reproductive mode in otherwise diapause-inducing short photoperiods (Ikeno et al., 2010, 2011a b, 2013).

In addition to season, insect reproduction is often taking place during a defined time of day; clock disruption strongly affects mating success in *Drosophila melanogaster* (Beaver et al., 2002; Sakai and Ishida, 2001), locust (Tobback et al., 2011) and several lepidopteran species (Giebultowicz et al., 1989; Kotwica et al., 2009).

Given the reported tissue-specific plasticity in JH reception, the crosstalk of JH signaling with photoperiodism and the circadian nature of reproduction in some insects, we aimed to explore which factors regulate reproductive/diapause physiology in *P. apterus* males.

2. Methods and materials

2.1. Insect rearing

P. apterus bugs (short-winged form) were maintained at 25 °C on dry linden seeds and were supplemented with water. The cultures were kept at either of two photoperiod regimes: long-day (LD; 18 h light, 6 h dark) that permits reproduction, or short-day (SD; 12 h light, 12 h dark) that induces adult reproductive diapause. Adult males of specific age after adult ecdysis (AAE) were selected for experiments.

2.2. MAG measurement

The male accessory glands (MAGs) were dissected in Ringer's solution. Each gland was photographed under a stereomicroscope (magnification 20× and 63×) from ventral and dorsal side. The area of MAG was measured in ImageJ (Schindelin et al., 2012) and the size of left and right MAG was averaged. For clear presentation, the size of MAG was plotted as a fold change referring to the size of MAG in 1 week AAE diapause males (size 1).

2.3. cDNA cloning, RNA interference (RNAi) and methoprene treatment

Cloning of *P. apterus* cDNAs encoding Met (Konopova et al., 2011), Tai, Cyc, Clk and Cry2 (Bajgar et al., 2013b) was described in the cited references. Double-stranded RNA (dsRNA) was prepared using the T3 and T7 RNA polymerases with the MEGAscript kit (Ambion) from plasmids containing the appropriate gene fragments and injected into *P. apterus* adult males as described previously (Bajgar et al., 2013b; Smykal et al., 2014). Adult diapause males (five days AAE, SD photoperiod) received 3 μl of dsRNA at a concentration of 2–4 μg/μl in Ringer's solution; control animals were injected with heterologous dsRNAs derived from bacterial *β-galactosidase* (lacZ) or with the Ringer's solution alone.

For JH mimic treatments, diapausing SD adult males were anesthetized under CO_2 and treated topically on the dorsal surface with 4 μl of 0.3 mM methoprene (VUOS Pardubice, Czech Republic) dissolved in acetone; controls were treated with acetone only. When RNAi and JH mimic treatments were to be combined, methoprene application followed dsRNA injection by four days. Males were then kept in SD conditions for an additional 10 days and then sacrificed and subjected to MAG measurement.

In the photoperiod transfer experiment, diapausing SD males 5 days AAE were injected with dsRNA as described above and after 48 h of rest in SD conditions, males were shift to LD conditions. The male accessory gland was dissected and measured 21 days after the transfer.

2.4. Statistical analysis

The differences between MAG size were tested for statistical significance using Graphpad6 (Prism) and Statistica 12 (StatSoft, 2013) software. Student's *t*-test was used for all pairwise comparisons (Graphpad6). For comparisons of multiple samples, General linear models (GLM) in Statistica 12 were applied.

2.5. mRNA quantification

Analyzed tissues (MAG, fat body) were dissected in RNAse-free Ringer's solution. Total RNA was isolated with Trizol reagent (Invitrogen). After Turbo DNAse (Ambion) treatment, 1 μg of total RNA was used for cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen). Relative transcript levels were measured

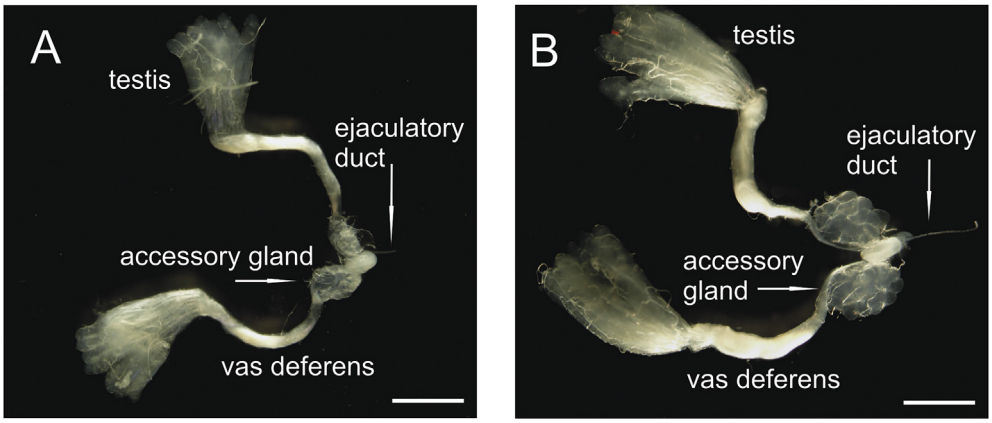

Fig. 1. The internal reproductive organs of *P. apterus* males. Short photoperiod (12:12) induces the diapause state for which small male accessory glands are typical (A), while long days (18:6) promote development resulting in larger accessory glands (B). In both examples adult males 14 days after adult ecdysis grown at a constant temperature of 25 °C are shown. Scale bar: 1 mm.

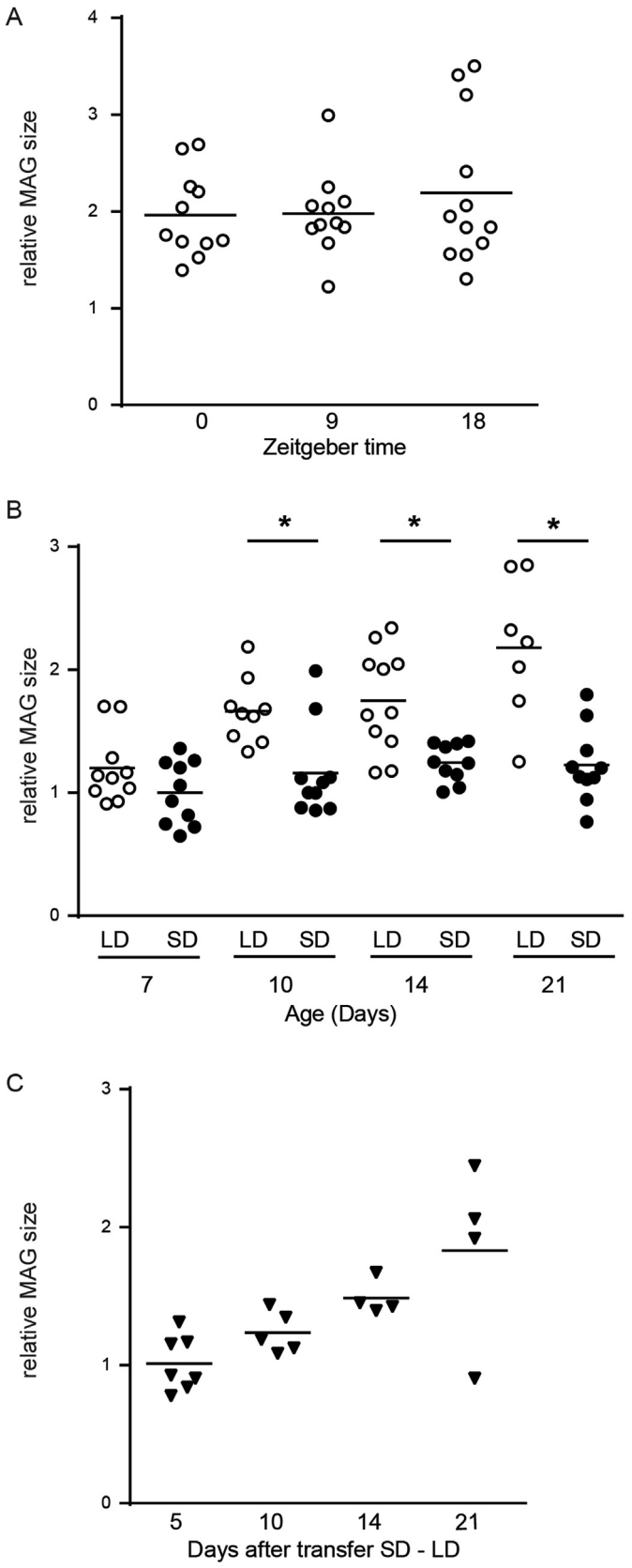

Fig. 2. Size of male accessory glands (MAG) in *Pyrrhocoris apterus*. (A) Relative MAG size (shown as a fold change compared to diapause MAG) does not significantly vary during three time points in 14 days after adult ecdysis (AAE) reproductive males. (B) Diapausing males (full circles) retain MAGs of comparable size irrespective of age (data from 7, 10, 14 and 21 days AAE are shown), while MAGs of reproductive males (open circles) gradually grow being significantly larger from day 10 AAE. (C) MAGs of males transferred from diapause-inducing condition to long day, gradually grow being significantly larger (*) then diapause MAGs 14 and 21 days after transfer.

by quantitative PCR using qPCR 2× SYBR Master Mix (Top Bio) and a C1000 Thermal Cycler (both Bio-Rad). All measured transcripts were normalized to relative levels of ribosomal protein (*rp49*) mRNA as described previously (Dolezel et al., 2007). Sequences of primers were published previously (Smykal et al., 2014; Bajgar et al., 2013a).

2.6. Locomotor activity measurement and analysis

Adult males were accommodated in a glass test tube (diameter 25 mm, length 150 mm) equipped with a water reservoir on one side and a peeled linden seed (*Tilia cordata*) wrapped in textile mesh attached to the other side. Tubes were placed in LAM25 monitors (Trikinetics, Waltham, MA, USA) horizontally with infrared beams crossing the tube in the middle. Monitors were placed in an incubator (MIR 253, Sanyo) with 25 °C and either an SD or LD regime (approximately 500 lux) and locomotor activity was recorded in 5 min bins.

To compare activity under LD and SD conditions, respectively, locomotion was recorded in 5 min bins for at least 15 days. Days 13–15 served as confirmation of a particular bug's survival; only bugs surviving to day 15 were used for further analysis. The locomotor activity of each bug was determined for day 3–12 using ActogramJ software (Schmid et al., 2011). Data from 10 days of at least 10 bugs (≥100 days in total) per treatment were used in the statistical analysis (Graphpad6, Prism).

To assay the ability to adjust to a new regime, bugs were reared in SD from early development; three day AAE males were injected with dsRNA and transferred to LD for locomotor activity measurements. Activities of control, ds *Met* and ds *Clk* animals were compared for each day separately in the statistical analysis (Graphpad6, Prism).

2.7. Mating activity

dsRNA was injected to 3 days AAE males which were kept individually in Petri dishes (diameter 70 mm) with access to water and food. Once per two days, males were placed together with two receptive virgin females (5–10 days AAE, from LD) for 30 min. Males that did not mate during 30 min (reproductive male starts copulation within 3 min, data not shown) were return to Petri dish and the test was repeated two days later. In transfer experiment, dsRNA was injected to 3 days AAE males reared in SD and bugs were transferred to LD. Offspring of mated females was kept for 5 days to test its viability.

3. Results

3.1. MAGs differ between reproductive and diapause males in age-dependent manner

First, we tested conditions which could influence MAG size, particularly time of day, age after adult ecdysis (AAE) and diapause/reproductive status. Comparison of three time-points (0, 9, and 18 h after light on) in reproductive males two weeks AAE, kept in LD regime suggests that MAG size does not fluctuate during the day (Figs. 1 and 2A). Therefore, further measurements were performed on animals sacrificed only at one time-point (0–1 h after lights on). Measurements of males 7, 10, 14 and 21 days AAE indicate that MAGs retain a constant size during diapause (Fig. 2B; SD animals).

In contrast MAG, size gradually increased with age in reproductive males kept in LD, with a significant difference from day 10 AAE and later (Figs. 1 and 2B). Consistent with these findings, MAGs grew after transfer of diapause males from SD to LD, a condition inducing reproduction (Fig. 2C).

3.2. Methoprene induces MAG growth through Met and Tai

To investigate the impact of JH on MAG size we applied the synthetic JH mimic methoprene to diapausing SD males. Application of acetone only (vehicle) had no influence on MAG size, while addition of methoprene resulted in significant enlargement of MAGs 10 days after application (controls in Fig. 3A–F).

To identify the JH receptor responsible for MAG growth, we have functionally evaluated the role of putative JH receptors *Met* and *tai*, circadian clock genes *cyc, Clk, cry2* and *tgo*, which is homologous to the aryl hydrocarbon receptor nuclear translocator and served as a control unrelated to insect circadian clock or JH signaling.

Depletion of either *Met* or *tai* eliminated the impact of JH mimics on MAGs, which retained diapause-like size (Fig. 3A and B). Removing either *Clk*, *cyc* or *cry2* did not prevent MAG progression after methoprene administration, as their size was comparable to control groups (Fig. 3D–F). In the case of *tgo*, a statistically significant difference between control and JH mimic treated males was only observed in ANOVA test (Table S1).

qRT PCR confirmed that expression of *Met* and *tai* was higher in MAG than in fat body of 10 days AAE reproductive males, while Clk was expressed in MAG in lower amounts (Fig. 4).

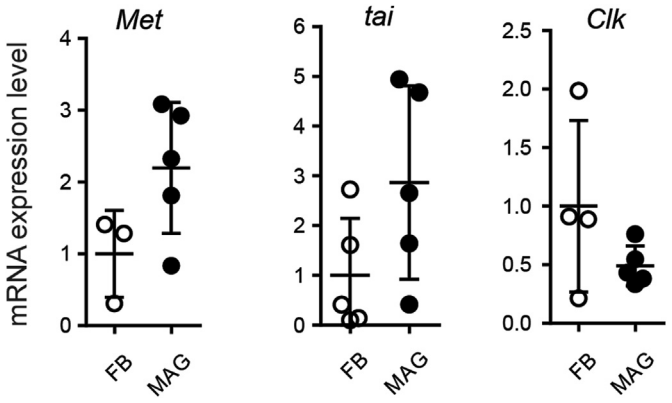

Fig. 4. Met, tai and Clk are expressed in the MAG. The relative gene expression is compared to levels in fat bodies of the same males (arbitrarily set to 1). All data were normalized using expression of rp49 mRNA.

3.3. Photoperiodic regulation of MAG size requires several circadian gene homologs

To reveal genes involved in photoperiodic regulation of MAG, we transferred diapause males to reproduction-inducing LD conditions. While MAGs were significantly larger 21 days after transfer in control males [injected with *β-galactosidase* (*lacZ*) dsRNA, Fig. 5], no growth was observed after depleting JH receptor *Met* and circadian gene *Clk*. Knockdown of either *tai* or circadian factor *cry2* significantly reduced growth of MAGs after transfer to LD (Fig. 5).

3.4. Neither Met knockdown, nor JH mimic administration influence locomotor activity

We have explored the overall locomotor activity as a parallel readout of diapause/reproductive status. It was reported previously, that reproductive *P. apterus* females show higher activity than diapause ones (Hodkova et al., 2003). Our data confirmed the same

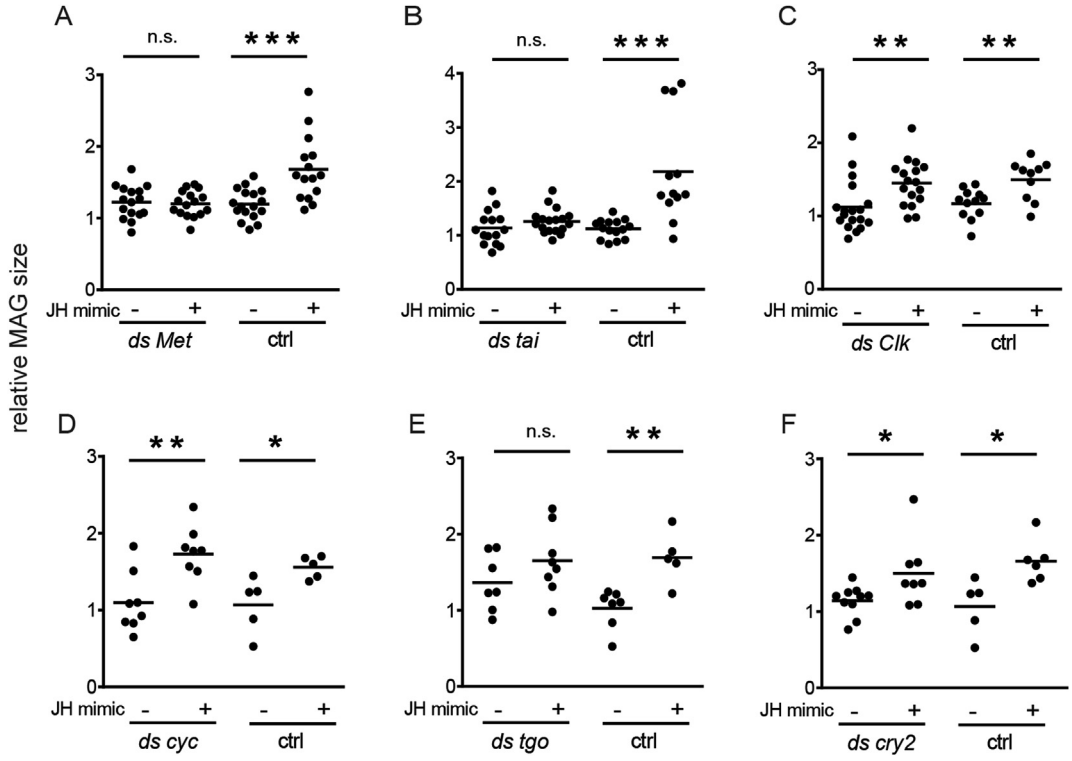

Fig. 3. JH mimic induces MAG grow through Met and Tai in short days. Diapausing males under short-day conditions (SD) were injected with dsRNAs targeting the indicated genes, and treated with methoprene (+) or acetone only (−) four days later. After 10 days, males were sacrificed and MAG size was measured. Asterisks indicate statistical significance of differences against the control values as determined in Student's t-test: *, $P < 0.05$.

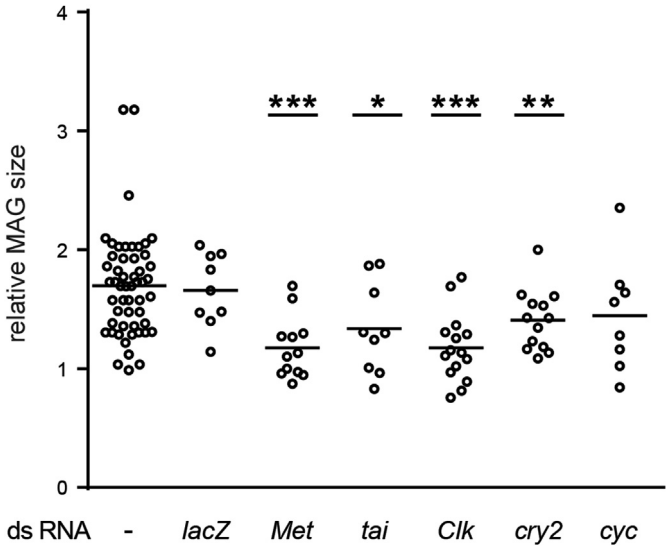

Fig. 5. MAG size 3 weeks after transfer from SD to LD. Data from individual males represented as dots, the horizontal bar corresponds to mean size. Control males (intact or injected with lacZ dsRNA) have MAGs larger than corresponding males in SD (size 1 on y-axis). Knocking down Met or Clk had retained small MAGs. Lesser, yet significant impact on growth was observed after tai and cry2 RNAi, while the smaller size of MAG after cyc RNAi was not significantly different from controls. T-test, two-tailed. ***-$p < 0.0001$, **- $p < 0.004$, *- $p < 0.026$.

trend in males (Fig. 6A) when control males in LD showed significantly higher activity (approximately 5-fold) than diapause males in SD. Interestingly, addition of the JH mimic methoprene did not change activity in diapause males and consistent with this finding, depletion of *Met* in LD males did not reduce locomotor activity (Fig. 6A).

3.5. Photoperiod-dependent locomotor activity change requires Clk

Since *Clk* knockdown prevented MAG growth after transfer from SD to LD (Fig. 5), we have explored role of *Clk* in regulation of locomotor activity. Indeed, activity of control bugs slightly increased after transfer from SD to LD, whereas activity of ds *Clk* males slightly declined after the transfer. Controls where significantly different from ds *Clk* on days 17 and 18 (Fig. 6B and Table S2). Surprisingly, activity of ds *Met* males increased even more, although difference was significant between ds Met and controls only on day 15 after transfer (Fig. 6B and Table S2).

3.6. Mating behavior does not require either Met or Clk

Since *Met* knockdown did not reduce locomotion, we were wondering whether *Met* influences mating behavior. While diapause males did not mate at all (data not shown), a proportion of control reproductive males were mating 6 days AAE and all males 13 days AAE were mating (Fig. 6C). Neither *Clk* nor *Met* knockdown reduced mating ability of LD males. In a second experiment, diapause males from SD (control, ds *Met* or ds *Clk*) were transferred to LD and mating behavior was tested every other day. First control males were mating 7 days after the transfer and 100% mating was observed on day 13 (Fig. 6D). Remarkably, *Clk* knockdown males did not mate at all even 17 days after the transfer. In contrast, *Met*

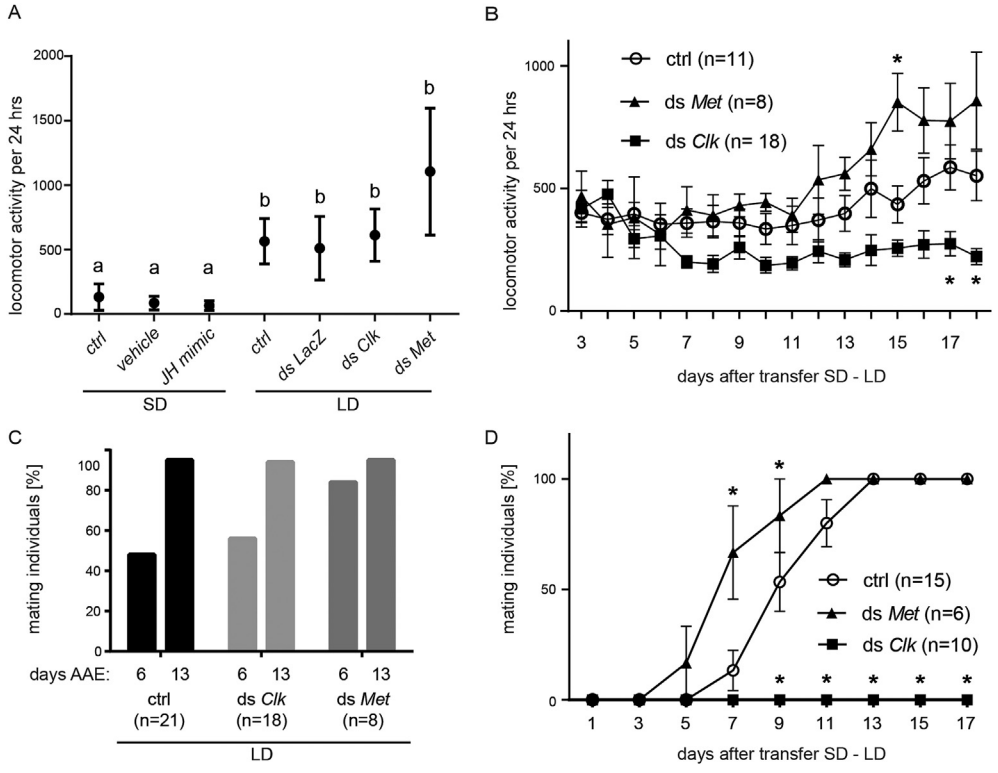

Fig. 6. Locomotor activity and mating behavior of males. (A) Average locomotor activity per individual male per 24 h recorded under short (SD) and long (LD) photoperiods. 15–20 individual males per treatment were individually recorded over a period of 10 days. The average numbers of beam crosses per 24 h are plotted with SEM on y-axis. Males exposed to SD show a significantly lower activity compared to LD males regardless of JH mimic administration or Met RNAi (see "a" and "b" identifying two statistically distinct groups, One way ANOVA – multiple comparison). (B) Locomotor activity in LD after transfer of diapause males from SD. Average locomotor activity per individual male per 24 h is plotted for control, ds Met and ds Clk males, respectively. (C) Mating behavior in LD is not affected by Clk or Met, respectively. (D) Clk knockdown males fail to switch from reproductive diapause to mating behavior after transfer to LD. * indicates significant difference from control (2way ANOVA; see Table S2 and S3 for statistical analysis).

knockdown males were mating even slightly earlier after the transfer when compared to controls. Notably, fertility of both *Clk* and *Met* knockdowns from LD was comparable to control and larvae seemed to have normal survival rate (data not shown).

4. Discussion

This study explored factors regulating male reproductive diapause in adult *P. apterus*. While MAGs of diapause individuals remain small irrespective of age, MAGs of reproductive males gradually grow and their size in the presence of JH is positively correlated with the successful reproductive activity in the males (Socha, 2006; Socha and Hodkova, 2006). Similarly, the short day- and/or JH-dependent growth was documented also in accessory sexual glands of *P. apterus* females (Jedlicka et al., 2009). The suppressive role of short photoperiod is overridden by the JH mimicking compound methoprene, which requires *Met* and its partner *tai*, further supporting JH as the factor regulating growth of MAG. Hence, the regulation of MAG is consistent with female reproduction, which also relies on JH through *Met* and *tai* in several insects (Guo et al., 2014; Marchal et al., 2014) including *P. apterus* (Smykal et al., 2014). RNAi knockdowns then suggest that JH acts through canonical Met/Tai reception, although nothing is known about downstream factors. Consistently, Met depletion in SD diapause males prevented MAG growth even after transfer of these males to reproduction-promoting conditions (see Fig. 7 for a summarizing model).

The actual mechanism how Met and Tai influence MAG's growths was not addressed in this study. Since previous work identified these two factors essential for *vitellogenin* expression in the female fat body (Smykal et al., 2014) and our data indicate that expression of *Met* and *tai* in MAG is even higher than in the fat body (Fig. 4), a local role of these two factors in this gland is possible. The variability of *Met*, *tai* and *Clk* levels in the fat body (Fig. 4) is consistent with expression of circadian genes *period* and *Pdp1* in this tissue, as was reported previously (Dolezel et al., 2007, 2008).

Ikeno et al. identified two circadian genes, *period* and *cycle*, as the essential components regulating development of *R. pedestris* inner male reproductive organs (Ikeno et al., 2011a). The work we present confirms the role of additional circadian genes in mediating photoperiodic information in another hemipteran species, *P. apterus*. Since circadian factors Clk, Cyc, and Cry2 are not involved in MAG growth upon JH mimic administration to males in SD, we suggest, as the most plausible explanation, that these circadian homologs are involved in the regulation of JH hormone synthesis or activity of the corpus allatum (Fig. 7).

These data, however, do not explain the relationship of circadian and photoperiodic clocks, respectively. While the first regulate daily activity, even in constant darkness, the latter are essential for measuring the ration of day-to-night (the photoperiod). The presented data are therefore an addition to the already long list of examples, where different circadian genes were connected to diapause in various insects, including flies (Goto et al., 2006; Kobelkova et al., 2010; Stehlik et al., 2008; Yamada and Yamamoto, 2011), mosquitoes (Meuti et al., 2015) and Heteroptera (Ikeno et al., 2013, 2011a, 2011b, 2010).

Whether this involvement includes all clock components or if only some of the circadian genes were recruited independent of their circadian function (gene pleiotropy, for more detailed discussion see Bradshaw and Holzapfel, 2010; Goto, 2013; Kostal, 2011), requires further studies. An important aspect is the anatomical localization of both circadian and photoperiodic clocks. To shed at least some light suggesting future research direction, we tested whether JH and its reception contributes to another photoperiodic phenotype – the overall locomotor activity and the mating

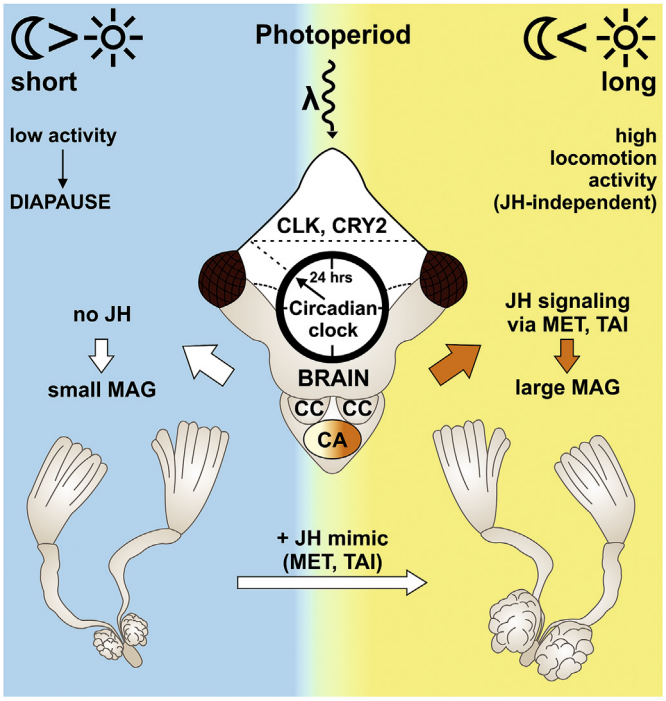

Fig. 7. Model summarizing current data on MAG regulation in *P. apterus*. Under short days (left, blue background), males enter diapause characterized by JH absence resulting in small MAGs. Administration of JH mimic induces growth of MAGs to sizes observed under a long photoperiod (right, yellow background). In both situations, the growth requires expression of Met and Tai. When males are transferred from short to long days, expression of circadian genes Clk and Cry2 is needed to initiate MAG growth. The locomotor activity differs between males in LD and SD conditions and is not influenced by either JH mimic administration or Met knockdown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

behavior. Our data indicate high activity in reproductive males and low activity of diapause males to be independent on JH presence or absence, respectively (Fig. 6A). Activity increase, although mild, is observed when diapause males were transferred to LD. Importantly, activity of *Clk* knockdown males remained low after the transfer (Fig. 6B). Similarly, *Clk* RNAi did not affect reproduction of LD males, but destroyed ability of diapause male to switch to reproductive mode after the transfer to LD regime (Fig. 6C and D). These data further support role of *Clk* in photoperiodic time measurement. Future studies should therefore aim at identifying factors regulating both JH-dependent and JH-independent diapause phenotypes. This factor then will contribute in localizing the photoperiodic clock anatomically.

Surprisingly, knocking down *Met* did not reduce mating behavior. One possibility is that *Met* was knocked down insufficiently, allowing for residual protein expression. However, given the rise in overall locomotor activity and earlier mating behavior in *Met* knockdowns, we favor explanation that Met is not necessary for male reproduction. Noteworthy, *Met* RNAi males were fertile producing comparable number of offspring. Microsurgery ablation of *corpus allatum*, JH-producing organ, might further address role of JH in male's reproduction and behavior.

Author contribution

V.U.: designed and performed experiments, analyzed data.

O.B.: performed locomotor activity and mating experiments, qRT PCR, analyzed data.

H.V.: performed experiments.

D.D.: designed experiments, wrote the manuscript.

Acknowledgments

We thank Martina Hajduskova (www.biographix.cz) for Fig. 7 and graphical abstract, Dr. Dostalkova and Dr. Eva Holá for advice on statistical analysis, Joanna Kotwica-Rolinska for advices on mating behavior experiments and critical reading of the manuscript, and Pavel Jedlicka for critical reading of the manuscript. We also appreciate advice from two anonymous reviewers. This work was supported by LH14029 (MSMT) and 14-32654J (CSF). We acknowledge the use of research equipment funded by the European Union program FP7/2007–2013, grant No. 316304. V.U. was supported from Postdok_BIOGLOBE (CZ.1.07/2.3.00/30.0032).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2016.01.003.

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Cryptochrome 2 mediates directional magnetoreception in cockroaches

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Edited by David L. Denlinger, Ohio State University, Columbus, OH, and approved December 18, 2015 (received for review September 19, 2015)

The ability to perceive geomagnetic fields (GMFs) represents a fascinating biological phenomenon. Studies on transgenic flies have provided evidence that photosensitive Cryptochromes (Cry) are involved in the response to magnetic fields (MFs). However, none of the studies tackled the problem of whether the Cry-dependent magnetosensitivity is coupled to the sole MF presence or to the direction of MF vector. In this study, we used gene silencing and a directional MF to show that mammalian-like Cry2 is necessary for a genuine directional response to periodic rotations of the GMF vector in two insect species. Longer wavelengths of light required higher photon fluxes for a detectable behavioral response, and a sharp detection border was present in the cyan/green spectral region. Both observations are consistent with involvement of the FADox, $FAD^{\bullet-}$ and $FADH^{-}$ redox forms of flavin. The response was lost upon covering the eyes, demonstrating that the signal is perceived in the eye region. Immunohistochemical staining detected Cry2 in the hemispherical layer of laminal glia cells underneath the retina. Together, these findings identified the eye-localized Cry2 as an indispensable component and a likely photoreceptor of the directional GMF response. Our study is thus a clear step forward in deciphering the in vivo effects of GMF and supports the interaction of underlying mechanism with the visual system.

magnetoreception | cryptochrome | light spectrum | locomotor activity | circadian genes

Behavioral evidence for sensitivity to geomagnetic fields (GMFs) has been found in numerous vertebrate and invertebrate taxa (1); however, the underlying mechanisms remain a biological and biophysical enigma. In the late 1970s, the effect of light on the orientation of birds inspired Schulten and colleagues (2) to suggest that reactions of radical pairs (RPs) formed by photosensitive biological processes may be susceptive to external magnetic fields (MFs), and thus provide the basis for in vivo chemical magnetoreception. Since then, ample studies have supported this hypothesis (reviewed, e.g., in refs. 3 and 4).

In the past decade, proteins from the Cryptochrome/Photolyase family (CPF) have been widely discussed as being relevant to the light-dependent biological compass relying on the RP mechanism (5–7). Plant Crys mediate sensitivity to blue/UVA light (8), and this sensitivity was reported to be influenced by a MF (9), although later verification failed (10). Crys are essential for circadian clock function in mammals, but are likely not directly involved in light reception (11). In the fruit fly, *Drosophila melanogaster*, Cry mediates the light entrainment of the circadian clock (12). Both fly circadian rhythmicity and geotaxis turned out to be Cry-dependent and were also affected by a MF (13–15). Curiously, some insect species have only a *Drosophila*-type of Cry (Cry1 or animal type I Cry), whereas others have a mammalian-type of Cry (Cry2 or animal type II Cry) or both (16).

The validity of the RP mechanism was proven in the carotenoid–porphyrin–fullerene triad (17). In CPF proteins, the change in redox state of their flavin adenine dinucleotide (FAD) cofactor can result in magnetosensitive RPs (18). Although the RPs studied in two CPF proteins were magnetosensitive (19, 20), RP-based GMF effects and anisotropic MF effects have not been shown in CPF proteins. In contrast, ultrafast GMF effects on transient FAD fluorescence in an apparently purified Cry from birds was reported in a recent study (21), suggesting the existence of a GMF-sensitive reaction that differs from spin-selective RP recombination.

The biological output of the RP–GMF interaction might hypothetically be generated when a particular redox status of a FAD cofactor is reached, changing the configuration of the Cry protein (22) or its C terminus, which switches the Cry to a signaling state (23). Concerning possible downstream effects, Cry activation was shown to control permeability of potassium channels in *Drosophila* (24).

In terms of Cry-mediated in vivo chemical magnetoreception in general, an organism's sensitivity to the presence of GMF should be considered separate from its sensitivity to the GMF's orientation (25). Although the sole detection of the presence or intensity of a GMF can be accomplished in vitro via a disordered RP system (17), a number of additional critical requirements should be met to function as a sensor of magnetic direction, from the anisotropy of electron–nucleus interactions to the anatomy of a sensory organ (see *Discussion*).

Significance

The photosensitive protein Cryptochrome (Cry) is involved in the detection of magnetic fields (MFs) in *Drosophila*. However, Cry-dependent responses to natural MF intensities and to the direction of the MF vector have not been demonstrated previously in any insect. Birds, monarch butterflies, and many other species perceive the direction of geomagnetic field (GMF) lines, but the involvement of Cry has not been rigorously proven using genetic tools. In this study, by combining behavioral and genetic approaches, we provide the first unambiguous evidence to our knowledge of a Cry-dependent sensitivity to the direction of GMF in two cockroach species. Furthermore, by eye-covering experiments and by immunolocalization of a crucial mammalian-type Cry2 under the retina, we clearly show that the eye is an indispensable organ for the directional GMF response.

Author contributions: I.S., D.D., and M.V. designed research; M.K., O.B., T.V., P.S., P. Bartos, R.N., K.T., P. Braeunig, M.D., J.P., and D.D. performed research; H.-J.L. contributed new reagents/analytic tools; O.B., J.P., D.D., and M.V. analyzed data; and I.S., R.P., D.D., and M.V. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1518622113/-/DCSupplemental.

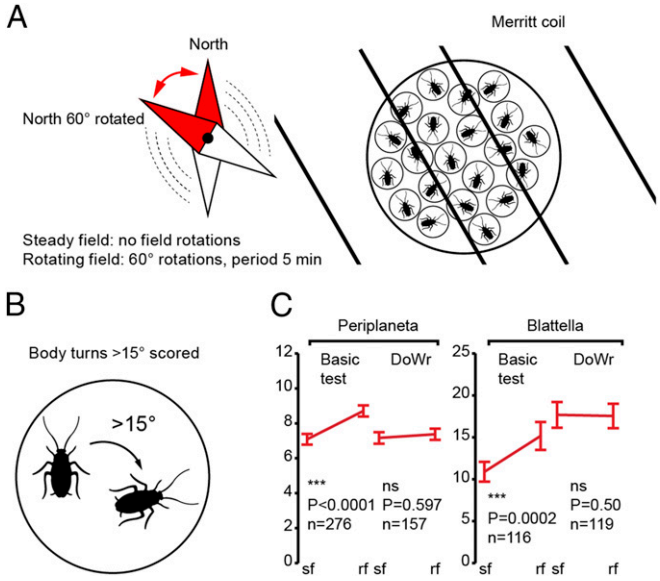

Fig. 1. MIR. (*A*) Schematic illustration of the magnetoreception assay: When the geomagnetic horizontal vector is rotated back and forth 60° periodically, the cockroaches change their resting positions more frequently compared with during steady MF periods. Magnetoreception assay setup: Cockroaches were placed individually into Petri dishes with opaque walls (small circles) accommodated in arena (large circle). On the next day, MF was changing its direction every 5 min during 135-min intervals. The four black lines depict the position of the Merritt coil frames. (*B*) Body turn was scored if body rotation exceeded 15°. (*C*) MIR essay is selective to magnetic direction: The activity was scored as a number of body turns per 135-min interval during control (steady field, sf) and treatment (rotating field, rf) periods. Red line depicts average paired levels of all individual activities (±SEM) between control and treatment periods. In the Basic test setup, significant elevation of activity (Wilcoxon Match Pair Test) was found. Nonspecific effects of electric feeding of coils were eliminated using a double-wrapped design (DoWr) that allowed us to feed the coils without producing any external MF. The red lines indicate the mean values of all animals between control and treatment; intra- and intersample variations are irrespective of pair test significance. n, number of animals.

The most convincing evidence of Cry-dependent magnetosensitivity was provided on *Drosophila* (26, 27). The ability to recognize the presence of a MF relies on functional Cry1, and this magnetoreception in Cry-deficient fruit fly mutants could be rescued using mammalian-like Cry2 (28). The flies were trained to recognize the local magnetic anomaly up to 10 times stronger than the natural GMF in T-shape maze experiments. Although the choice of one of two arms involved orientation, the actual physiological effect was consistent with nondirectional magnetic sensitivity (29), as was discussed for plants (9, 10), fruit fly geotaxis (14), and the fruit fly circadian clock (13, 15). Therefore, rather than demonstrating a genuine directional sensor serving as a GMF compass, these studies proved that Cry mediated detection of a rather intense, artificial magnetic anomaly.

Here, we have taken advantage of an assay enabling us to test directional magnetic sensitivity in insects at naturally occurring GMF intensities (30) and functionally confirmed that mammalian-like Cry2 is necessary for sensing the directional component of MFs of natural intensities by two different species of cockroaches.

Results

Previously we developed an assay enabling us to test directional magnetic sensitivity in insects at natural GMF intensity (30) (Fig. 1 *A* and *B*) in a nonconditioned, spontaneous behavioral reaction to slow shifts of the magnetic North position. We first used the American cockroach, *Periplaneta americana*, but soon realized that this species most likely contains only Cry2. Thus, we added another cockroach species, *Blattella germanica*, which has both Cry types (see *SI Appendix*, Fig. S1 for phylogenetic analysis and *SI Appendix*, Fig. S2 for details on Cry1 search in *P. americana*).

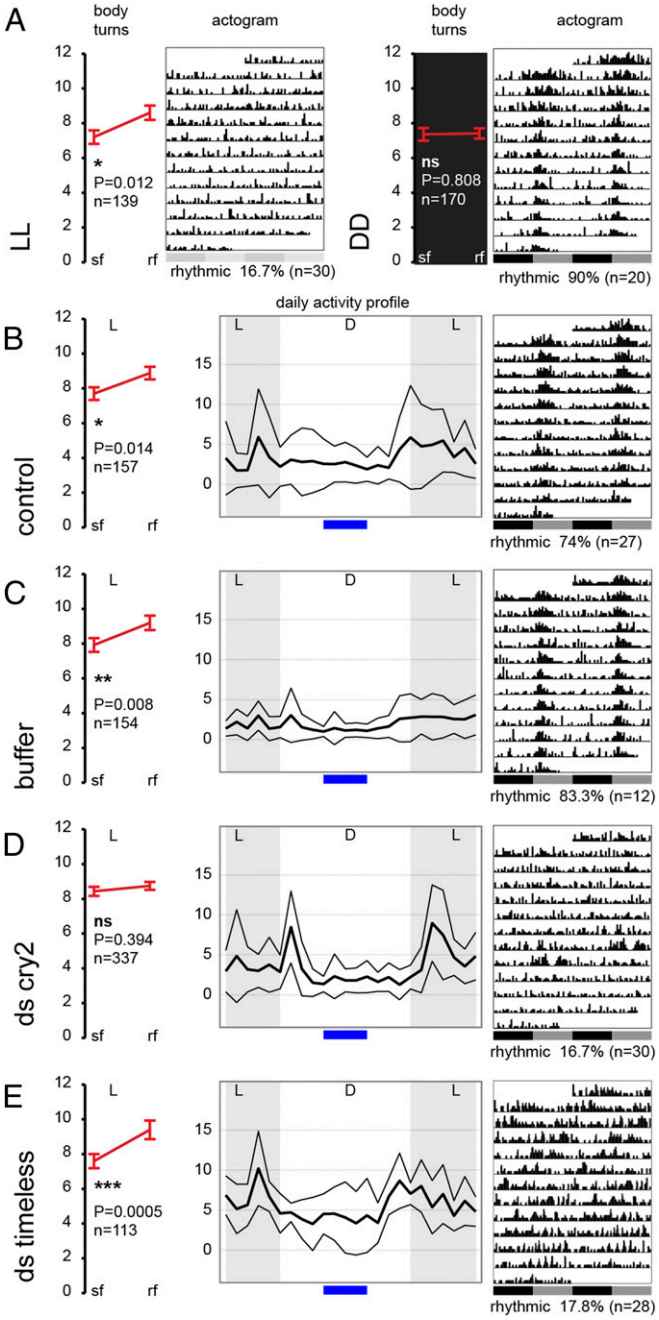

Fig. 2. Phenotypes of *P. americana*. Magnetoreception scored as body turns under sf and rf (*A–E*, *Left*); red line depicts the average change of body turns (±SEM). Daily activity profiles at a 12 h light:12 h dark cycle are shown as a black line (±SD, thinner lines); horizontal blue bar under daily activity profiles indicates the time during which magnetoreception was assayed (*B–E*, *Middle*). Circadian activity in constant conditions is shown as double-plotted actograms (*A–E*, *Right*). (*A*) Constant light (LL) abolished circadian rhythmicity, leaving magnetic sensitivity intact, whereas DD abolished magnetic sensitivity with unaffected circadian locomotor activity. (*B*) The control RNAi and (*C*) buffer injected animals displayed normal magnetoreception, as well as circadian rhythmicity. (*D*) *cry2* RNAi-treated animals lost both magnetoreception and circadian rhythmicity. (*E*) *timeless* RNAi cockroaches showed unaffected magnetoreception, but their circadian behavior was disrupted.

During their resting time around noon, cockroaches display minimal locomotor activity (Fig. 2*B*). However, when the direction of the horizontal magnetic vector of the natural GMF is rotated periodically (Fig. 1 *A* and *B*), the animals change their resting positions more frequently. We term this phenomenon magnetically induced restlessness (MIR) (Fig. 1*C*). As a control, a double-wrapped coil that does not generate any MF had no effect on MIR in any of these two species (Fig. 1*C*).

Cry2 Is Necessary for Directional Sensitivity. The fact that no MIR was observed in complete darkness (DD; Fig. 2*A*, *Right*) supports the involvement of photosensitive processes in *P. americana* magnetoreception. To test the causal involvement of Cry2 in cockroach directional magnetoreception, we used our behavioral assay under 365 nm UV light in combination with a reverse genetic approach. Injections of double-stranded (ds) cry2 RNA (RNAi) significantly reduced the cry2 mRNA and protein levels (*SI Appendix*, Figs. S5 and S8) and completely abolished MIR behavior (Fig. 2*D*). Control injections of the nonspecific dsRNA or buffer alone (Fig. 2 *B* and *C*) had no effect on the response to changes of the MF vector. Importantly, the assays were conducted during the middle of the photophase, when a drop of activity was observed in all treatment groups. This indicated that the MIR was not an artifact caused by endogenous activity patterns of the cockroaches (Fig. 2 *B–E*, *Middle*). The silencing of Cry2 was also accompanied by severe disruption of circadian rhythmicity in constant dark conditions (Fig. 2*D*, *Right* and *SI Appendix*, Table S1) compared with control animals (Fig. 2 *B* and *C*). The overlap of the circadian clock mechanism and magnetic sensing was further tested under constant light conditions known to interfere with proper clock function. As expected, the constant light regime resulted in arrhythmic circadian behavior, but the sensitivity of the animals to the changes in the MF direction was unaffected (Fig. 2*A*, *Left*). In addition, injection of dsRNA targeting the clock gene *timeless* had no effect on MIR, but did abolish cockroach circadian rhythmicity (Fig. 2*E* and *SI Appendix*, Table S1). These results clearly support the separation of GMF sensing from the circadian clock.

The following experiments confirmed that the locomotor activity in photophase is lower after any treatment in the second cockroach species, *B. germanica* (Fig. 3 *A–E*, *Middle*). Although control dsRNA or buffer injection had no effect on MIR and circadian phenotypes in DD (Fig. 3 *A* and *B*), cry2 knockdown abolished MIR and reduced circadian rhythmicity (Fig. 3*C*). Remarkably, cry1 reduction did not affect MIR, whereas circadian rhythmicity was reduced even more than in cry2 RNAi animals, indicating that cry1 RNAi was efficient (Fig. 3*D* and *SI Appendix*, Fig. S5). Notably, cry1 knockdown resulted in significant up-regulation of cry2 levels (*SI Appendix*, Fig. S5); nevertheless, the detailed mechanism, how cry1 depletion affects behavioral rhythmicity, is beyond the scope of this study. Consistently, cry1+cry2 double RNAi abolished MIR and reduced circadian rhythmicity (Fig. 3*E*). Cry2 therefore represents a prerequisite for magnetic susceptibility in both cockroach species.

Magnetoreception Is Dependent on Light from UV to Cyan/Green 505 nm. To further characterize magnetoreception, we tested seven wavelengths of light under different intensities (*SI Appendix*, Table S7). The minimal light intensity needed for MIR was at UVA 365 nm. The sensitivity of the MIR gradually dropped to 4×10^{16} photons $m^{-2} \cdot s^{-1}$ under two blue light wavelengths, followed by a local boost of sensitivity at 505 nm. Under 505 nm, the MIR was still significant under dim light (7×10^{15} photons $mm^{-2} \cdot s^{-1}$), but the MIR response dropped precipitously at higher wavelengths; no MIR was observed at 528 nm, even with 1,000× stronger light (6×10^{18} photons $m^{-2} \cdot s^{-1}$; Fig. 4*A*). Enlarged samples were tested to exclude possible errors (*SI Appendix*, Table S7), but the steep threshold of MIR sensitivity was upheld. There was also a negative result at 407 nm light and its maximal intensity

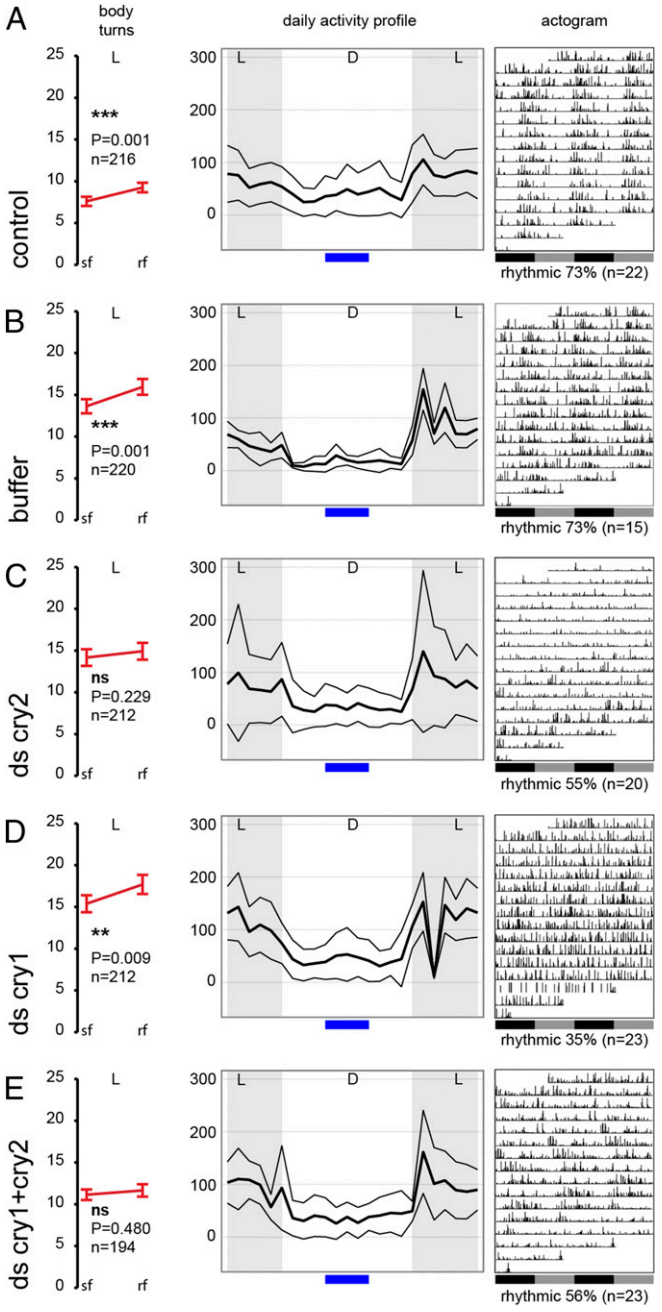

Fig. 3. Phenotypes of *B. germanica*. Magnetoreception scored as body turns under sf and rf; red line depicts the average change of body turns (±SEM). Daily activity profiles (*Middle*) at LD 12:12 are shown as a black line (±SD, thinner lines); horizontal blue bar indicates the time during which magnetoreception was assayed. Circadian activity (*Right*) in DD is shown as double-plotted actograms. (*A*) The control RNAi- and (*B*) buffer-injected animals displayed normal magnetoreception as well as circadian rhythmicity. (*C*) *cry2* RNAi-treated animals lost magnetoreception and have reduced rhythmicity in DD. (*D*) *cry1* RNAi cockroaches showed unaffected magnetoreception, but their circadian behavior was disrupted. (*E*) *cry1, cry2* double RNAi-treated animals lost magnetoreception and had reduced rhythmicity in DD.

2×10^{17} photons $m^{-2} \cdot s^{-1}$, likely showing the upper border of a functional window shown previously [e.g., from experiments on birds (31)].

Magnetoreceptor Is Most Likely Located in the Compound Eyes. Because light is necessary for magnetoreception (Fig. 2*A*), we attempted to localize the magnetoreceptive organ anatomically by shielding/painting the compound eyes. The animals with compound

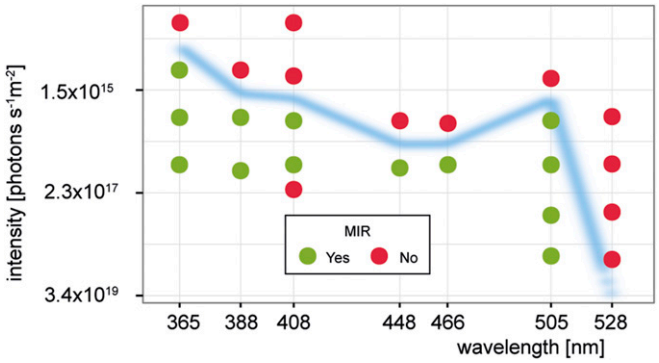

Fig. 4. Light sensitivity of *Blattella* magnetoreception is wavelength-dependent and restricted to the region from UV to cyan/green light. Green dots indicate functional MIR; red dots no MIR reaction. Blue line approximates low threshold of illumination necessary for MIR. *y* axis, light intensity; *x* axis, wavelength of light used in experiment. For details, see *Discussion, Spectral Effects and Magnetic Signaling*.

eyes covered by transparent enamel retained MIR, whereas black enamel prevented any magnetoreceptive response, suggesting eyes as the necessary organ for magnetoreception (Fig. 5*A*).

Laser-scanning confocal microscopy localized Cry2 to a hemispherical multicellular structure immediately beneath the cockroach retina (Fig. 5 *B* and *C*). Colocalization of Cry2 with antisera raised against the alpha subunit of Na/K-ATPase (32) (*SI Appendix*, Fig. S10 *A–C*) resulted in a pattern similar in structure to *Drosophila* epithelial glial cells (33, 34), suggesting these cells are glia localized underneath the retina between the two basement membranes (35). Higher magnification revealed an orderly columnar alignment of the Cry2-positive cells (Fig. 5 and *SI Appendix*, Fig. S9), with the strongest signal localized in the close vicinity of the plasma membrane (*SI Appendix*, Fig. S10).

Discussion

Cry Phylogeny in Blattodea. In this study, we explored the role of Crys in magnetoreception, using two cockroach species. Cry1 was not found in *P. americana* by examination of its assembled transcriptome, or in raw RNA reads even when Cry2 coding sequences or Cry unrelated genes were identified (*SI Appendix*, Fig. S2). Because the Cry protein structure is remarkably well conserved, we suggest that Cry1 is absent in *P. americana*. This hypothesis is further supported by phylogenetic analysis of insect Crys, where Cry1 was not identified either in cockroach *Cryptocercus* or in two termite species, suggesting the loss of Cry1 in multiple species of the Blattodea lineage. Therefore, the most plausible explanation is the existence of different *Cry* genes in *B. germanica* and *P. americana.*

Detection of Directional Changes of MF in Cockroaches. Even though the MIR assay does not monitor directional locomotion from point A to point B, which might be expected if compass abilities are investigated (e.g., in migratory birds), it gives unequivocal evidence of sensitivity to the direction of the magnetic vector. The simplicity of the behavioral output is a particular strength of this assay compared with orientation tests made on birds, where unimodal versus axial orientation under different light conditions are results that should be considered separately (36). Another merit of our unconditioned assay is its relative insensitivity to changes in motivation under different light regimens, which may alter more complex behavioral programs such as migration behavior.

Spectral Effects and Magnetic Signaling. The dependence of magnetoreception on specific wavelengths and intensities of light has been documented extensively in birds (summarized in refs. 31, 36, 37). The FAD cofactor of CPF proteins is capable of both ground-state (dark) and light-driven redox reactions. One-electron reduction of neutral fully oxidized state (FAD_{ox}) and one-electron oxidation of anionic fully reduced state ($FADH^-$) produce anionic semiquinone ($FAD^{\bullet -}$) and neutral semiquinone ($FADH^{\bullet}$) radicals, respectively, that are capable of magnetosensitive spin-correlated RPs (18) (*SI Appendix*, Fig. S11). The most frequently discussed model supposes spin-correlated RPs consisting of $FAD^{\bullet -}$ and a cationic radical of Trp or Tyr initiated by the UVA/blue light excitation of FAD_{ox} (*SI Appendix*, Fig. S11, Scheme 1). MF modulates interconversion of the singlet and triplet states of RP, hence changing the proportions of two competing pathways yielding different reaction products (5).

FAD changes its spectral absorption properties as it goes through several redox states, during its redox cycle (38) (*SI Appendix*, Fig. S12). Examination of spectral limits during in vivo magnetoreception may help clarify which redox forms of FAD and which radical partners participate in the signaling conformation of Cry proteins (39).

Our MIR spectrum for *Blattella* (Fig. 4) shows a steep decline of sensitivity in the cyan region 505–528 nm, remarkably matching the decline of light absorption of three redox forms of flavin: FAD_{OX}, $FADH^-$ and $FAD^{\bullet -}$ (40) (*SI Appendix*, Fig. S11). Such a coincidence points to possible involvement of flavin in cockroach magnetoreception. The role of the $FADH^{\bullet}$ redox form, which absorbs in wide spectrum including wavelengths above cyan (*SI Appendix*, Fig. S10), is not clear. The MIR spectrum (Fig. 4), however, does not fully conform to a direct light absorption by FAD_{ox} (*SI Appendix*, Fig. S11). Although two peaks of magnetosensitivity of *Blattella* at UV 365 and green/cyan 505 nm are apparent, the absorbance of FAD_{ox} peaks at about 450 nm. As a possible explanation, an indirect FAD_{ox} excitation via energy transfer from a UVA-absorbing antenna cofactor may occur. Such a cofactor (e.g., methenyltetrahydrofolate) is well-known for many CPF proteins, and was discussed also for *Drosophila* Cry (41). Because both wavelength peaks of MIR response exactly match the peaks of visual sensitivity of *Periplaneta* (42) and *Blattella* (43), they might reflect a generally close association between magnetoreception and vision.

The drop in MIR sensitivity is surprisingly sharp in the wavelength range between 505 and 528 nm, where magnetoreception is lost even under exposure to three orders magnitude of light intensity (Fig. 4 and *SI Appendix*, Table S7). Such a sharp cutoff cannot easily be attributed to the spectral sensitivity limit of a single crucial photopigment. As Wiltschko et al. (44) state from a comparable phenomenon in birds: "It rather seems to reflect some

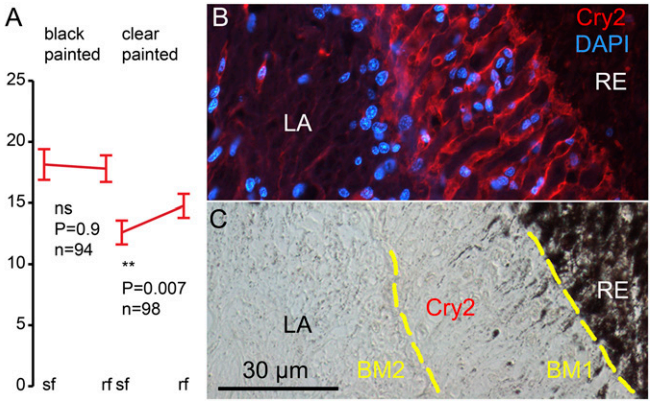

Fig. 5. *P. americana* eye participates in magnetoreception and expresses Cry2. (*A*) Cockroaches with clear-painted eyes responded to MF rotation, whereas individuals with black-painted eyes did not. (*B*) Immunofluorescence and (*C*) Nomarski contrast of *P. americana* eye. Cry2 immunoreactivity (in red) is localized underneath the retina (RE) between the two basement membranes (BM1, BM2), according to Ribi (35). BM1, first basement membrane; BM2, second basement membrane; LA, lamina; RE, retina; blue, cell nuclei (DAPI stained).

antagonistic interactions with receptor activated by longer wavelength light." As short and long wavelengths may have opposite effects on the relative concentration of FAD_{OX} versus semiquinone $FADH^{\bullet}$ redox states, changes in illumination color can also result in antagonistic changes of cryptochrome activation (36). If wavelengths from UV to blue stimulate MIR while green light 528 nm suppresses it, then the semiquinone $FADH^{\bullet}$ (or other still-unknown pigment) could be considered an antagonistic candidate in our MIR assay.

Reports of light-dependent magnetoreception beyond 528 nm are not unique within behavioral reports on birds (green/yellow) (45). However, the finding that preexposure of European robins to white light not earlier than 30 min before testing is necessary for proper orientation suggests a dependence on the short-wavelength part of spectra as well (39), meaning that orientation under green/yellow light is only a transient phenomenon (45).

A contrasting situation was reported in the fruit fly (*D. melanogaster*) and monarch butterfly (*Dannaus plexippus*), which lost magnetic compass orientation if the illumination wavelength was already above 420 nm, which is still well within the absorption range of FAD_{OX} (26, 27, 46). Because the studies did not seek thresholds for separate colors, the question remains whether the diurnal species such as *Dannaus* or *Drosophila* had already reached the absolute threshold of their sensitivity to light-driven magnetoreception. *Blattella* lost its magnetoreception under only 10-fold dimmer light than *Dannaus* or *Drosophila.* Provided they have denser cuticle or eye pigment shielding than night-active *Blattella*, brighter illumination might induce magnetoreception even for green and longer wavelengths. The inconsistencies among *Blattella*, *Drosophila*, *Dannaus*, and birds show that taxa-specific Crys may use different redox cycles and that the analysis of the long-wavelength tail of the light spectrum may be of particular interest to identify other yet-uncharacterized signaling partners. In summary, our behavioral data on the spectral dependence of Cry2-dependent insect magnetoreception are consistent with the involvement of electron transfer reactions of FADox, $FADH^{-}$, and $FAD^{\bullet -}$.

Directional Sensitivity. Local magnetic conditions that possibly control RP reactivity are a result of interplay between external MFs, nuclear spins of RP partners (47, 48), and electron spins of nearby radicals (49). To function as a directional sensor that changes the product yield solely after a shift in the magnetic vector, the RPs must respond anisotropically (2, 50). So far, there has been no demonstration that any Cry radical reaction responds to an Earth-strength MF, or that this response is anisotropic, as would be required for a magnetic compass (18).

Another important condition is that sensory molecules should optimally be aligned in the same direction within the receptor cells, in order that the stochastic effects of freely rotating molecules would be eliminated (51). Such a condition could be met by cytoskeletal anchoring to the membrane, or by at least partial immobilization within the cells (52).

Moreover, for sufficiently fast compass orientation, the magnetic direction-sensitive structure should ideally consist of spatially organized receptor cells, so that the signal from differentially activated cells can be compared, and the GMF vector can be directly identified. As suggested (48), the retina forms an ideal hemispherical organ for photoreception, with a potential for directional magnetoreception. If the Cry reaction products are involved in the visual signaling pathway, they could modulate the rate of light transduction to a neural signal (5).

Our behavioral–genetic evidence provides a link between truly directional magnetic sensitivity and mammalian Cry protein, supporting the so-far-hypothetical predictions of directional magnetic sensitivity discussed earlier.

Anatomical Localization of the Magnetoreceptor. The experiment with painted eyes suggested that the magnetoreceptive tissue is localized either within the compound eye or underneath it (Fig. 5*A*). Cry2-immunoreactive cells were localized immediately beneath the pigmented layer of the cockroach retina in a hemispherical multicellular structure (Fig. 5*B*). The cells were spread between the two basement membranes (Fig. 5*C*) separating the retina from the fenestrated layer of lamina neuropil (35). High-magnification analysis identified Cry staining strongest in the close vicinity of the plasma membrane (*SI Appendix*, Fig. S10). Indeed, association of Cry with the scaffolding protein close to the membrane was reported recently for *Drosophila* (53). Such immobilization would further support Cry as a directional magnetoreceptor, as discussed earlier. Furthermore, microinjection of a fluorescent neuronal tracer into the cockroach ocular photoreceptor cells revealed an intimate contact between the neuronal output from the retina and the Cry2-positive cells (*SI Appendix*, Fig. S9), suggesting their possible interplay.

Taken together, the results of our study establish the role of a mammalian-type Cry in light-dependent magnetoreception in two insect species phylogenetically distant to *Drosophila.* More important, our study delivers original evidence that the Cry2 protein is involved in the detection of GMF vector direction at natural intensities, which is a crucial feature of a biological receptor providing genuine compass bearings; by means of immunolocalization, we identified the eye and subretinal region as a likely site of magnetoreception in insects; and we show that spectral dependency of mammalian Cry-linked magnetoreception is in line with involvement of flavins. Altogether, the work provides original genetic proof of the previously hypothesized chemical reactions that may underlie a functional compass in animals.

Materials and Methods

Behavioral Tests: MIR. Cold-immobilized cockroaches, regardless of sex, were transferred individually into glass Petri dishes with white opaque walls and placed into a white arena with a translucent lid. On the next day, a camera–PC system underneath a glass pane holding the dishes sampled the silhouettes of the animals illuminated from above every 1 min. Frames taken between 10:00 and 14:30 were downloaded and divided into six 45-min intervals: the first two (1, 2; 10:00–11:30) before magnetic North rotation, the middle three intervals (3–5; 11:30–13:45) when the field was periodically rotated, and the last interval (6; 13:45–14:30) after this magnetic treatment. The temperature varied between 21 °C and 24 °C in the testing room.

Photic Conditions. A set of three UV LEDs 365 nm (Nichia NCSU033A) illuminated the arena through a translucent lid that diffused light so that its intensity was 4.04×10^{16} quanta $m^{-2} \cdot s^{-1}$ in the center of the arena and 3.12×10^{16} quanta $m^{-2} \cdot s^{-1}$ along the wall line (radiometer International Light IL700, SHD 033 probe). Types and spectral characteristics of all LEDs used are given in *SI Appendix*, Fig. S6 and Table S7.

Magnetic Conditions. The natural geomagnetic background within the testing space was as follows: horizontal component 18 μT, total vector 45 μT, and inclination 66°. The spatial variation in the arena region was <2% (measured by HMR 2300 magnetometer; Honeywell). During periods of magnetic North rotations (periods 3–5), only the horizontal component of local GMF was rotated by 60° by means of a horizontal four-element double-wrapped Merritt coil, making an angle with the *N*–S axis of 120° (*SI Appendix*, Fig. S3).

Evaluation and Statistics. For both MIR and circadian activity, the number of body axis changes >15° was determined visually using Screen Protractor software (Iconico.com Software) for *Periplaneta*. In the case of *Blattella*, MIR recordings were done automatically with image analysis software RoachLab. In both cases, the personnel scoring the activity and doing the statistical analysis were not aware of which set of images they were evaluating. For MIR analysis, the activity of every animal was given as a pair of numbers: activity in steady field – sf (control periods 1 + 2 + 6) versus activity in rotated field – rf (treated periods 3 + 4 + 5). The experiment was principally designed as "paired," where individual animals represented objects producing mutually consecutive outcomes from measurement periods. In such a design, the outcomes must be compared on the basis of paired statistical tests, which can then adjust the internal correlation (individuality) of primary data. Because of the pair design of the test, we did not compare statistically groups among each other.

Antibodies and Immunodetection. The synthetic peptide CHSPSYRENIKSGIHFHR corresponding to the C-terminal region of the *Periplaneta* Cry2 was used to generate a custom-made specific antibody (Moravian Biotech). Cry2 primary antibody was used at a dilution of 1:1,000 or 1:1,500, with similar results. Immunofluorescent detection and microinjection of Alexa Fluor-conjugated dextran neuronal tracer were carried out as described earlier (54). The alpha5 mouse monoclonal antibody (DSHB Hybridoma Product a5; *SI Appendix*, Fig. S10) was used at a dilution of 1:50, as described in refs. 32 and 34.

Verification of RNAi Efficiency. *cry2*, *cry1*, *timeless*, or *lacZ* dsRNA was injected, and the cockroaches were euthanized 14 d later. Their brains were dissected and used for RNA isolation and subsequent quantitative RT-PCR analysis (*SI Appendix*, Fig. S5). Alternatively, dissected brains were surgically divided into two hemispheres, including the optic lobes and eyes. One hemisphere was used for RNA isolation and subsequent qRT-PCR analysis (*SI Appendix*, Fig. S8*B*). The second hemisphere was fixed and used for immunocytochemistry (*SI Appendix*, Fig. S8 *A* and *C–F*). In this case, both control (*lacZ* RNAi) and *cry2* RNAi brain sections were processed on the same microscope slide. The analysis was performed double blind, and the reduction of protein was evaluated by Image J analysis software (*SI Appendix*, Fig. S1*E*).

ACKNOWLEDGMENTS. We thank Ladislav Dusek (Institute of Biostatistics and Analyses, Brno) for advice on statistics and Vladimir Benes and Dinko Pavlinic (European Molecular Biology Laboratory) for help with *P. americana* transcriptome. This work was funded by Grant Agency of the Czech Republic projects (206/07/J041 and 13-11908J) (to M.V. and I.S.). P.B. acknowledges the support by the DFG (LO 797/5-1). H.-J. L. was supported from Ministry of Science and Technology of Taiwan (NSC 103-2923-B-002-005-MY3) and D.D. was supported from Ministry of Education, Youth and Sports (LH14029).

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Supporting Information

Materials and Methods

Insects. Cockroaches were kept in plastic buckets and were fed with cat food pellets and water *ad libitum*. The animals were housed under a 12-hr light/dark cycle, and those subjected to a permanent dark or permanent light series were kept under respective illumination conditions for at least one month.

Photic conditions. A set of three UV LEDs 365 nm (Nichia NCSU033A) illuminated the arena through a translucent lid that diffused light so that its intensity was 4.04×10^{16} quanta m^{-2} s^{-1} in the center of the arena and 3.12 $x10^{16}$ quanta m^{-2} s^{-1} along the wall line (radiometer International Light IL700, SHD 033 probe, USA). Types and spectral characteristics of all LEDs used are given in Fig. S6 and Table S7.

Behavioral tests - circadian phenotypes. Cold immobilized *Periplaneta* adults were placed individually into glass Petri dishes and housed into a thermostat (23°C±0.3°C). Constant darkness (DD) or constant light (LL) was kept inside, respectively. Locomotor activity was monitored by a PC-camera system under IR light illumination. Frames were automatically taken every 4 min. *B. germanica* adults were cold immobilized, placed individually into glass test tubes (diameter 2.5 cm, length 15 cm) and activity was measured in Large Activity Monitors (Trikinetics). Free running periods and daily activity profiles were determined in ActoJ software (1).

Controls. To exclude possible non-specific effects of the electric feeding of coils, a double-wrapped coil design was used, which made feeding of coils without any externally produced magnetic field feasible (Fig. 1). During field rotation phases, the horizontal magnetic intensity temporarily dropped to 87% (Fig. S3A). To test the possibility that magnetoreception behavior is induced by changes of GMF intensity instead of direction change, two control series were performed: reducing the horizontal intensity to 87% and to 5% (Fig. S3B) while leaving the direction of the vector intact. In these two series, the Merritt coil was oriented in parallel with the N-S geomagnetic axis.

Evaluation and statistics. We performed tests of normality of all primary variables and found highly significant differences from a normal (Gaussian) distribution. Data revealed highly heterogeneous types of profiles, including bimodal patterns and outliers; the shape of the underlying distribution was asymmetric. Consequently, a parametric model like an ANOVA could not be applied. That was why we adopted straightforward testing based on a rank-sum, non-parametric Wilcoxon Matched Pairs test, which has no assumptions regarding shape and type of data distribution.

Cryptochrome sequences. Sequences and alignment used for phylogenetic analysis are available upon request from D.D. *B. germanica's* Cry1 and Cry2 protein sequences were retrieved from i5K genome drafts and the sequences were confirmed by PCR and sequencing. To identify *P. americana cry1* transcript, or its fragment, we have first BLAST searched GenBank sequences and our *P. americana* transcriptome assembly retrieving only Cry2 coding transcript. Then we have BLAST searched database of 2.2×10^8 raw pair-end 100bp illumina reads obtained from RNA sequencing of *P. americana* brains. Cry1 protein sequence from *B. germanica* served as a query for tblastn program, with parameters: '-evalue 50000 -num_descriptions 10000000 -num_alignments 10000000' (2). This search returned 29,108 hits, which were visualized in mview software (3) and sequences were controlled by eye. Hits that did not match Cry2 sequence were further analyzed (BLAST search in *P. americana* transcriptome and eye inspection, if sequence might possibly contain any similarities suggesting its relatedness to cryptochromes).

mRNA Quantification. Total RNA was isolated from brains of adult *P. americana* with the TRIzol reagent (Invitrogen, Carlsbad, CA). After TURBO DNase (Ambion, Austin, TX) treatment, 1 µg of total RNA was used for cDNA synthesis with Superscript III reverse transcriptase (Invitrogen). Relative transcript levels were measured by quantitative RT-PCR using the iQ SYBR Green Supermix kit and the C1000 Thermal Cycler (both from Bio-Rad Laboratories, Hercules, CA). All data were normalized to the relative levels of actin mRNA. Primer sequences used for qRT-PCR are listed in Table S3 and Table S5.

RNA interference (RNAi). dsRNAs were synthesized with the T7/T3 Megascript system (Ambion) from plasmids containing the appropriate gene fragments (see Table S4 and Table S6 for primer sequences). The RNAs were annealed to form dsRNA, and its quality was controlled by gel electrophoresis. Experimental animals were injected with either 10 µl (*Periplaneta*) or 4 µl (*Blatella*) of 5-10 µg/µl dsRNA solution. The efficiency of RNAi-mediated depletion of each targeted mRNA was verified by qRT-PCR.

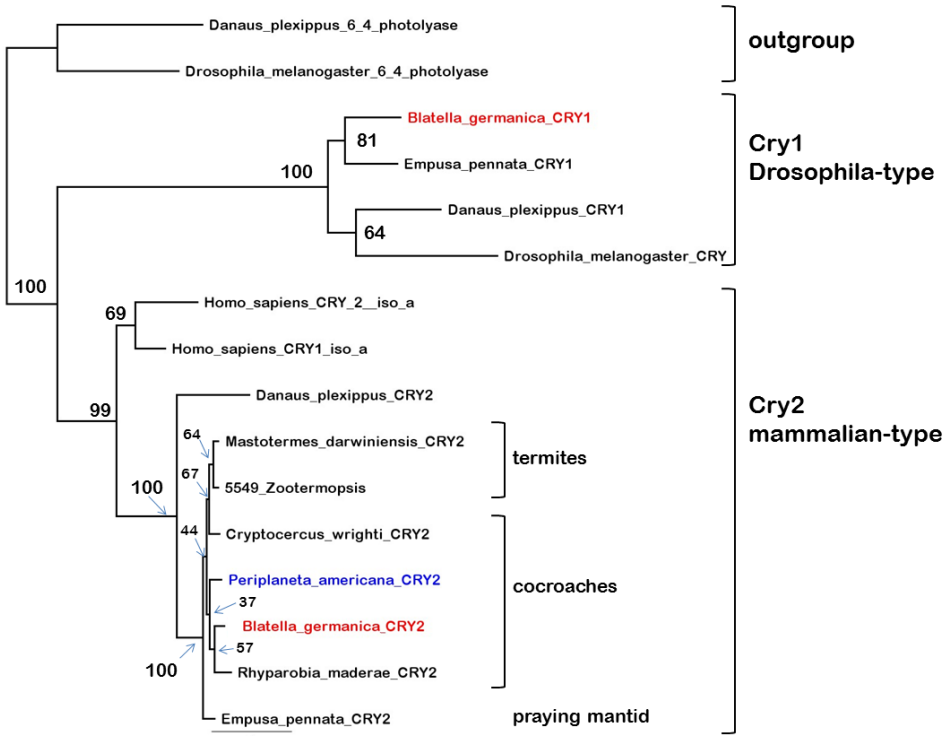


Fig. S1. A phylogenetic tree showing relationships among cryptochromes (Crys). Cry proteins were aligned with ClustalW algorithm and analyzed in the RAxML program (Geneious 7, WAG substitution matrix, (4) to construct a maximum likelihood tree. Bootstrap values are given at the nodes as % (1000 replicates). Sequences are unambiguously clustered into three groups: (*i*) 6-4 photolyases serving as an outgroup in this analysis, (*ii*) type 1 cryptochromes containing *Drosophila* Cry and insect Cry1 genes, (*iii*) type 2 cryptochromes containing insect Cry2 and mammalian Crys. Note, that *Blatella* and *Periplaneta* Cry2 proteins cluster together with two cockroach species (*Rhyparobia* and *Cryptocercus*) and two termites (*Zootermopsis* and *Mastotermes*). Mantid *Empusa* forms a sister taxon to cockroach/termite clade; this topology is completely consistent with current view of Blattodea and Mantodea evolution (5, 6). However, Cry1 clade contains only one cockroach representative (*Blatella* Cry1) branching together with mantid (*Empusa penata* Cry1). Since no Cry1 was found even when searching complete genome of Zootermopsis, the most plausible explanation is a loss of Cry1 gene in termites and some cockroach species. Sequence alignment is available upon request from D.D.

Fig. S2. 2.2 x 10^8 raw pair-end 100bp illumina reads from brain transcriptome of *P. americana* were inspected for presence of any similarities to Cry1 protein using BLAST-X algorithm with *B. germanica* Cry1 protein sequence serving as a query. This alignment shows representative hits completely covering regions to which best 1904 hits were mapped. Note that all of these hits correspond to Cry2 sequence and map to regions where Cry1 and Cry2 show conserved sequence features. Inspection of remaining (non-Cry2) hits did not reveal any pattern similar to Cry/Photolyase sequence motif.

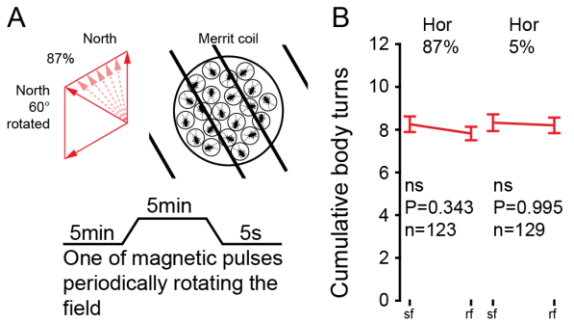


Fig. S3. Magnetoreception assay setup and controls. (A) Cockroaches were placed individually into Petri dishes with opaque walls. 11 Petri dishes (*Periplaneta*) or 18 Petri dishes (*Blatella*) were simultaneously exposed to magnetic field. The Merritt coils (depicted by black bars) axis made an angle 120° with the N-S axis (red solid arrows). During the rotating field period, ten trapezoid pulses fed the coil, changing the position of the geomagnetic horizontal vector by 60° every 5 min. During the 5 s ascending and descending phases, the intensity of rotating vector (red dotted arrows) temporarily dropped to 87%. To check the selective sensitivity to field direction change, only the intensity of horizontal field was adjusted either to 87% or even to 5%, leaving the direction of the field intact. (B) These manipulations did not elicit any significant behavioral response confirming the sole change in the horizontal GMF direction being fully responsible for the observed light-dependent MIR. (vertical bars indicate +/- SEM). (sf - steady magnetic field; rf - rotating magnetic field)

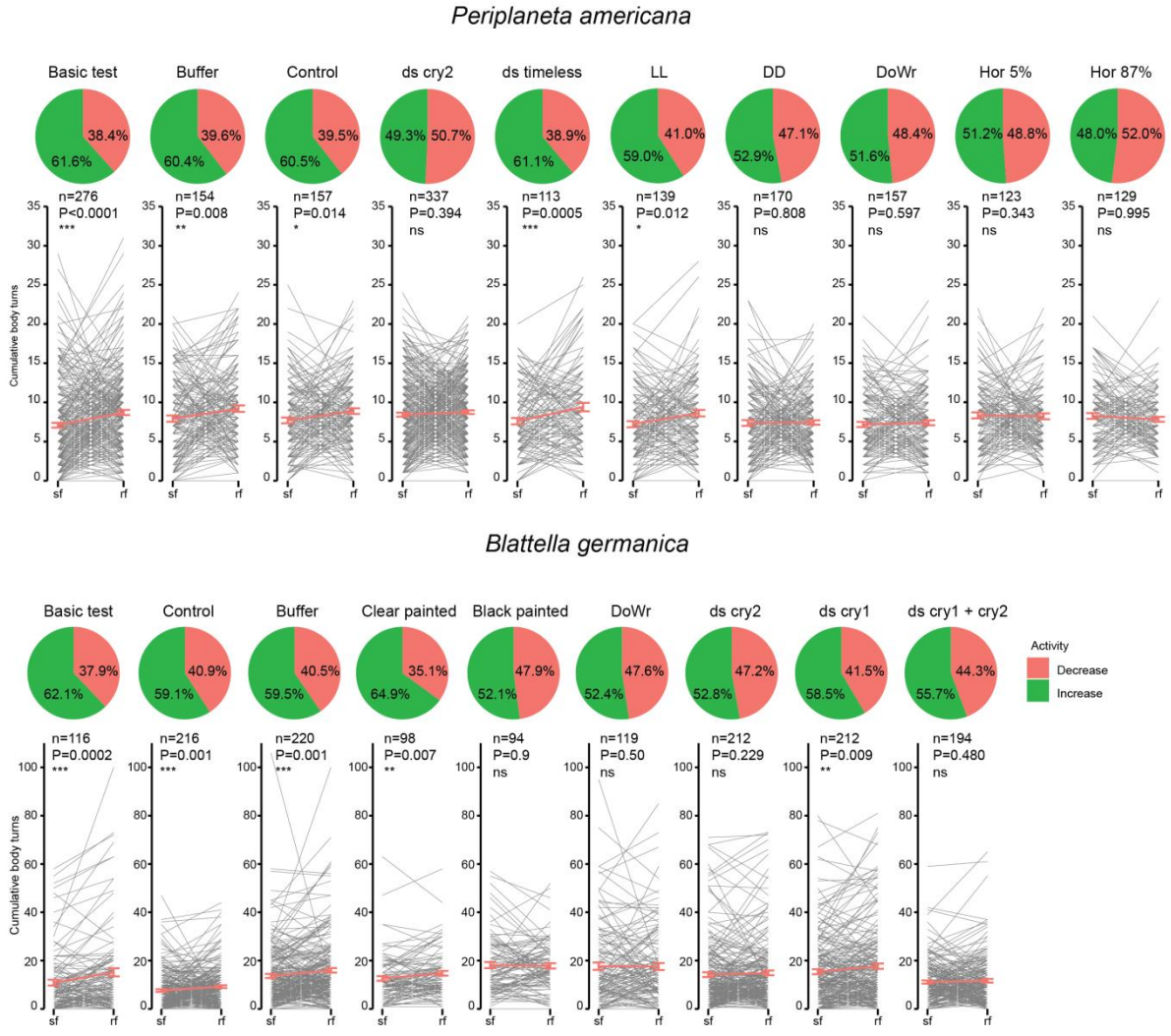
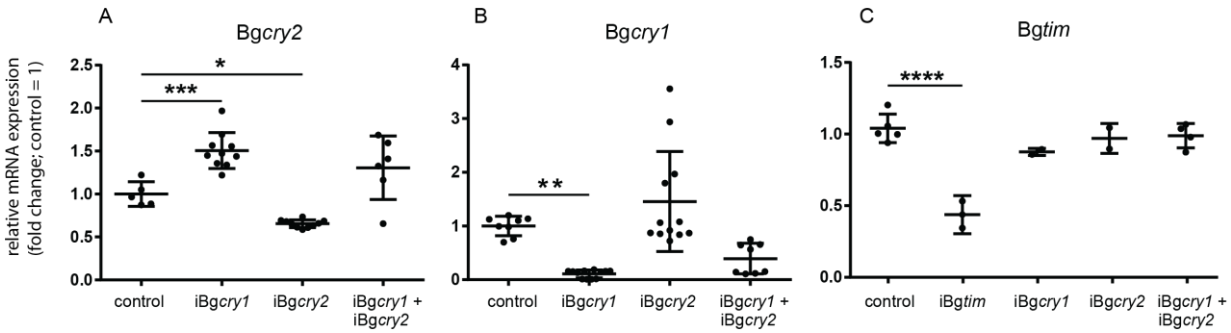


Fig. S4. Summary of all magnetoreceptive phenotypes in the two examined cockroach species, *P. americana* and *B. germanica*. Number of body turns (bottom graph) in steady magnetic field (sf) and rotating magnetic field (rf) are delineated by grey lines for each individual cockroach. The average trends are depicted by red lines (+/-SEM). Pizza graphs (top) summarize percentage of animals with increased (green) or decreased (magenta) activity in rotating magnetic field, respectively. (n - number of individuals; P - outcome of Wilcoxon Match Pair Test; ns – not significant)



Dunnett's multiple comparisons test – **Bg*cry2* expression**	Mean Diff.	95% CI of diff.	Significant?	Summary
control vs. i cry1	-0.5059	-0.7908 to -0.2210	Yes	***
control vs. i cry2	0.3451	0.06023 to 0.6300	Yes	*
control vs. i cry 1+2	-0.3060	-0.6210 to 0.008918	No	ns

Dunnett's multiple comparisons test – **Bg*cry1* expression**	Mean Diff.	95% CI of diff.	Significant?	Summary
control vs. iBg cry1	0.8914	0.2897 to 1.493	Yes	**
control vs. iBg cry2	-0.4566	-1.058 to 0.1452	No	ns
control vs. iBg cry1 + iBg cry2	0.6075	-0.05161 to 1.267	No	ns

Dunnett's multiple comparisons test – **Bg*tim* expression**	Mean Diff.	95% CI of diff.	Significant?	Summary
control vs. tim	0.6020	0.3489 to 0.8551	Yes	****
control vs. cry1	0.1644	-0.08870 to 0.4175	No	ns
control vs. cry2	0.06961	-0.1835 to 0.3227	No	ns
control vs. cry1+2	0.04901	-0.1539 to 0.2519	No	ns

Fig. S5. Expression data of *cry1*, *cry2* and *timeless* (*tim*) in *B. germanica* heads and statistical analyses of expression levels. Adult cockroaches were injected with dsRNA identically as was done for animals used in behavioral experiments. 5 days later, total RNA was isolated and expression levels were measured together with *actin* transcript serving as a reference. The expression levels are plotted as fold changes relative to the expression levels of control animals (+/-SEM). Statistical significance is shown for one way ANOVA (Dunnet's test).

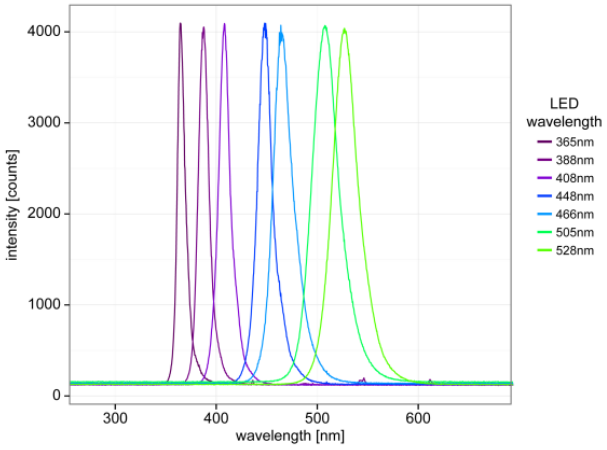


Fig. S6. Emission Spectra of LEDs used for results presented in Fig. 4 and Table S7

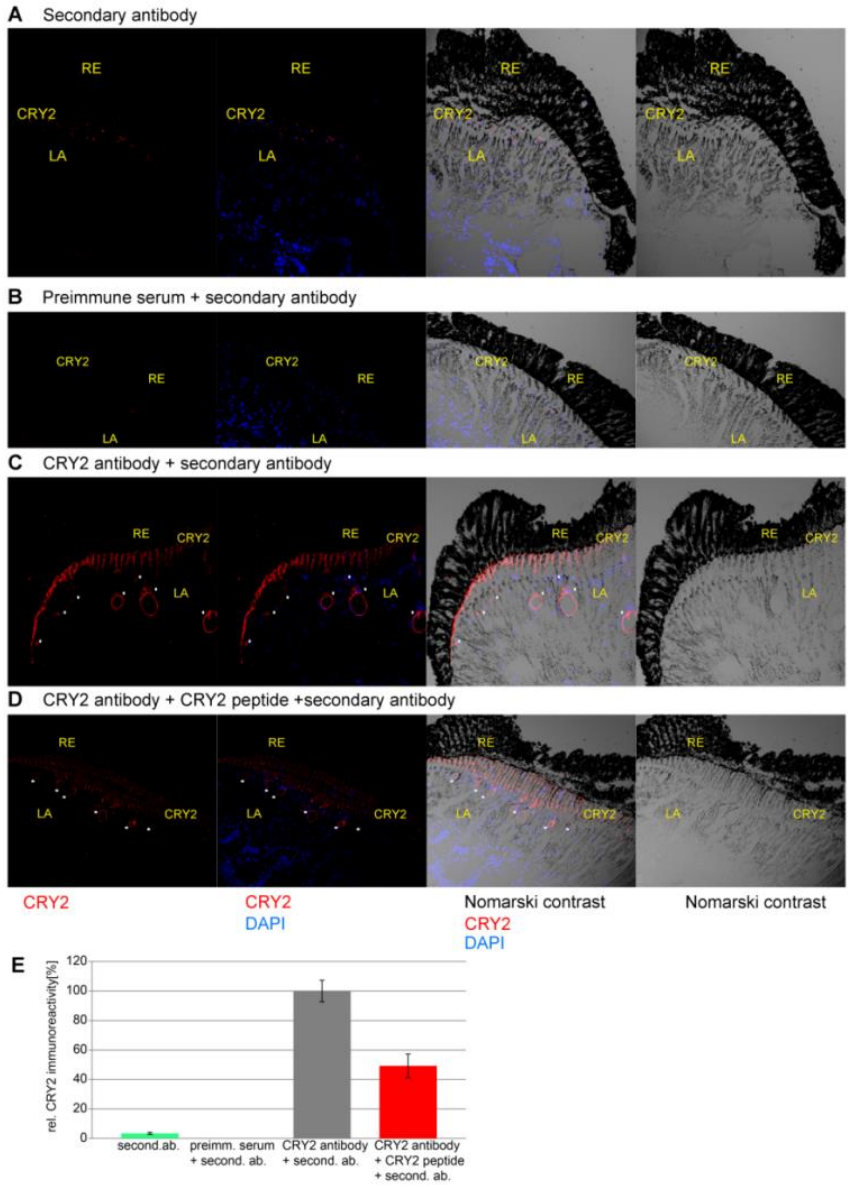


Fig. S7. To test specificity of Cry2 immunolabeling the following experiments were performed on *P. americana* sections. The secondary antibody was used alone (A) or in combination with pre-immune serum (B). Only the combination of Cry2 antibody with secondary antibody resulted in significant signal (C), which was reduced by incubation of Cry2 antibody with Cry2 peptide overnight (5 fold molar peptide excess), although only to 50% (D). The staining intensity was assessed in Image J software, error bars correspond to standard deviation of 4 independent experiments (E).

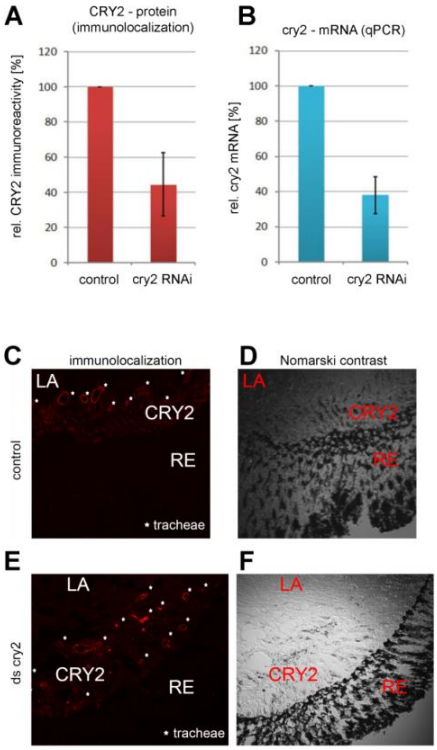


Fig. S8. *cry2* RNA interference reduces both *cry2* mRNA levels and Cry2 protein in *Periplaneta* eye. (A) Cry2 protein signal in control and *cry2* RNAi cockroaches (average of 4 independent experiments with standard deviation). (B) Relative *cry2* mRNA levels in the same animals (average of 4 independent experiments with standard deviation). (C) Cry2 immunolocalization in the eye of a control cockroach. (D) The same specimen under the Nomarski contrast. (E, F) Cry2 immunofluorescence is reduced in the RNAi treated cockroaches. White dots label nonspecific antibody binding to tracheae.

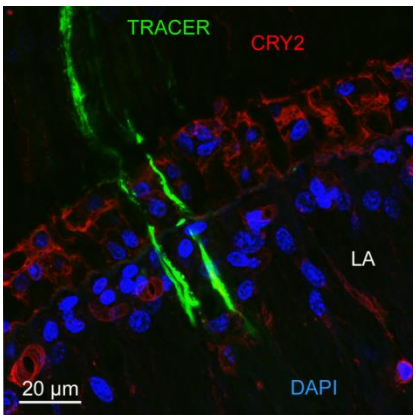


Fig. S9. The neuronal projections (green) from the *P. americana* eye ommatidia penetrate through the layer of Cry2-positive cells (red), which are arranged in columns; blue - cell nuclei (DAPI stained)

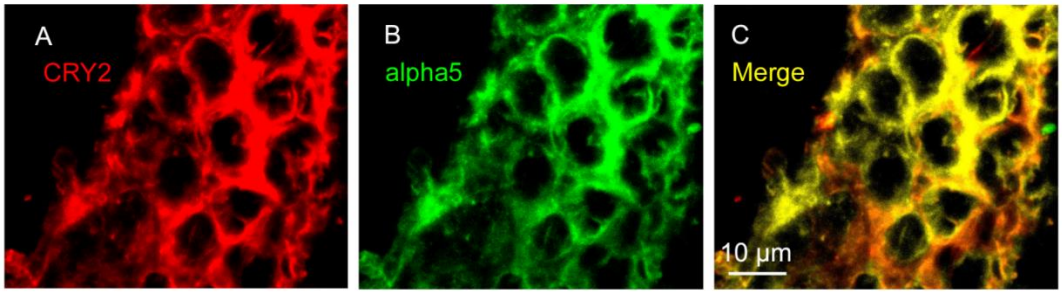


Fig. S10. Cry2-positive cells in *P. americana* (A, C) also co-express alpha subunit of sodium-potassium pump, which in visual system is mainly expressed in glial cells (B, C).

$$FAD_{ox} + X \xrightarrow{hv} FAD_{ox}^{*} + X \longrightarrow {}^{(1)}_{(3)}\left[FAD^{\bullet -} + X^{\bullet +}\right] \qquad \text{(scheme 1)}$$

$$FAD_{ox} + X \xrightarrow{hv} FAD_{ox} + X^{*} \longrightarrow {}^{(1)}_{(3)}\left[FAD^{\bullet -} + X^{\bullet +}\right] \qquad \text{(scheme 2)}$$

$$FAD_{ox} + X \longrightarrow {}^{(1)}_{(3)}\left[FAD^{\bullet -} + X^{\bullet +}\right] \qquad \text{(scheme 3)}$$

$$FADH^{-} + X \xrightarrow{hv} FADH^{-*} + X \longrightarrow {}^{(1)}_{(3)}\left[FADH^{\bullet} + X^{\bullet -}\right] \qquad \text{(scheme 4)}$$

$$FADH^{-} + X \xrightarrow{hv} FADH^{-} + X^{*} \longrightarrow {}^{(1)}_{(3)}\left[FADH^{\bullet} + X^{\bullet -}\right] \qquad \text{(scheme 5)}$$

$$FADH^{-} + X \longrightarrow {}^{(1)}_{(3)}\left[FADH^{\bullet} + X^{\bullet -}\right] \qquad \text{(scheme 6)}$$

Fig. S11. Schemes 1-6. Electron transfer (ET) reactions between the FAD cofactor of a Cry protein and an unspecified non-radical species X resulting in a radical pair. ET can be driven by light (hv) as in Schemes 1, 2, 4 and 5 or can proceed in the ground state (Schemes 3 and 6). Asterisk indicates the excited state. X can stand for a different species in each scheme. For simplicity, X is stated as a neutral species but can be also charged (negatively in Schemes 1-3 and positively in Schemes 4-6). Magnetic field affects the inter-conversion between singlet (scheme 1) and triplet (scheme 3) states of a radical pair as long as the two radicals are in a spin coherency.

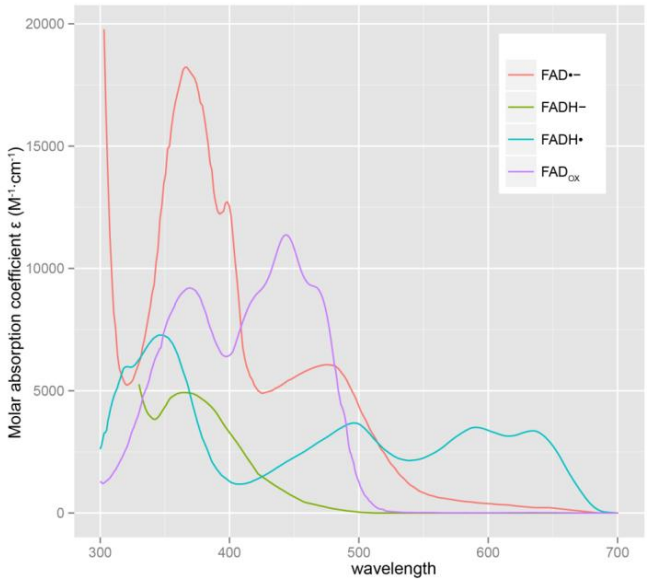


Fig. S12. Molar absorption coefficient spectra of FAD in four different redox states of Cry. Reference spectra were taken from Islam *et al* (7) for FAD_{ox}, from Palfey and Massey (8) for $FAD^{\bullet -}$ and $FADH^{\bullet}$ and from Müller (9), for $FADH^{-}$. Corrections to spectra were applied in order to reflect a protein-bound state as found in DASH-type cryptochromes (10, 11), nevertheless, the spectra are similar to those given for cryptochromes and photolyases elsewhere (12).

Table S1. Quantification of Behavioral Rhythmicity in Constant Dark – *Periplaneta americana*

	Rhythmic individuals (%)	Rhythmic (n)	Arrhythmic (n)	Tested individuals (n)
buffer	84.3	10	2	12
control dsRNA	74	20	7	27
ds cry2	16.7	5	25	30
ds tim	17.8	5	23	28

Table S2. Quantification of Behavioral Rhythmicity in Constant Dark – *Blatella germanica*

	Rhythmic individuals (%)	Rhythmic (n)	Arrhythmic (n)	Tested individuals (n)
buffer	73.3	11	4	15
eGFP dsRNA	72.7	16	6	22
ds cry2	55	11	9	20
ds cry1	34.8	8	9	23
ds cry1, ds cry2	56.5	13	10	23

Table S3. Primers used for qRT-PCR analyses – *Blatella germanica*

gene	forward primer (5´-3´)	reverse primers (5´-3´)
elongation factor 1	ACCAATCTCTGGATGGCATGG	GAGGCTTCTCAGTGGGTCTG
cry1	ACCATGGAAAGCTCCCATTG	TCTCTCGAATTCGCTGCATAT
cry2	ACGGATGAAGCAGGTATACC	CTCTAACGGTGGCGATTTCT

Table S4. Primers used for cloning dsRNA template – *Blatella germanica*

gene	forward primer (5´-3´)	reverse primers (5´-3´)
cry1-frag.1	CCGCGACAGTTCAGTG	GGTGGCTCCCCTTTATG
cry1-frag.2	TACACAATGTCTGTCAACAATG	ACAACACAACCTGCCTTTTC
cry2-frag.1	CGGGAAGGATTAAAATGTGCTAC	GGAGGCCTTCAATATCGAAACC
cry2-frag.2	CGTTTGGTCGTCCGAAAATG	GTGGAAGAACTGCTGGAAGA

Table S5. Primers used for qRT-PCR analyses – *Periplaneta americana*

gene	forward primer (5´-3´)	reverse primers (5´-3´)
actin	ACAGGGAAAAGATGACTCAGATTA	CTTCATAAATAGGTACTGTGTGCG
tim	AGGTCCTGGTGCATCTCGTAAAG	GATGGTGACTATGTGAAGTGC
cry2-frag.1	TGTTTTCTTACACGAGGTGATCTTTG	GGACGCAAAGCTGATCCTAATGG
cry2-frag.2	TACAGAGGACCAGGCTTGTT	ACGATGAAAATGGATTCCACTTT

Table S6. Primers used for cloning dsRNA template – *Periplaneta americana*

gene	forward primer (5´-3´)	reverse primers (5´-3´)
tim-frag.1	GTGCTGGACATGTTTGCAGAGA	GTCACATCTGGATCGGC
cry2-frag.1	AAGTTAATTATGAGTGAGGAAAAA	AATAACAATTGACCAT
cry2-frag.2	TCTACTGTGCAGCAACAAAGA	TGCATTTCGCTGCTTTTTG

Table S7. MIR of *B. germanica* exposed to different light wavelengths and intensities, statistically significant response is presented as red text.

λ(nm)	Light intensity (Photons s^{-1} m^{-2})	n and P
365nm	6x10e13	n=60, P=0.329
	6x10e14	n=43, P=0.001
	6x10e15	n=98, P=0.001
	6x10e16	n=60, P=0.002
388nm	6x10e14	n=115, P=0.113
	6x10e15	n=116, P=0.006
	8x10e16	n=84, P=0.003
408nm	6x10e13	n=58, P=0.218
	8x10e14	n=60, P=0.299
	7x10e15	n=135, P=0.002
	6x10e16	n=86, P=0.007
	2x10e17	n=107, P=0.715
448nm	7x10e15	n=89, P=0.118
	7x10e16	n=91, P=0.020

466nm	8x10e15	n=155, P=0.187
	6x10e16	n=72, P=0.010
505nm	9x10e14	n=73, P=0.071
	7x10e15	n=131, P=0.006
	6x10e16	n=131, P=0.002
	7x10e17	n=85, P=0.002
	5x10e18	n=78, P=0.025
528nm	6x10e15	n=59, P=0.202
	6x10e16	n=145, P=0.571
	6x10e17	n=78, P=0.645
	6x10e18	n=195, P=0.333

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EDUCATION and EMPLOYMENT:

2000 – 2004 high school, Plasy

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14.7.2007 Bachelor´s degree – field: Biomedical Laboratory Techniques

4.6.2010 Master´s degree – field: Clinical Biology

4.6.2010 Dean´s price award for excellent diploma thesis

1.7.2010 – presence

Postgraduate student – field: Molecular Biology and Genetics

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INTERSHIPS & TRAINING ABROAD:

October 2010 (2 weeks in total) – University of Würzburg, Prof. Dr. Charlotte Helfrich-Föster Lab (learning FISH technique for insect´s whole mount samples)

November – December 2011 (6 weeks in total) – University of Leicester, Dr. Ezio Rosato Lab (learning Yeast Two-Hybrid technique)

March – June 2016 (7 weeks in total) – University of Warsaw, Dr. Joanna Kotwica-Rolinska lab (learning design and application of CRISPR-CAS9 technique)

TEACHING ACTIVITIES:

- Methods of Molecular Biology (KMB 770) – each summer of 2011, 2012, 2013, 2014 practical hands-on intensive training course (duration 2 weeks) introducing methods of molecular biology to undergraduate students (~20-25 students each year)
- Advanced Methods of Molecular Biology (KMB 603) – spring 2013, 2015 practical training from quantitative PCR, yeast-two-hybrid assay

SUPERVISION OF UNDERGRADUATE STUDENTS:

- Bcl. Marion Sieber (master thesis, defended 21.1.2014): Protein-protein interaction of photoperiodic clock factors in *Pyrrhocoris apterus.*
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RESEARCH ACTIVITIES – grants:

- Impact of temperature on the circadian rhythms of *Drosophila melanogaster in vivo* (grant from university for PhD students, GAJU), principal investigator
- The role of *cryptochrome2* in magnetoreception and circadian rhythms in insect (grant from university for PhD students, GAJU), principal investigator
- Protein-protein interactions of photoperiodic clock factors in *Pyrrhocoris apterus,* (grant from university for PhD students, GAJU), principal investigator
- Architecture of an Ancestral Insect Clock – Surprising Combination of Mammalian and Drosophila Genetic Components (Czech Science Foundation) – team member
- Juvenile hormone in Insect diapause and circadian rhythms (Ministry of Education CR, Kontakt II project) – team member

MEMBERSHIPS OF SCIENTIFIC SOCIETIES:

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* equal contribution

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PRESENTATION AT INTERNATIONAL CONFERENCES:

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- The impact of temperature on the circadian clock in housefly *Musca domestica*, 13th International EMBL PhD Symposium in Heidelberg : The Rhythm of Life: Cycles in Biology (November 17 - 19, 2011) – poster.
- The impact of temperature on the circadian clock in *Drosophila melanogaster* in vivo. 1st Molecular retreat in Bejčkův Mlýn, Czech Republic (October 14-16, 2011) – talk.
- *Cryptochrome2* and its possible role in circadian clock and magnetoreception in cockroach *Periplaneta americana*. 3rdMolecular retreat in Svatý Tomáš (March 22-24, 2013) – talk.
- Protein-protein interaction of photoperiodic clock factors in *Pyrrhocoris apterus.* 7th International symposium on Molecular Insect science (July 13-16, 2014) 13-16 July, Amsterdam – posters.

- Differential expression analysis of diapause and reproductive females of firebug *Pyrrhocoris apterus.* 7th International symposium on Molecular Insect science, Amsterdam (July 13-16, 2014) – poster.
- *Cryptochromes* and their possible role in circadian rhythms of ancient insect order. 14th EBRS Congress in Manchester, UK (August 02-06 2015) – poster.
- Geographic variability of circadian clock in the Linden bug, *Pyrrhocoris apterus*. Gordon Reasearch Conference, Chronobiology, Girona, Spain (July 2015) – poster.

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