Inter and intraspecific clock gene variations in Drosophila

Thesis submitted for the degree of Doctor of Philosophy at University of Leicester

By

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Abstract

Circadian clock genes have undergone many structural and functional modifications during their evolution. Even in closely related evolutionary lineages, the circadian molecules can be variable and perform the same or different tasks. I studied three important clock genes *Cryptochrome (cry), period (per)* and *timeless (tim)* in *D. melanogaster.* The study of the *cry* L232H polymorphism revealed no difference in the distribution of the two alleles across Europe. Population cages with different initial *cry* allelic frequencies, nevertheless converged to a ~1:1 ratio after 16 generation, mimicking natural population frequencies. The analysis of locomotor activity in the laboratory showed a temporal difference in the phase of activity for the males and females, with female cry^{HH} and male cry^{LL} active significantly earlier than other genotypes. If this increases the probability of disassortative mating, intermediate frequencies of the two alleles might be generated.

I also studied the intermolecular co-evolution between the two interacting circadian proteins TIM and PER. The individual *per* and *tim* transgenes from *D. pseudoobscura* in *D. melanogaster* mutant hosts showed more than 50% rhythmicity but very long (29h) period for *per* and very short period (21h) for *tim*. By combining them in the *D. melanogaster* double mutant background, the hemizygous flies showed no improvement in rhythmicity but an excellent rescue of periodicity of ~24h. This suggest that TIM and PER may form a heterospecific coevolved module that interacts more robustly with the other host clock proteins.

Finally, using my transgenes and null mutants I showed that *Par Domain Protein 1ε* (*Pdp1ε*) participates in the expression of residual rhythmicity of mutants for the negative limb of the circadian clock.

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List of Abbreviations

bp	base pair
DD	constant darkness
DNA	deoxyribonucleic acid
g	grams
h	hour
L	litre
LD	light dark cycle
DD	continuous dark
min	minute
mel	*D. melanogaster*
n.a.	not available
ns	not significant
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
ps	*D. pseudoobscura*
sec	second
SD	standard deviation
SEM	standard error of the mean
SNP	Single Nucleotide Polymorphism
ZT	zeitgeber time

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Chapter 1: Introduction

1.1 Circadian Rhythms

Circadian rhythms are the subset of biological rhythms that cycle with ~24 hour periodicity (Dunlap and Loros 2004). Most organisms from cyanobacteria to humans have circadian rhythms which adapt them to regular external environmental changes. The term 'circadian' was coined by Franz Halberg during the late 1950s meaning "approximately one day" (*circa* means "around" and *dies* means "day") (Pittendrigh 1954). Circadian rhythms are observed in the biochemical, physiological or behavioural processes of plants, animals, bacteria and fungi. The formal study of biological rhythms such as daily, tidal, weekly, seasonal, and annual cycles is called chronobiology, but circadian oscillations are the most extensively researched.

Although the earliest known account of circadian processes date back from the 4th century BC by Androsthenes, the first formal written account comes from Jean Jacques d'Ortous de Mairan in 1729 who was a French geologist. He studied the helioptrope plant in the total darkness and noticed that its leaves opened during the day and closed during the night showing a self-sustained rhythmicity of about 24 hours in the absence of any external cues such as light and temperature (Hall 1995). Carolus Linnaeus, who was the great 18th century botanist, also maintained a garden that

showed the time by planting flowers that opened or closed their blossom an hour apart (Hall 1995).

Circadian rhythms are ubiquitous features of life. They are important regulatory mechanisms which are almost universally used to harmonise physiological and behavioural processes with the 24 h geophysical cycles of light and temperature of our planet (Sandrelli et al. 2008). The presence of circadian control systems in the simple organism like cyanobacteria shows the fact they have evolved at the very early stages of life on Earth around two billion years ago (Markson and O'Shea 2009). During the Cambrian period (540 million years ago) circadian rhythms would probably have been around 21 h, reflecting the period of the rotation of the Earth at that time. However, modern organisms have evolved in response to their perpetual 24 h cycling environment and, under constant conditions in the laboratory, model higher organisms such as mice and flies show corresponding behavioural rhythms (Rosato and Kyriacou 2006). The evolution of the circadian clock has provided the advantage of predicting the environment rather than merely responding to the changes. This likely enhances Darwinian fitness and so the circadian clock became ubiquitous (Pegoraro and Tauber 2011). These clocks regulate sleep-wake behaviour, cognition, feeding, heartbeat, blood pressure, kidney functions, and almost all aspects of digestion as well as cell division (Gachon et al. 2004).

Clock have been sought in all three kingdoms, and although very infrequently found in the Archaebacteria, they exist in some cyanobacteria (Golden et al. 1998), are found frequently among the Eukaryota, and in almost all the taxa that evolved

during the Cambrian phylogenetic explosion, the Plantae, Fungi and Animalia (Sogin 1994).

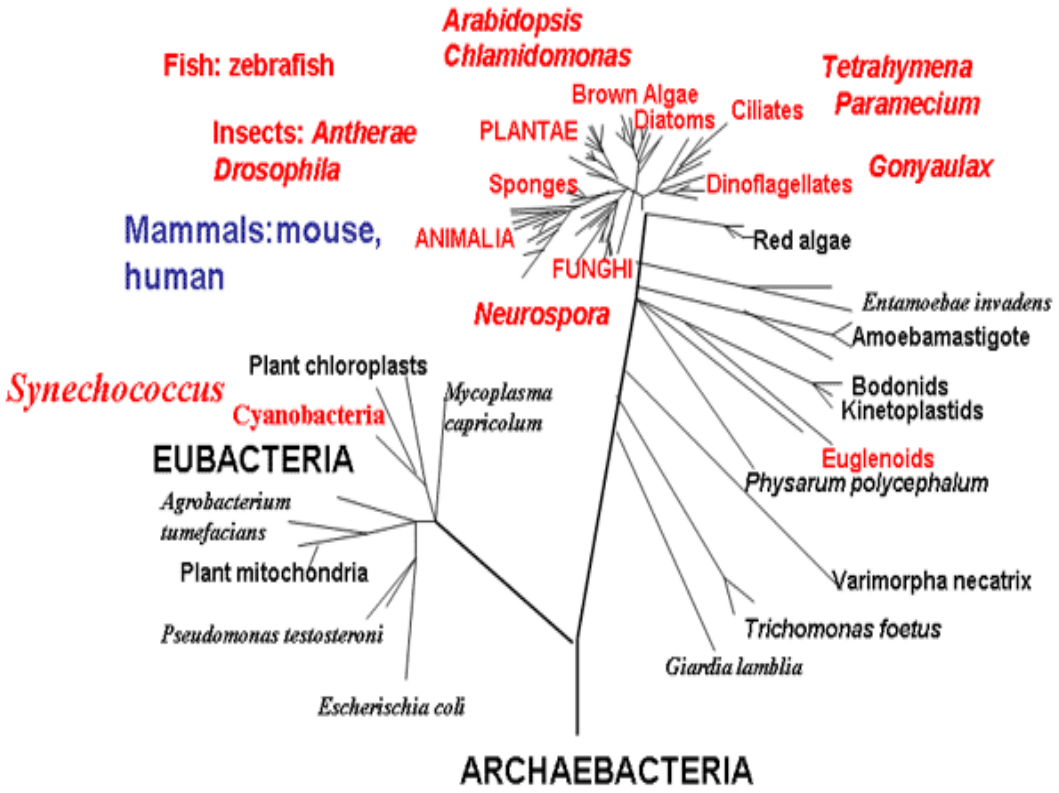


Figure 1.1: Circadian system in the universal tree of life. An unrooted universal phylogenetic framework reflecting a maximum-likelihood analysis for the relationships among organisms. The three major assemblages of organisms, Archaebacteria, Eubacteria, and Eukaryota, diverge from a single ancestor (Dunlap 1999). In blue and red are the groups in which the circadian clock has been described

The organism's internal molecular clock can be synchronized with the external cues of the 24 h light/dark cycle, a process termed entrainment. The environmental inputs that entrain the circadian clock include light, temperature, social stimuli and food availability, but light is the most potent ' Zeitgeber' (time-giver). The term "Zeitgeber" is German in origin and was used by Jurgen Aschoff (one of the founders of chronobiology) in 1960. Entrainment provides an animal the advantage to anticipate

environmental changes like dawn and dusk and modify its behaviour. This way metabolism, physiology and behaviour can be tuned to occur at the appropriate times (Dunlap and Loros 2004). Studies using cyanobacteria revealed that those strains with an endogenous circadian period that resonated with the periodicity of their light-dark environment were able to out-compete other strains which had different circadian periods (Ouyang et al. 1998). Similar observations have been reported from plants. Arabidopsis plants that were unable to predict day length were less viable as compared to the other wild type counterparts (Green et al. 2002). Cavefish which lived in total darkness for millions of years possess an internal clock which uses food as a timing cue and display an approximately 48 h period (Cavallari et al. 2011).

A genuinely endogenous circadian clock persists in constant conditions, such as darkness, and under such conditions, the clock “free-runs” (Pegoraro and Tauber 2011). This criterion allows a distinction between circadian rhythms from those "apparent" rhythms that are merely responses to external periodic cues. The recurrence of the circadian rhythms is approximately but not precisely 24 h. There are certain other criteria on the basis of which they can be distinguished from coincidental or apparent cycles. The rhythm is temperature-compensated, i.e. it maintains the same period over a range of temperatures (Pittendrigh 1954). The reason for this is to distinguish circadian rhythms from other biological rhythms arising due to the circular nature of a reaction pathway. At a low enough or high enough temperature, the period of a circular reaction may reach 24 h, but it will be merely coincidental and not temporally programmed. Also the rhythms can be adjusted to match local time: Travel across time zones illustrates the necessity to adjust the biological clock so that it can

reflect the local time. Until rhythms are reset, a person usually experiences 'jet lag' leading to desynchronization of physiological activities (Waterhouse et al. 2002).

The endogenous molecular oscillations that constitute the circadian clock drive rhythmic physiological and behavioural phenotypes. The latter are relatively easy to observe and quantify, and they provide a glimpse of their underpinning molecular cycles and of the neuronal organization of the circadian network. The circadian clock of Cyanobacteria controls many of the important mechanisms e.g. nitrogen fixation, photosynthesis and cell division. Up to 60% of the entire *Synechococcus elongatus* transcriptome is under circadian control (Vijayan et al. 2009). In plants both cellular and system physiology is regulated by the circadian clock, ranging from leaf movement, photosynthesis and flowering to the transport of nutrients and growth patterns (Adams and Carré 2011). In insects the adult's locomotor activity and the eclosion of the adults from pupae are the two extensively studied phenotypes which are controlled by the circadian clock. Among higher organisms, mice and humans are the examples of the most studied. The daily rhythms in their body temperature and the sleep/wake cycle are under circadian control (Young, 1998). There are individuals who suffer with the advance sleep syndrome with a short-period 22 h endogenous clock (Toh et al. 2001), delayed sleep phase syndrome (Archer et al. 2003) and also seasonal affective disorder (Murray et al. 2003). All these are known to be caused by circadian defects. Some other disorders which are associated with the circadian dysfunction are jet lag (disruption in the body's physiological rhythms after rapid transitions across the time zones) and shift work which results in the loss of appetite

and general body fatigue increasing the risk of disease and thus affecting life span (Waterhouse et al. 2002: Castanon-Cervantes et al. 2010).

1.2 Clock Genetics

The basic model for the circadian clock has three main components (Figure 1.2). The first component is the input pathway which will entrain the clock to external cues like temperature and light. Then there is a central oscillator which has a self-sustained pacemaker and functions continuously even in the absence of input and the third component is the output giving the physical or physiological behaviour enabling the organism to react to the daily changing environment. This is an oversimplified model but has been proven very useful to understand the basic concept. Sometimes the components of clock input pathways feed into the clock outputs e.g. photoreceptors (Lopez-Molina et al. 1997). It has also been observed that the clock output components affect the pace of the central oscillator through a feedback on the clock itself (Roenneberg and Merrow 1998).

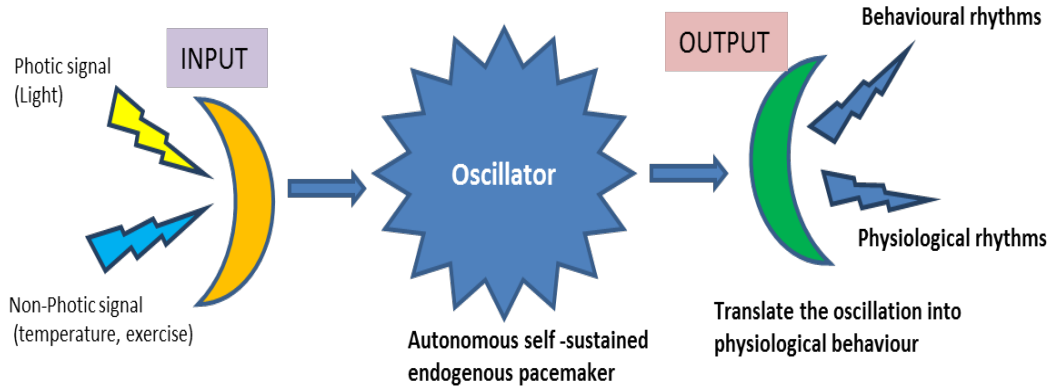


Figure 1.2: A basic model presenting the three components that generate circadian rhythms.

At the cellular level, molecules controlling the circadian clock are cyclically transformed. Phosphorylation, degradation and altered subcellular localization of clock proteins result in the modified expression of clock and clock controlled genes, ultimately coordinating the activities of the organism with its environment (Peschel and Charlotte Helfrich-Förster 2011; Jeffrey C Hall 2003; X Zheng and Amita Sehgal 2008). From the studies with cyanobacteria, *Neurospora,* Drosophila and mice, the basic molecular mechanisms underlying the circadian oscillators have been uncovered. Comparison of the sequence of the clock genes with each other shows that the circadian components of *Drosophila* and mice and even some of the components in plants show a high degree of sequence similarity (Bae et al. 2000).

1.2.1 Why Drosophila?

These insects have short generation time, the ease of screening large population of mutant animals. There are many other reasons which also speak in the favour of this little insect. Drosophila's genes have been studied for more than a century and many techniques and bioinformatics tools are available to study its behaviour at the molecular level. The locomotor activity of fruit fly is a very obvious and easily measurable circadian pattern of behaviour. Another important factor is the simplicity of the neuronal network underlying the circadian clock (Peschel and Helfrich-Förster 2011).

1.2.2 *period*

Neurogenetics as a field can be traced back to the behavioural screening of the different eclosion rhythms in Drosophila by Ronald Konopka and Seymour Benzer in

1971. They identified three mutants that eclosed at different times of the day, one had short period rhythms of 19h, and another was long (28h), while the third was arrhythmic. These allelic mutations defined the *period* gene, and were identified at the molecular level in 1984 (Bargiello et al. 1984; Bargiello and Young 1984; Reddy et al. 1984). These and later molecular analyses revealed important insights into the molecules and mechanisms underlying circadian oscillator function, that generalise to all rhythmic organisms.

per is located on the long arm of the X chromosome. It is the best characterized of all genes that control or influence the fly's circadian and ultradian (recurrent periods or cycles repeated throughout a day) rhythmic behaviour, specifically the circadian periodicity of locomotor activity and the emergence of adult from pupae (Hall, 2003). When the *per* transgene is transformed into per^{01} (arrhythmic) hosts, circadian rhythms are rescued (Bargiello et al. 1984). PER is a 1218 amino acid protein (Citri et al. 1987) and levels of PER protein and its mRNA oscillate out of phase with each other in a way which suggests that PER is a negative regulator of its own transcription (Hardin et al. 1990).

Figure 1.3 : A diagrammatic representation of PER showing major domains.

Comparison of the *per* coding region among *D. melanogaster*, *D. pseudoobscura* and *D. virilis* reveals that the translation products consist of interspersed blocks of six conserved (c1-c6) and five non conserved (n1-n5) regions (Colot et al. 1988). The longest c2 region includes the three classic *per* mutations. It also includes the PAS (named for PER, ARNT, and SIM, the first three proteins

identified with this motif) domains consisting of two degenerate 51 amino acid direct repeats. These regions are protein-protein dimerization domains (Crews et al. 1988) and are essential part of those proteins which acts as environmental sensors via light, oxygen and redox potential (Gu et al. 2000). The conserved region c2 also includes the cytoplasmic localization domain CLD involved in retaining PER in the cytoplasm (Saez and Young 1996), the n2 includes the Threonine-Glycine repeat region involved in temperature compensation (Sawyer 1997) and stabilization of PER (Dembinska et al. 1997). PER also contain a NLS (Nuclear Localization Signal) which leads to the down regulation of *per* transcription (Huang et al. 1993). A CLK: CYC inhibition domain (CCID) at the C terminal of the protein (Figure 1.3) is also known to be the part of that gene product which corresponds to the binding site for PER's molecular partners CLOCK and CYCLE (Chang and Reppert 2003).

1.2.3 *timeless*

timeless was the second clock gene to be discovered after *per*. It is located on the second chromosome (Sehgal et al. 1994) and the TIM product has 1389 amino acids including an NLS, a CLD and two PER interaction domains PER1 and PER2 (Saez and Young 1996; Kamae and Tomioka 2012).

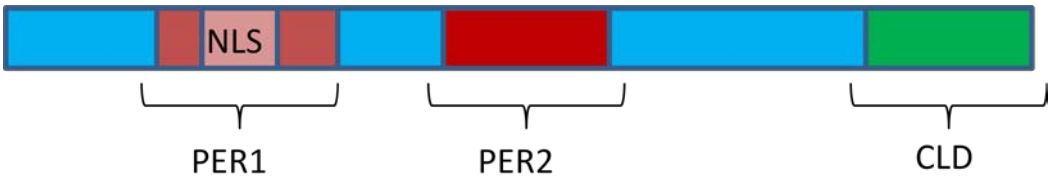


Figure 1.4: A diagrammatic representation of TIM with functional domains indicated and labelled.

There are two *timeless* paralogue in Drosophila, *timeless1* and *timeless2* (timeout) (Benna et al. 2000). *tim1* is known as *tim* and its function as a circadian clock gene is well established (Zheng and Sehgal 2008). *tim2* is required for the chromosome stability and circadian photoreception (Benna et al. 2010).

In nature, two major allelic variants of *tim* can be found in *Drosophila melanogaster* : one allele produces a 23 amino acid N-terminally shortened and more light-sensitive form of TIM (*s-tim*), the other allele encodes both the long L-TIM and truncated form (*ls-tim*) (Sandrelli et al. 2007; Tauber et al. 2007). TIM is a cardinal component of the circadian clock (Young 1998) and its light sensitivity is generated via its physical interaction with the circadian blue-light photoreceptor Cryptochrome (Ceriani 1999; Lin et al. 2001). A null mutation, tim^{01} produced by an intragenic deletion of 64 bp, produces an arrhythmic phenotype in constant darkness (DD). Like *per*, *tim* is also a negative regulator of its own transcription and for this reason no cycling of the *tim* mRNA has been observed in tim^{01} flies (Sehgal et al. 1994). PER cycling between the nucleus and cytoplasm is also very much reduced in tim^{01} mutants (Sehgal et al. 1994) revealing that TIM has some role to play here. In Drosophila TIM is required for the stability and cyclic expression of *per* (Zheng and Sehgal 2008). Studies both *in vivo* and *in vitro* suggested that PER and TIM form dimers, thought initially to be essential for the nuclear entry of the complex (Saez and Young 1996) but other studies shows that PER can enter nucleus alone and in fact it enters the nucleus earlier than TIM (Shafer and Rosbash 2002; Chang and Repper 2003). TIM has been reported to play a critical role in the timed nuclear entry of PER (Hara et al. 2011).

tim does not exist in the honey bee genome (Rubin et al. 2006) and is not an essential component of the circadian clock in the cricket *Gryllus bimaculatus* (Danbara et al. 2010) but its presence in the ancestral insect the firebrat suggest that *tim* was originally involved as an essential component in the insect circadian clock (Kamae and Tomioka 2012).

1.2.4 Drosophila CLOCK and CYCLE

The sequences of PER and TIM suggest that there is no DNA binding domain present in either so how do they regulate their own transcription? The identification of mouse *Clock (mClk*) mutant was an important step in answering this question (King et al 1997 a&b). The basic Helix Loop Helix (bHLH) –PAS containing mCLK suggested that it may be driving the cycle of *mPer* (King et al. 1997). In Drosophila a homologue of *mClk* was identified by mutagenesis and the mutant called *Jrk* was found to be arrhythmic in the homozygous condition so this gene was renamed as $dClk^{jrk}$ (Allada et al. 1998). A second transcription factor containing a bHLH-PAS domain was identified in Drosophila which was again found to be essential for the transcriptional activation of *per* and *tim.* This was called *cycle* (*cyc*) and homozygous mutant flies for this gene were observed to be arrhythmic (Rutila et al. 1998). This gene was found to be the homologue of mammalian *Bmal1* whose product was found to form a heterodimer with mCLK (Hogenesch et al. 1998). dCLK and CYC also interact with each other (Rutila et al. 1998) and this heterodimer activates the transcription of *per/tim* through the E-box sequences in their promoters. *dClock* and *cyc* are located on the long arm of chromosome 3 (Allada et al. 1998).

Another interesting finding was that like *per* and *tim*, *dClock* is also expressed rhythmically through an autoregulatory feedback loop (Lee et al. 1996). The two basic zipper (bZIP) transcription factors VRILLE (VRI) and Par Domain Protein 1ε (PDP1 ε) were found to affect the repression and activation of *dClk* expression respectively (Blau and Young 1999; Cyran et al. 2003). The level of *vri* RNA was found to be oscillating in similar phase as *tim* and was also expressed in per^{01} flies (Blau and Young 1999) suggesting that *vri* may also be CLK/CYC controlled. Being an essential developmental gene, the homozygous mutants for *vri* are lethal but the heterozygotes have short locomotor activity periods (Blau and Young 1999). The heterozygotes for the *Pdp1* ε mutants showed a longer period suggesting *vri* and *Pdp1* ε have opposite effects on *Clk* (Cyran et al. 2003).

1.3 The feedback loops

The Drosophila circadian oscillator is composed of three intracellular feedback loops in gene expression: a *per/tim* loop, a *Clk* loop (Hardin et al. 1990; Glossop, 1999). and a *Clockwork orange* (CWO) loop (Kadener et al. 2007). Within these feedback loops, rhythmic transcription of particular clock genes is controlled via feedback from their own protein products (Hardin 2005). These feedback loops utilize a range of mechanisms to regulate transcription in different phases of the circadian cycle.

1.3.1 Post-translational modification

A critical requirement for the negative transcription/translation feedback loops is to allow the appropriate time-lag so all these process work on a ~ 24h cycle. The posttranslational modifications of these clock proteins play a very important role for

this purpose. They are suggested to regulate the period, phase and amplitude of the circadian clock rhythms (Bae and Edery 2006). The most important modification is the phosphorylation of proteins like PER and TIM which regulates their timing of nuclear entry, their degradation and also their interaction with other proteins to form complexes (Mehra et al. 2009). The kinases and phosphatases which perform post translational modifications also work rhythmically resulting in production of degradation signals. They are also important for the recruitment of a variety of additional factors that regulate their binding (Kojima et al. 2011).

In Drosophila Casein Kinase 1 (CKI) also called DOUBLETIME (DBT) reduces the transcriptional activity of CLK by phosphorylating it (Yu et al. 2006). CK1 also phosphorylates PER which reduces its stability (Eide et al. 2005). Another kinase which effect Drosophila clock proteins is CKII (Casein Kinase 2). It is responsible for the phosphorylation of PER and TIM. It is basically a complex of two subunits and the mutation in either of them leads to a delay in the nuclear translocation of PER resulting in a longer period (Akten et al. 2003). SHAGGY (SGG) is encoded by the gene *shaggy/GSK-3* and plays an important role in phosphorylating TIM. SGG facilitates the nuclear transport of the PER/TIM complex. Lowered SGG activity leads to decreased TIM phosphorylation resulting in a longer period (Martinek et al. 2001). Protein phosphatase 1 (PP1) was also found to be important for the stability and nuclear localization of TIM. The inhibition of this phosphatase results in a delay in nuclear localization of TIM and the fly shows a longer period of activity (Fang et al. 2007). Protein phosphatase 2A (PP2A) is important for the stability of PER. Its overexpression results in longer periods (Sathyanarayanan et al. 2004). SLIMB (SUPERNUMORARY LIMBS) is an F-box protein. PER phosphorylation by DBT produces an atypical SLMB

binding site where SLMB binds and PER is ubiquitinated and rapidly degraded through the ubiquitin-proteosome degradation pathway (Chiu et al. 2008). Another ubiquitin ligase CULLIN-3 (CUL-3) also physically interact and forms a complex with the hypophosphorylated version of TIM in the absence of PER, allowing its accumulation over the night (Grima et al. 2012).

1.3.2 The core feedback loop

For initiating this loop, CLK-CYC heterodimers bind E-box regulatory sequences (usually *CACGTG)* from mid-day (~ZT4 to ~ZT18) through early night, thereby activating transcription of *per* and *tim* (Allada and Chung 2010; Darlington et al. 1998; McDonald et al. 2001; Wang et al. 2001). The level of *per* and *tim* transcription peaks early in the night, whereas their respective proteins do not accumulate to peak levels until four to six hours later (Hardin et al. 1990; Zeng et al. 1996). This delay is the result of phosphorylation dependent destabilization of PER by DBT (DOUBLETIME) and possibly also CK2 (Casein Kinase 2) resulting in the low level of monomeric PER (Akten et al. 2003; Kloss et al. 1998; Nawathean and Rosbash 2004). Recent data suggests that post-translational regulation may mediate the delay in accumulation of PER in the cytoplasm (Lim et al. 2011). TIM concentration increases in early night, which stabilizes PER by preventing PER degradation and binds to the DBT/PER complex forming PER/DBT/TIM (Kloss et al. 2001; Lin et al. 2001). By midnight this whole complex is translocated into the nucleus upon SGG-dependent TIM phosphorylation and CK2 dependent PER phosphorylation (Lin et al. 2001; Martinek et al. 2001). SGG plays a vital role at this stage by phosphorylating TIM, and speeds up the process of nuclear entry of the complex. (Martinek et al. 2001). The dephosphorylation at this stage

balances the nuclear localization of PER and TIM and is performed by protein phosphatase 2A (PP2A) and protein phosphatase 1(PP1) (Fang et al. 2007; Sathyanarayanan et al. 2004). The speed of the oscillation depends upon the accumulation of PER/TIM in the early night and their degradation in the late night. Their stability at this stage is controlled by SLMB E3 ubiquitin ligase which marks the phosphorylated PER for degradation (Grima et al. 2002). Its role is negatively regulated by NEMO which phosphorylates PER at the S47 site (Chiu et al. 2011).

PER phosphorylation continues even inside the nucleus which potentiates PER's ability to repress transcription (Nawathean and Rosbash 2004). Late at night TIM dissociates from the TIM/DBT/PER complex and PER/DBT represses CLK-CYC dependent transcription of *tim* and *per,* by binding to CLK and inhibiting the DNA binding activity of CLK-CYC dimers from~ZT18 to ~ZT4 (Menet et al. 2010; Rothenfluh et al. 2000). Nuclear degradation of PER by DBT kinase results in a decline in its level, which reactivates the positive elements CLK and CYC the following day which bind to *per/tim* E-boxes and the cycle starts again. The nuclear localization of PER and its repressor activity can occur independently of TIM as well (Rothenfluh et al. 2000). Drosophila S2 cells shows PER both in cytoplasm as well as in nucleus (Chang et al. 2003). The repressor activity upon CLK/CYC also continues but at a low level (Nawathean and Rosbash 2004). TIM is essential for the stability of PER (Vosshall et al. 1994) so at ZT0 when the lights are turned on, TIM is degraded and PER is no more protected (Lee et al. 1996; Zeng et al. 1996). Inside the nucleus progressive phosphorylation of PER by DBT triggers the binding of E3 ubiquitin ligase SLIMB which results in the proteosomal degradation of PER at ~ZT4 (Grima et al. 2002; Kloss et al.

1998). Once PER is degraded CLK starts accumulating again, binding to CYC and CLK-CYC dimers binds the E-boxes and start transcription of *per* and *tim* again.

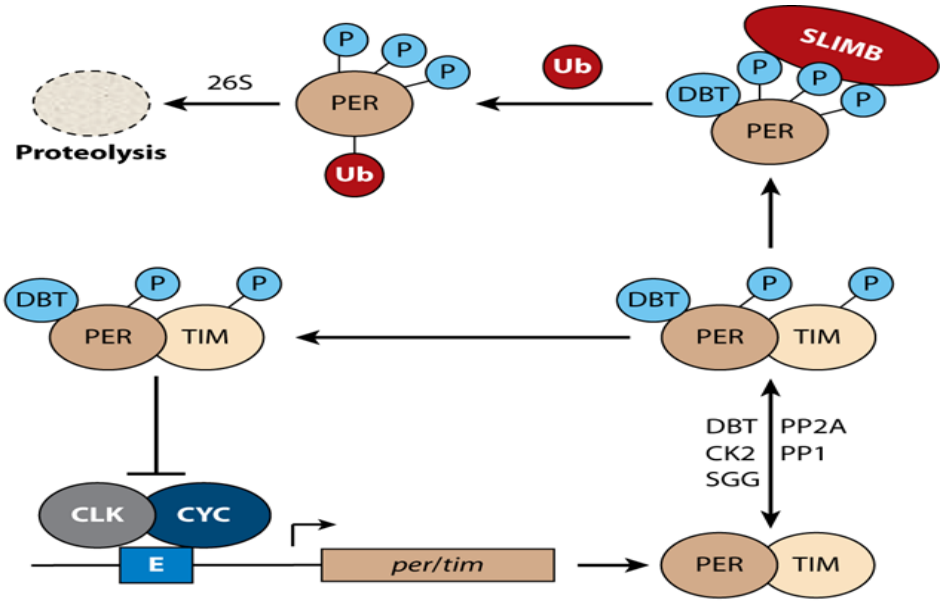


Figure 1.5: The core molecular clock in Drosophila. CLOCK/CYCLE (CLK/CYC) bind to E-box elements (E) contained in the promoters of *period (per) and timeless (tim*). PER and TIM proteins are modified by the kinases DOUBLETIME (DBT), CASEIN KINASE 2 (CK2), and SHAGGY (SGG) and the phosphatases PROTEIN PHOSPHATASE 2A (PP2A) and PROTEIN PHOSPHATASE 1 (PP1). PER and TIM dimerize and transition to the nucleus, where they repress CLK/CYC activity. Phosphorylated PER and TIM also bind the E3 ubiquitin ligase SUPERNUMERARY LIMBS (SLIMB), which leads to ubiquitination and ultimately proteolysis by the 26S proteasome (Figure from Allada and Chung 2010).

1.3.3 The CLK feedback loop

CLK/CYC also activates the *Clock* loop, which plays an important role in the phase and amplitude of the rhythmic output through the core oscillators (Allada and

Chung 2010). CLK/CYC directly activates the transcription of *Pdp1ε* and *vri* (Blau and Young 1999; Cyran et al. 2003). Both proteins bind to the V/P boxes in the promoter region of *Clk,* where *vri* inhibits and *Pdp1ε* activates *Clk* transcription (Cyran et al. 2003). The two proteins VRI and PDP1ε peak with different phases. The peak of VRI before PDP1ε results in the repression of *Clk* transcription but after few hours PDP1ε displaces VRI and activates *Clk* transcription (Cyran et al. 2003; Glossop et al. 2003). Due to the delay in the accumulation of PDP1ε activator relative to the VRI repressor the cycle of *Clk* transcription is in antiphase to that of *per/tim* (Cyran et al. 2003). The peak of *Clk* mRNA is observed in the early day in contrast to the *per/tim* peak which is early night.

1.3.4 The CWO feedback loop

CLK/CYC also activates a bHLH repressor*, clockwork orange (cwo).* Its role is to bind the CLK/CYC target E-box and repress CLK/CYC mediated transcription (Lim et al. 2007; Richier et al. 2008). CWO also acts as an activator. Studies show that it has an important role in the circadian clock function as *cwo* mutants have long periods with a low amplitude cycle (Kadener et al. 2007; Richier et al. 2008). The expression of CLK appears to be dependent on CWO and for this reason the level of CLK was reduced in the *cwo* mutants (Matsumoto et al. 2007). As CLK/CYC has a role in activating the components of many feedback loops, this suggests that CLK may act as a master regulator (Kilman et al. 2009).

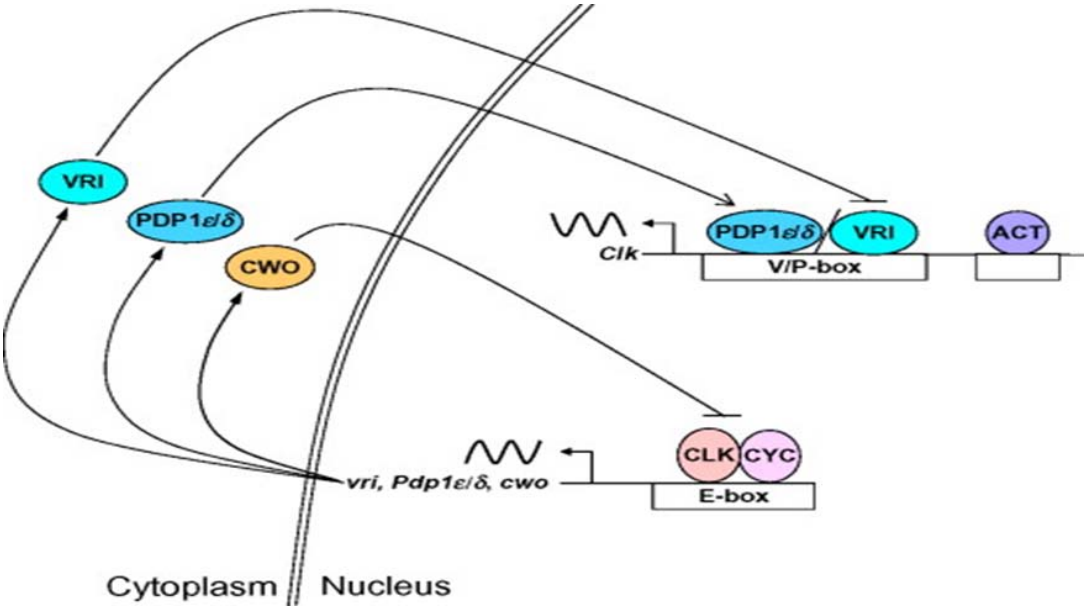


Figure1.6: The *Clk* feedback loops (Hardin 2011). (For detail see text)

1.4 Light Entrainment

The environmental cycles of light, temperature and social stimuli entrain the circadian oscillators and enable the physiological and behavioural rhythms to occur at appropriate time of the day (Hardin 2011). Light is the principal stimulus that re-sets biological clocks each day. Left to their own devices, such clocks are, indeed, only *circa*-dian in terms of the pace at which they run (Hall 2000). However, light input at dawn will advance a clock and the same input at dusk will delay it. Second, one imagines from first principles that a 'light-to-clock system' would have distinct attributes that differ from those of the visual system components that are involved in image formation (Hall 2000).

Against this background, it was perhaps not surprising that specialized photoreceptive molecules, *Cryptochromes* (CRYs) a term originally invoked for plant photoreceptors that underlie various responses to blue light, were identified (Stanewsky, et al. 1998; Ivanchenko et al. 2001). These molecules are relatives of

photolyases-DNA repair enzymes that are activated by the absorption of blue light (300-500nm) (Cashmore, 1999). Photolyases use blue light to reverse the deleterious effects on DNA induced by far-UV light (200-300nm) whereas *Cryptochromes* acts as light sensors able to reset and synchronize the circadian clock to the solar day. These molecules were first identified in *Arabidopsis thaliana* and then in prokaryotes, archaea, and many eukaryotes. They are also known for their putative role as magnetoreceptors in migratory birds (Chaves et al. 2012).

In fly heads, CRY protein level increase in the dark and decline in the light (Emery et al. 1998; Lin et al. 2001) meaning that CRY abundance is thus driven by the environmental LD cycle: in constant light (LL) CRY is completely degraded. CRY levels thus cycle in LD, but in constant conditions, only *cry* mRNA cycles, and so is driven by the circadian mechanism (Busza et al. 2004; Dissel et al. 2004). CRY is regulated both at transcription and posttranslational levels.

CRY contains a conserved photolyase domain and a unique carboxy-terminal domain (Cashmore, 1999). On stimulation by light, the carboxy terminal domain is thought to change conformation or release an inhibitor to reveal a TIM binding site (Busza et al. 2004; Dissel et al. 2004; Rosato et al. 2001). CRY then binds TIM which is associated with the PER/DBT complex, triggering its degradation by the proteasome (Naidoo et al. 1999). CRY also interacts with JETLAG (JET) an F-box protein involved in the resetting mechanisms of the circadian clock. The affinity of JET for TIM is greater than for CRY (Peschel et al. 2006).

Light induces a conformational change in CRY allowing it to bind to TIM. As a result of this interaction, a posttranslational modification occurs in TIM which enables it to bind to JET. The affinity of S-TIM (from the *s-tim* allele) to JET is greater compared

to L-TIM, which leads to more efficient degradation of S-TIM by JET and stabilization of CRY. As long as JET triggers the degradation of TIM, CRY would be spared. After TIM levels have decreased to a critical amount, CRY becomes the prime target of JET. Possibly the degradation of CRY allows the re-accumulation of TIM in the next circadian cycle and for that reason TIM levels start to increase during the late day (Peschel et al. 2009).

Light acts through the eye and extraocular pathways (Stanewsky et al. 1998; Suri et al. 1998; Yang et al. 1998) resulting in the rapid turnover of TIM protein, and since TIM stabilizes PER, PER also disappears. Thus, in the late day and early evening, a time when PER and TIM are increasing, a light-induced decrease in PER/TIM results in a delay, back to the low point of PER and TIM in the day. Conversely in the late night and early subjective morning when PER/TIM levels are normally falling, the same light induced destruction of PER and TIM results in their premature disappearance and results in an advance into the next day (Hunter-Ensor et al. 1996; Lee et al. 1996; Myers et al. 1996; Yang et al. 1998; Zeng et al. 1996).

In *D. melanogaster*, there appears to be only one cryptochrome-encoding gene that encodes 569 amino acid residues (Emery et al. 1998; Ishikawa et al. 1999; Stanewsky et al. 1998). Drosophila's cry^b mutant, caused by a missense mutation that results in barely detectable levels of CRY protein, results in the doubly-defective nature of this mutant: nonresponsiveness of TIM on the one hand, and lack of free-running molecular rhythmicity of TIM in the eyes on the other (Stanewsky et al. 1998).

Most research work that led to the spectacular advances in understanding the molecular mechanisms of circadian clock was conducted under simplified laboratory conditions involving controlled cycle of the light and dark condition and temperature.

However under natural conditions there is a continuous variation of light condition and temperature during the day and season (Vanin et al. 2012). The cycling in clock proteins and the rhythms in the locomotor activity was both observed to be dramatically different from those described under laboratory conditions. Neither PER nor TIM could be detected in November and flies were found to be completely inactive (Menegazzi et al. 2013). Drosophila has been always described as crepuscular because the activity profile in the laboratory conditions has a morning and evening peak only (Rieger et al. 2007). Vanin et al. (2012) compared the amount of activity in the twilight against the total amount of activity during the day under natural conditions. Their finding suggested these flies were diurnal because the total amount of activity during twilight was less than 25% of the total activity.

1.5 Temperature entrainment: *per* splicing

The studies using *D. melanogaster* as model organism showed that over a wide range of photoperiod and temperature, the activity profile is roughly aligned with Light: Dark conditions (Rieger et al. 2003). The main factor which tunes the temporal distribution of morning and evening activity is "temperature". There is an increase in the amount of nocturnal activity with increasing temperature which is certainly an adaptive response (Majercak et al. 1999). For example in colder days the flies will increase their midday activity to take advantage of the warmer temperature. In hot days they shift their activity towards the cooler night time to decrease the risk associated with the expenditure of energy during the hot part of the day (Low et al. 2012). This temperature dependent behavioural adaptation is controlled by

temperature dependent splicing of a 3'-terminal intron from *D. melanogaster period (per*) transcript (Majercak et al. 1999; Low et al. 2012). Two types of transcripts are produced by *per* which differ from each other in the alternatively spliced intron with in the 3'untranslated region (UTR) named dmpi8 (*D. melanogaster per* intron8); Type A contains the 89bp intron while the type B does not have this intron (Figure 1.7).

per splicing is the key aspect of the temperature response by circadian clock. Relatively more of spliced variant B is produced at lower temperature than type A variant which means more splicing of the *per* RNA 3' intron at lower temperature. This leads to the advance in accumulation of PER and thus a change in the phase of activity. The flies increase their day time activity in the colder temperature (Low et al. 2008). When the splicing by dmpi8 was prevented in the transgenic flies, they displayed preferential nocturnal activity even during the colder days (Majercak et al. 1999). *per* splicing by dmpi8 is also effected by clock and photoperiod and it has been observed that the longer days prevent the intron removal (Collins et al. 2004; Majercak et al. 2004). As the change in the day length is associated with temperature change so this interplay between the photoperiod and temperature suggest that dmpi8 splicing plays a central role in the seasonal adaptation of *D. melanogaster* (Low et al. 2008). The splicing is reduced at the warmer temperature which changes the accumulation of *per* mRNA and leads to a longer mid-day siesta (Majercak et al. 1999; Low et al. 2008). The activity of dmpi8 is a result from suboptimal 5' and 3' splicing signals (ss) which results in the binding of the dmpi8 to the spliceosomes. This process is inefficient at the higher temperatures, so the transgenic flies which had their dmpi8 5' and 3' ss optimized, had nearly all of the dmpi8 introns removed at all temperature and the flies displayed less robust mid-day siesta than the control flies (Low et al. 2008).

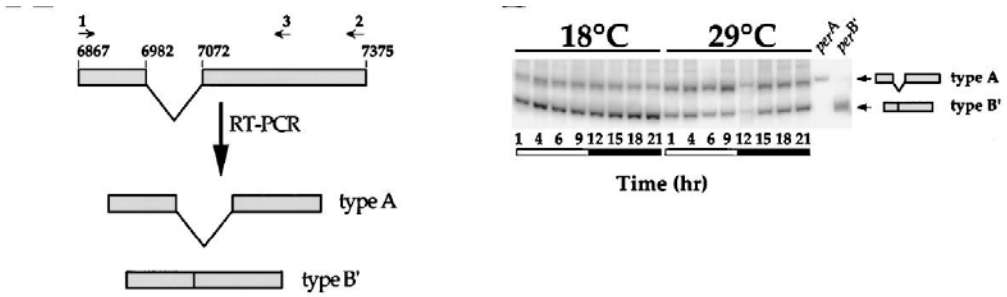


Figure 1.7: Graphical representation of the per splicing showing two types of variant also the RT-PCR products are shown in an agarose gel which differ from each other in size.

Findings by Vanin et al. (2012) suggest that the dependency of dmpi8 splicing over the temperature and photoperiod could be only part of the molecular mechanism underlying behavioural adaptation to seasonality in *D. melanogaster.* A linear relationship was seen in nature between *per* 3'splicing and temperature over the entire range from 7-30^{o}C, but only at temperatures > 22^{o}C did the locomotor behaviour correlate with the splicing. This probably means that only at very hot summer temperatures is the splicing mechanism of any adaptive value.

1.6 The neuronal pathway

Drosophila circadian behaviour is controlled through ~ 150 identifiable neurons in the brain (Helfrich-Förster 2005; Nitabach and Taghert 2008). They are organized into two clusters the lateral and dorsal neurons which are further divided in to six sub-clusters. The first cluster made of three dorsal neurons (DN1-3); the dorsal lateral neurons cluster (LNd) ; the lateral posterior neurons (LPNs) and then are the ventral lateral neurons (LNv) which are basically in the form of two clusters, the large (lLNv) and the small (sLNv) (Dubruille and Emery 2008). There is a specific role for each cluster in controlling either one or other aspects of circadian rhythms. PDF (Pigment

Dispersing Factor) is the critical neuropeptide effector for ~20 LNv. The other groups of neurons do not express PDF (Dubruille and Emery 2008). PDF is secreted in the vicinity of dorsally located neurons through the dorsal projection of sLNv and most of the DNs respond to the PDF neuropeptide (Park et al. 2000). The LNds form a heterogeneous group of six cells, with one cell larger than the others and only three to four of them express the photoreceptor CRY (Picot et al. 2007).

The sLNv neurons determine the behaviour of the flies in free-running conditions (DD) and are important for the morning peak of activity in LD12:12 conditions, so they are referred to as 'morning cells' or 'M' (Grima et al. 2004). The M-cells required PDF for their function (Renn et al. 1999). There is also a set of neurons called "evening cells", or 'E'. They include the LNds, the PDF-negative fifth sLNv and also a subset of DNs. The E-cells control the phase of activity in continuous light (LL) (Picot et al. 2007). The role of these two groups of neurons reflects adaptive seasonal responses to the short winter and long summer days (Stoleru et al. 2007). The role of light seems to be important here again, and it has been observed that E-cells process light information and function as master clocks in the presence of light while M-cells need darkness to cycle autonomously and dominate the neuronal network. The results indicate that the network switches control between these two centres as a function of photoperiod (Stoleru et al. 2007). The l-LNv cells are involved in the sleep/wake cycle and phase shifting in the dawn-advance zone (Shang et al. 2008; Sheeba et al. 2008). In the larval brain temperature entrainment is also controlled by DN2 cells (Picot et al. 2009).

Surprisingly, observations of genetically "clockless" genotypes in the wild have revealed both morning and evening components which suggest that in nature the

canonical clock genes, particularly *per* and *tim,* are not essential for quasi-normal entrainment (Vanin et al. 2012). However some subtle effects of per^{01} in the wild have been detected recently (Menegazzi and Yoshii 2013). It appears that in the wild other neurons outside the circadian circuit may be recruited (Vanin et al. 2012).

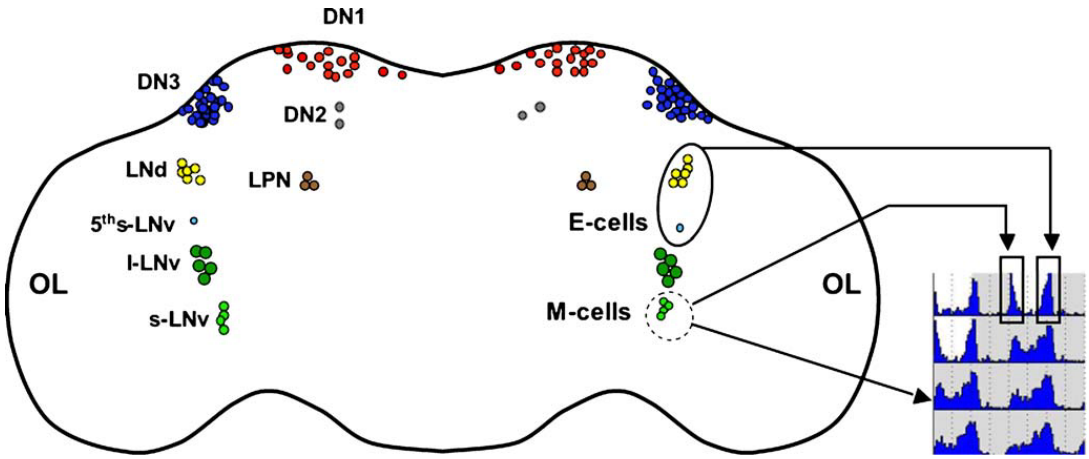


Fig. 1.8: The circadian clock neurons in the Drosophila brain. The small ventrolateral (s-LNvs) and the large ventrolateral neurons (l-LNvs) are the only PDF-positive cells. The fifth s-LNv, the dorsolateral neurons (LNds), the lateral posterior neurons LPNs, and the three groups of dorsal neurons (DN1s, 2s, and 3s) do not express PDF. The s-LNvs are the M-cells, since they are necessary for the morning peak of activity under LD conditions. They are also necessary for free-running locomotor activity rhythms in DD. The fifth s-LNv and the LNds generate evening activity (E cells) under LD conditions OL Optic lobe (Dubruille and Emery 2008).

1.7 Clocks in other organisms

The regulation of clock proteins by negative feedback loop was also described in the fungus, *Neurospora crassa*, where the negative feedback loop of the FREQUENCY (FRQ) protein was shown to autoregulate its own transcription (Aronson et al. 1994). The fungal clock proteins WC1-2 and VIVID share close homologies with mammals and Drosophila in certain domains such as the PAS domain, also found in PER, CLOCK. The main feedback loop mechanism also operates through a similar

pathway (Baker et al. 2012). The transcription of *frq* is driven by white collar (WC) complex that is made of WC1 and WC2 proteins. The interaction of its protein (FRQ) with an RNA helicase, FRH leads to its repression (Figure 1.9 A). The photoreceptor VIVID has roles similar to Drosophila CRY By interaction with both repressor FRQ/FRH complex and activator WC complex, it promotes the clock proteins degradation and modulates transcription (Brown et al. 2012).

Unlike Drosophila; mammals have most of the clock components present in multiple copies which increases the complexity of the system (Clayton et al. 2001). CRY1 and CRY2 have replaced TIM of Drosophila and act as the main transcriptional repressors (Gotter et al. 2000). The mammalian TIM orthologue has more similarity with the Drosophila TIM2 (TIMEOUT) but its role in the mammalian circadian clock is still not clear (Gotter et al. 2000). In Drosophila, the studies on TIM2 show that it has a limited role in the light entrainment of the circadian clock yet as it is the ancestral TIM protein, it illuminates the evolution of the paralogue, TIM, which has taken over the main circadian light entrainment pathway (Benna et al. 2010).

In mammals CLK and BMAL1 (the dCYC Homologue) dimerize through their PAS domains at the start of the day and activates the transcription of the *mPer1-3* genes and *Cry1-2.* The corresponding proteins accumulated over the day and enter the nucleus in the evening where they inhibit the activity of mCLK-BMAL1. This way the transcription of *mPer* and *Cry* is repressed resulting in the decline in the level of their mRNA allowing a new transcription cycle to start (Figure 1.9 B) (Gallego and Virshup 2007; Green et al. 2008). Another transcription loop also participates here where the expression of BMAL1 is activated by RORa and inhibited by REV-ERBα (Emery and

Reppert 2004). Mammalian-like clock mechanisms are found in other insects which suggests their divergence from a common design (Sandrelli et al. 2008).

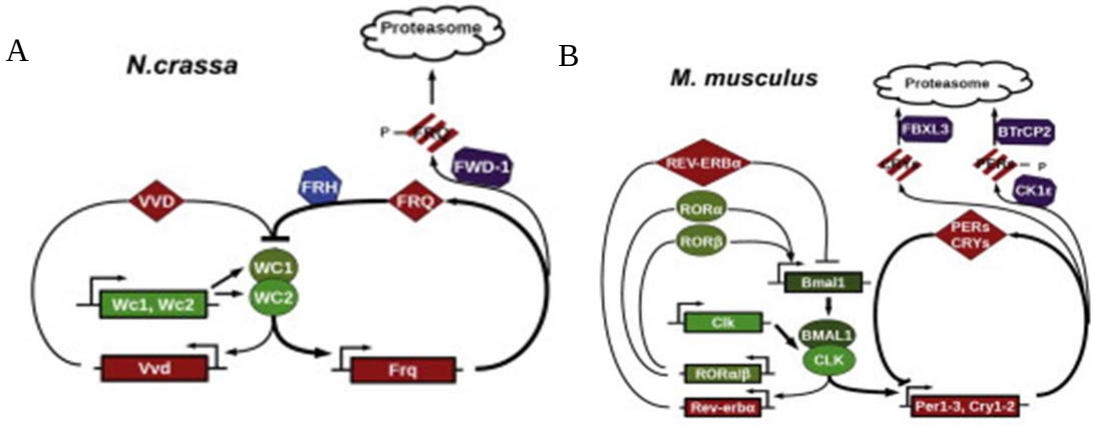


Figure 1.9: circadian clock feedback loops in *Neurospora crassa* (A) and *Mus musculus* (B) (Brown et al. 2012).

A number of insects have been used to compare circadian clock mechanisms including the yellow fever mosquito *Aedes aegypti,* sandfly *Lutzomya longipalpis*, giant silkworm *Antheraea pernyi*, the domestic silk moth *Bombyx mori,* monarch butterfly *Danaus plexippus*, coackroach *Leucophaea maderae*, cricket *Gryllus bimaculatus* and extensive studies have been conducted on the circadian clock of honeybees as well (Rubin et al. 2006). The comparison of circadian clock components in all these insect gives an idea of the ancestral clock of insects which must have consisted of two CRYs, two TIMs (mammalian and Drosophila as discussed earlier), and CYC/BMAL1 which was the main transcriptional activator (Sandrelli et al. 2008). The circadian clocks of Lepidoptera have two *cry* genes, one is Drosophila like which acts as a photoreceptor (*cry-d*) and the other is mammalian type *cry-m* which has the ability to repress CLK/CYC mediated transcription (Yuan et al. 2007). Both types of *cry* genes are also found in the

Anopheles gambiae and *Aedes aegypti* while honey bee *Apis mellifera* and *Tribolium castaneum* have only mammalian like *cry-m* (Rubin et al. 2006; Yuan et al. 2007).

Two sibling species of Queensland fruit fly *Bacterocera tyroni* and *Bacterocera neohumeralis* show temporal separation by timing of their mating rhythms (Smith 1979) mediated via CRY levels in day vs dusk mating (An et al. 2002). Similar observations were obtained with *B. cucurbitae* (Miyatake et al. 2002). They showed a highly significant change in their free running circadian periods when millions of sterile males were released in the successful Okinawa Prefectural Fruit Fly Eradication program (Koyama et al. 2004; Shimizu et al. 1997). In the process of generating millions of these flies, there was an inevitable selection for a fast developing line, which also displayed a short period of 22h (S-line). A slower developing line has a longer period of 29h (L-line) (Shimizu et al. 1997). These remarkable and somewhat fortuitous observations resonate with the Drosophila *per* mutants per^{S} and per^{L} which showed a difference of ~5h in their period but which also change development time in a parallel fashion (Kyriacou et al. 1990) in *B. cucurbitae*. This difference in period and the associated change in mating time was the only factor responsible for reproductive isolation (Miyatake et al. 2002). Difference in the time of the mating was also observed while comparing the mating rhythms of *D. melanogaster* and *D. pseudoobscura* (Tauber et al. 2003). The mating rhythms of *per-null D. melanogaster* flies carrying *D. pseudoobscura per* transgenes were later in the day as in *D. pseudoobscura*. When mixed together transformants carrying *melanogaster* or *pseudoobscura per* transgenes showed clear temporal isolation in their mating rhythms (Tauber et al. 2003). A study

of *D. melanogaster* and *D. simulans* also showed a clear species specific mating time that was determined by *per* (Sakai and Ishida 2001).

Among other Diptera the clock genes of *Musca domestica* have studied in detail (Codd et al. 2007; Piccin et al. 2000). The cycling of *Musca per, tim, Clk* and *vri* mRNA were the same as described for *Drosophila* except MdCRY which like Bacterocera and unlike Drosophila is not cycling in DD. MdTIM degrades immediately in LL while MdPER remains highly stable which suggest that unlike Drosophila, MdPER does not need MdTIM for stability (Sandrelli et al. 2008).

Orthologues of Drosophila *per* have been identified in many dipterans, including the housefly *Musca domestica* (Piccin et al. 2000), the sheep blow fly *Lucilia cuprina* (Warman et al. 2000), the melon fly *Bactrocera cucurbitae* (Miyatake et al. 2002) and two related species, the Queensland fruit fly *Bactrocera tryoni* and its sibling *Bactrocera neohumeralis* (An et al. 2002). *per* gene has also been identified in non-Dipteran species *Antheraea pernyi*. In this giant silk worm *per* is only 39% identical to that of the Drosophila at the amino acid levels but still it could rescue the circadian rhythms of per^{01} *D. melanogaster* mutants (Levine et al. 1995). The *mps1* transgenes (Peixoto et al. 1998; Petersen et al. 1988) contained the full *per* coding sequence from *D. pseudoobscura* attached to the *melanogaster* promotor generates very weak rhythms in per^{01} transformants whereas the *Musca per* transgenes showed 80-100% rescue for behavioural rhythmicity (Piccin et al. 2000). Phylogenetic analysis shows surprisingly, that the PER-PAS domain of *Musca* PER is more similar to *Drosophila melanogaster's* than is *D. pseudoobscura* PER. This is even though *Musca* last had a common ancestor with *D. melanogaster* 100 Mya, whereas *D. pseudoobscura* diverged

about 30 Mya (Piccin et al. 2000). Could it be that *Musca* PER interacts with the other clock components in the Drosophila heterospecific host (dTIM, dCLK, dCYC) more efficiently than does *D. pseudoobscura* PER, giving rise to these anomalous interspecific rescues? Perhaps if some of these other clock components from *D. pseudoobscura* were co-transformed with PER, rescue might become more robust? Such a scenario would suggest that these clock proteins co-evolve with each other in their protein-protein dimerization domains.

1.8 The present project

The present work includes comparative genetic and behavioural studies related to three important clock genes; *per, tim* and *cry.*

cry in *D. melanogaster* is highly polymorphic in the coding region (Tauber E pers comm.). A survey carried by Pegoraro et al (2012, submitted) to study genetic variation in *cry* alleles from European and North-American populations revealed the presence of many nucleotide polymorphisms within the coding region of the gene. One of these (nucleotide position 695) leads to an amino acid substitution in which the codon *CTT* at position 694-696 corresponding to Leucine (L), is altered to *CAT,* (Histidine, H). Flies harbouring either the L232 or the H232 allele showed significantly different circadian light responses (M. Pegoraro, pers comm.). In these initial studies, fly populations from different latitude were amplified by PCR to investigate the distribution of the two *cry* alleles. The preliminary results showed a cline in cry^{HL}. My project aims to further investigate this cline by genotyping more populations. The effect of this SNP on the phenotype of the flies will also be studied by comparing

locomotor activity period, circadian photosensitivity and eclosion rhythms of the different populations carrying cry^H and cry^L.

The second part of my research work involves intermolecular coevolution between *per* and *tim* genes. As mentioned earlier, *per* transgene from *Musca domestica* rescue the locomotor activity rhythms *of D. melanogaster* per^{01} mutant more efficiently than the *D. pseudoobscura per* transgene (Piccin et al. 2000). In an attempt to explain the differential success of the interspecific transformation of the clock genes in the present study I attempted to improve (or reduce) the levels of rescue over those seen by Peixoto et al. (1998) in the *D. pseudoobscura mps1* transgene by transforming these flies simultaneously with *D. pseudoobscura per* and *tim*. If rescue is improved, this would support the molecular coevolution idea mentioned earlier.

Chapter 2: Materials and Methods

2.1: Fly stocks

Fly stocks were maintained in the laboratory in plastic vials containing maize food. The maize food was prepared by mixing 72 g maize meal, 80 g, glucose, 50 g brewer's yeast and 8.5 g agar with one litre of water. When the food was cooling down, 2 g/L of Nipagine (methyl p-hydroxybenzoate], dissolved in 10 ml 100% ethanol) was also added to prevent the fungal growth. Flies were raised under two types of temperature condition; under 18^{o}C where they had a long developmental time of about 25 days, and 25^{o}C where they could complete their development from egg into an adult in 11-12 days. The light: Dark conditions were 12:12 LD in both of the rooms.

In first part of the project regarding *cry*, two lines were used. One of them was a Canton-S line while the other one was a natural line from Rende (Italy). For the study of the intermolecular coevolution between the biological clock genes *per* and *tim;* a total of 12 *D. melanogaster* transgenic lines were used. The details of these fly lines are as follows.

2.1.1 *per* transgenics

The *mps1* transformant lines *I20* and *I26* were used in this study. They express the *D. pseudoobscura per* transgenes coding sequence fused to the promoter of *melanogaster per* and carry the *rosy^{+} (ry^{+})* eye marker (Peixoto et al. 1998; Petersen et al. 1988). The transgene is located on chromosome II in I20 and chromosome III in I26.

2.1.2 *tim* transgenics

Three transformant lines containing *pseudoobscura-tim* were used for this study. These lines made by Dr. Eran Tauber and they had full length *psuedoobscura* TIM coding sequence attached to *melanogaster* promoter. All of them were marked with *w+*. One strain was *w; pseudoobscura-tim219 (19)* on a tim^{01} background. The insert is also located on the second chromosome. The second strain labelled *w; pseudoobscura-tim219 (21)* is on the tim^{01} background and is located on II chromosome. The third line used was *w; pseudoobscura-tim35 (14)/CyO* on a tim^{01} mutant where the insert was located on chromosome II. The insert was balanced with *CyO* because the homozygotes are non-viable. I had inherited all these lines and considerable preliminary effort was required to confirm their genotypes using PCR and genetic crosses.

2.1.3 *D. melanogaster* controls

Two transgenic strains containing the *D. melanogaster* transgenes for *per* and for *tim* in the corresponding null background and carrying *white* eye markers were also used. These were per^{01},*w/Y ;* per^{mel} /+ (*2A*) (Peixoto et al. 1998) and *w/Y;* tim^{01} tim^{mel} / tim^{01} (*t28s*) (Sandrelli et al. 2007) respectively. Three lines represented negative controls *w*; tim^{01} and *w*, per^{01} and the double mutant *w,* per^{01}*;* tim^{01}

2.1.4 The "HU" fly lines

Houton (HU), is a natural isolate from The Netherlands (52^{o}N) collected in the early 2000's (Tauber et al. 2007). By genetic crossing and PCR genotyping a homozygous line was created for the polymorphism that carried *s-tim* (Sandrelli et al. 2007; Tauber et al. 2007). Mutant genes were "Houtenized" by repetitive backcrossing

for 8 generations with the *s-tim* HU flies. The following HU lines were used in this project.

***per*^{01} HU**: This is the classic clock mutant characterized molecularly by a nucleotide substitution which results in the stop codon (Q464STOP) (Yu et al. 1987). These mutant do not produce any protein.

***tim*^{01} HU**: These flies are obtained by P- element mutagenesis (Sehgal et al. 1994). The mutation is the result of the frame shift in the coding sequence caused by the deletion of 64bp (Myers et al. 1996).

***Clk*^{jrk} HU**: This mutant was isolated by DNA mutagenesis (EMS induced) with a nucleotide substitution resulting in the STOP codon (Allada et al. 1998).

***cyc*^{01} HU**: An EMS induced mutation resulting in the stop codon (K159STOP) (Rutila et al. 1998).

***per*^{01}*; tim*^{01} HU**: This line was created by crossing the *per*^{01} and *tim*^{01} lines together using chromosome marker and balancer.

2.2: Behavioural Analysis

Circadian locomotor activity of flies was recorded in Trikinetics monitors (Waltham, Ma, USA). Individual male flies were loaded into glass tubes containing sugar food. The one end of the tube was closed with a cap and other end with cotton plug. Behavioural analysis was performed for all strain both at 25^{o}C and 18^{o}C. Initially the flies were kept for three to four days in LD12:12 and in DD for a further 7-10 days. The activity events were arranged into 30 min bins using DAM (Drosophila Activity Monitoring) file scan. CLEAN, a high resolution spectral analysis was used to obtain the

period, as well as autocorrelation, and actograms of individual flies were also inspected (Rosato and Kyriacou 2006). A fly was called rhythmic only when the activity profile was observed to be significantly rhythmic by both methods (Peixoto et al. 1998; Sawyer 1997). A fly was classified as having complex rhythms if it had multiple significant rhythms. A rhythm was considered weak if the autocorrelation was significant beyond 95% level but fell below r=0.1 after one or two peaks (examples are shown in the Figure 2.1). The average genotype locomotor activity profiles over 24 h were constructed in excel by using "Befly!" software created by Dr Ed Green (Allebrandt et al. 2010).

Circadian phase shifts were probed by a 20 minute light pulse at ZT15 and ZT21 after which flies were maintained in DD for several days to examine the change in phase.

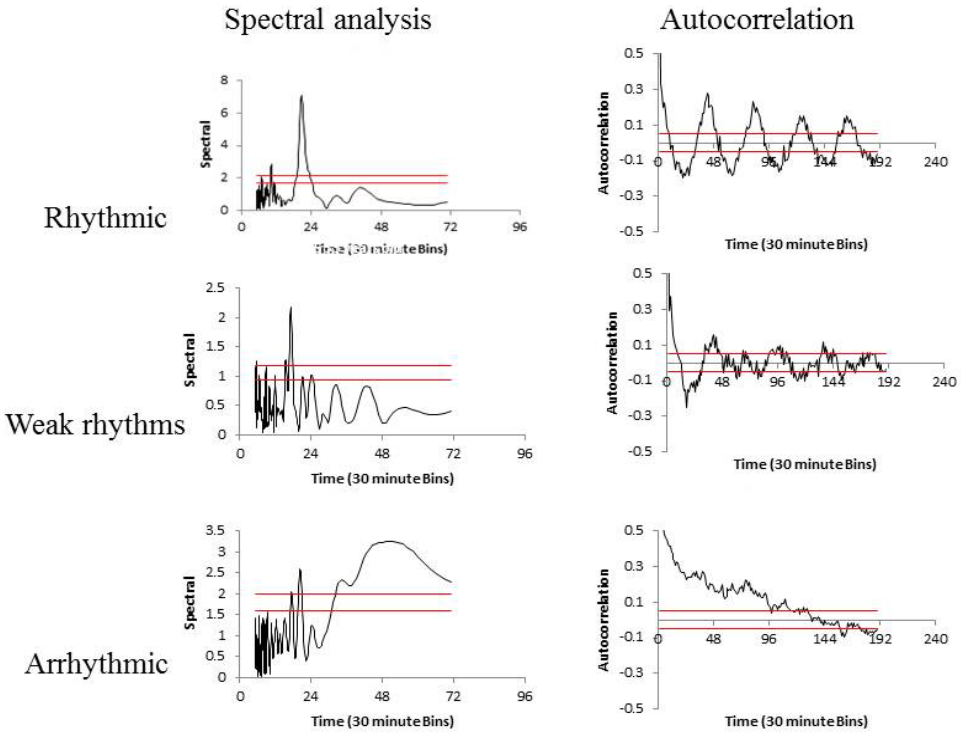


Figure 2.1: Examples of the spectral analysis and autocorrelation graphs used to assess the pattern of activity.

2.3: DNA extraction

Individual flies were preserved in ethanol and stored at -20^{o}C. For DNA extraction ethanol was taken out and flies were crushed with a yellow pipette tip which was already filled with 100µl of DNA extraction buffer (10mM of Tris HCl, 0.25mM of EDTA, 25mM of NaCl, 1.6µl of Proteinase K diluted from the frozen stock). These samples were incubated at 37^{o}C for 25-30 minutes and then heated up to 95^{o}C to inactivate the Proteinase K. 1-2.5µl of this mixture was usually used for 10 µl of PCR reaction volume.

In case of on-going genetic crosses where the fly was required to be genotyped and then crossed, the wings were used for the genotyping. For this purpose wings were cut off from the bodies of the unconscious flies using a needle and forceps. These wings were then transferred to the vials contain 20ul of the DNA extraction buffer. They were incubated in them over night on 37^{o}C. Next day these wings were soft so they were chopped with a tip in the same solution and then incubated for 1-2 h again on 37^{o}C. The enzyme was inactivated after this by heating up the vials on 95^{o}C for 5min.

2.4 Polymerase Chain Reaction (PCR)

This technique was used for the amplification of genomic DNA. It was used for the amplification of cry^{H} and cry^{L}, *ls-tim* and *s-tim*, per^{+} and per^{01}, tim^{+} and tim^{01}, *psuedoobscura-tim* and *pseudoobscura-per* genes. The primers were designed using primer3 version 0.4.0 (Rozen and Skaletsky 2000). They were first tested on a temperature gradient to find out the optimum temperature for their amplification. A total volume of 10µl of master mixture was prepared per individual reaction. The

relative quantity of the reagents used in each of them varied for each gene. The generalized method used for setting up the mixture was as follows:

The buffer used for amplification contained the following components.

Buffer (5X): 500µl of reaction buffer, 300µl of magnesium chloride, 10µl dATP'sdCTP's, dTTP's, dGTP, 160 µl of H_2O

4.25µl of PCR H_2O

0.5 µl each of forward and reverse primers

0.25 µl of Taq

7.5 µl of the mixture was added to the wells of PCR plate.

2.5 µl of genomic DNA (to be tested) was added in each so the total volume became 10 µl.

Then the plate was placed in PCR machine. The protocol used was

Step	Temperature	Time duration
1	92^{o}C	2minutes
2	92^{o}C	30 seconds
3	54.5^{o}C(varies for different amplification)	30 seconds
4	72^{o}C	1 minute
5	Repeat step 2 for 35 cycles	
6	72^{o}C	10 minutes
7	10^{o}C	Forever

After the completion of PCR, the amplified DNA segments were incubated for digestion for 2-3 h if required. The product was then loaded to gel to check/ separate the product.

2.5: Gel Electrophoresis

An agarose gel of 1-2% concentration was used for the separation of the amplified DNA. It was prepared by dissolving 2.25g of standard agarose in 150ml of 1xTBE buffer in microwave, a small quantity of Ethidium bromide (3-4.5µl) was added before solidifying it. 2µl of loading dye (0.25% Bromophenol Blue and 0.25% Cyanole) was laded in to the gel, along with the amplified DNA. Phi marker was diluted by adding 6µl of loading dye and 3µl of it was used with each gel. Gels were run in 1 x TBE at the voltage of 100 for 50-60 minutes and then analysed under UV light, the molecular weight was find out by comparing to the marker.

2.6: Cage Experiment

Four population cages were used for this experiment. In each of them 500 flies were placed in the following genotypic frequencies 90% *s-tim; cry*^{HH} and 10% *s-tim; cry*^{LL} (225 *s-tim; cry*^{HH} virgins, 225 *s-tim*; *cry*^{HH}; males; 25 *s-tim* ; *cry*^{LL} ; virgins and 25 *s-tim* ; *cry*^{LL} ; males) flies were set for the experiment. In cage 3 and 4 the proportion of the two genotypes was reversed i.e. 90% *s-tim; cry*^{LL} and 10% *s-tim; cry*^{HH}.

The cages were kept at 25^{o}C in LD 12:12. Food was changed at the rate of 6 vials per 10 days. The cage takes 12 vials and so no food vial remained for more than 20 days. After one month the old food vials removed are kept in 25^{o}C room. About 48 flies (half PCR plate) that emerged in them were sampled and genotypes for the frequency of *cry*^{H} and *cry*^{L} each month.

2.7: Statistical Analysis

For the analysis of the phase data “Oriana” was used (Kovach Computing Services, UK). It is a statistical programme which works with directional data measured

in degrees. It is most useful for looking at patterns in the time of day, week, month or year. It expresses the data in the form of circular statistics (Figure 2.2). Circular statistics were used to draw the graphs for representing the delay, advance as well as phase data. The statistical package "R" was also used for ANOVA. R is a statistical program which can be downloaded as freeware. It provides a wide variety of statistical (linear and nonlinear modelling, classical statistical tests, time-series analysis, classification, clustering) and graphical techniques, and is highly flexible. Prism and statistics-5 was also used for the analysis of data.

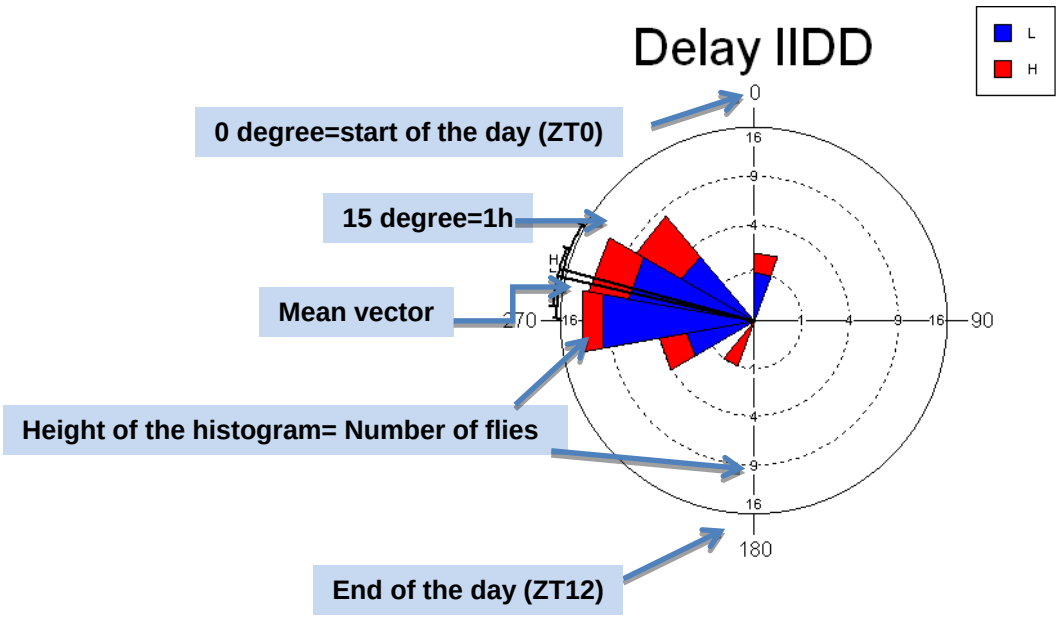


Figure 2.2: An example of the chart made in "Oriana". These graphs are used for the comparison of particular phase of activty in the 24h day. The whole 360° cycle is equal to the 24h day. Day starts at 0, goes clock wise and 15° is equal to 1h. Height of the histogram shows the number of flies observing a particular phase at that time.

2.8 Western blots

2.8.1 Protein extraction

About 50 Flies were collected in liquid nitrogen in sealed tubes. The tubes were than vortexed with maximum speed in order to break the frozen heads off. The tubes were kept back in the liquid nitrogen and the heads were separated from the rest of the body parts through a sieve used over dry ice. Frozen heads were passed in to the eppendorf. About 100µl of protein extraction buffer was added in to each eppendorf. The mixture was homogenized with micropestles and centrifuged on 4^{o}C for 5 minutes. The supernatant was transferred in to a new eppendorf and frozen on -20^{o}C. The composition of the buffer was as following.

20mM Hepes (pH7.5), 0.1MKCl, 10mM EDTA, 1mM MgCl2, 5% glycerol, 1mMDTT, 0.1%Triton X-100, 10ug/ml aprotinin, 5ug/ml leupeptin, 5ug/ml pepstatin and 0.5 mM PMSF

The concentration of protein in each sample was measured by Bradford assay (BIORAD) and OD was adjusted to 600nm. The concentration of the sample containing lowest amount of protein was noted down and the rest of samples were diluted with the extraction buffer accordingly to bring to equal concentration. All protein samples were heated on 95^{o}C for 5 minutes after the addition of β-mercaptoethanol.

2.8.2 SDS PAGE Gel

A total of 25ul solution was prepared for each sample and was loaded in to 8% Acryl amide gels. The gel was prepared in two layers. The lower one was resolving gel while the one above was the stacking gels in which the protein samples were loaded. The composition for the resolving gel was as following.

2.8.2.1 SDS-PAGE

The following Stock solutions were prepared for making gels.

Solution A : Acrylamide stock solution 30% (w/v) acrylamide, 0.8%(w/v) bis-acrylamide (protogel: National diagnostics).

Solution B: (4X separating Gel Buffer) 100ml, 75ml 2M Tris-HCL (pH 8.8) 0.5M, 4ml 10% SDS 0.4%, 21ml H_2O.

Solution C : (4X stacking Gel buffer) 100ml, 50ml 1M Tris-HCl (pH 6.8) 0.5M, 4ml 10% SDS 0.4%, 46ml H_2O, 10% ammonium persulphate (w/v)

2.8.2.2 Separating gel 8% 8cmX6cm

Bio-Rad Mini protean II apparatus was used for the preparation and running for SDS PAGE. The following solution was enough for two gels.

2.7ml Solution A, 2.5ml Solution B, 4.8ml H_2O 50ul of fresh prepared 10% ammonium persulphate and 5ul TEMED was added when ready to pour gels. The gel was covered with water until polymerised.

2.8.2.3 Stacking Gel

For two 5% 8cm X 6cm stacking gels the amount used was as follows; . 2.3ml H_2O, 0.67ml solution A, 1ml solution C. Then 30ul of 10% ammonium persulphate and 5ul TEMED was added to this mixture and poured on the top of the separating gel after

removing the water. At this stage the comb was inserted in to the gel and left for polymerization.

2.8.3 Running buffer

The next step was to prepare the running buffer for the gels. For this purpose every time fresh buffer was prepared. The formula used was as following in 1L with distilled water. 144g Glycine, 30.3g Tris base, 25ml 20% SDS. Samples were loaded in to the wells. At the end 5ul of rainbow marker was loaded to mark the position of the protein bands. The gel was run on 150 volts for about 1.3 h.

2.8.4 Transfer buffer

36.4g Tris Base, 14.66g Gylcine, methanol and 0.4ml 20% SDS was mixed and water was added till it becomes 1L. Semi dry method was used for the transfer of the proteins to the nitrocellulose membrane (Protran BA 85 nitrocellulose membrane, Schleicher and Schuell). The Biorad semidry transfer equipment was used for this purpose. The membrane was run for 15 minutes on 15volts.

2.8.5 Washing buffer

Fresh TBS was prepared for washing the membrane and also making the blocking solution. For the preparation of 1L, 2.42g Trise Base and 8.78g NaCl was mixed with dH_2O till it was 1L. For washing the membrane 0.5ml of Tween-20 was added to it.

2.8.6 Immunodetection

2.8.6.1 Milk buffer

5% non-fat dry milk solution was prepared using TBS and milk powder was dissolved thoroughly. The membrane was washed with this for 1h on a shaker then washed with the washing buffer TBST three times for 5 min to remove extra milk from the membrane. Antibodies were dissolved in the same solution and incubated overnight with the primary antibodies. Next day the membrane was washed again for 3 x 5 min and incubated with milk buffer containing secondary antibodies from 1-2 h.

2.8.6.2 Chemoluminescence

Solution A	**Solution B**
0.5ml 1M Tris-HCl pH 8.5	0.5ml m Tris-HCl pH 8.5
50ul luminal stock (sigma stock-25mM in DMSO)	3.4ul 30% Hydrogen peroxide (H2O2)
22ul p-coumerics acid (sigma. Stock 90mM in DMSO)	4.5ml dH2O
4.43ml dH2O	

At the end of the incubation with the secondary antibodies the membrane is washed. Solution A and B were mixed and poured over the membrane, left there for 1 min and then transferred the membrane on a piece of cling film folding the ends of the

cling film over the back of the membrane. The membrane was than fixed in a film cassette avoiding folds. It was then taken to the dark room and exposed for 5 sec to 30 min and developed manually.

2.9 RNA extraction

The whole procedure is carried out using double autoclaved equipment on dry ice. The extraction was performed using heads only which were extracted using the procedure mentioned earlier. Heads were transferred in to RNase free 1.5 ml microcentrifuge tubes. They were than homogenized in 500ul of Trizol reagent using plastic pestles. An additional 500ul of Trizol was added after this and the mixture was incubated on room temperature for 5 min to allow the complete dissociation of nucleo-protein complex. After incubation 200ul of chloroform was added and mixed on vortex for short while. They were then incubated for further 10min on room temperature. The next step was to centrifuge this tube on 12000g for 15 min on 4^{o}C. This will lead to the separation of the mixture into an upper phase and a lower denser phase. The upper phase was separated in to a new tube carefully and 500ul of isopropanol was added to it. The sample was incubated on room temperature for 10 min. The mixture was centrifuges on 12000g for 10 min on 4^{o}C. The RNA will be separated in a form of a tiny pellet in the bottom of the eppendorf tube. The supernatant was removed and the pellet was washed with the 1ml of 75% cold ethanol, centrifuged for 1 min on 7500g, the ethanol was removed and pellet was dried. Finally the pellet was dissolved in 15-20ul of DEPC water and stored at -80^{o}C.

2.10 DNase treatment

The contamination caused by the presence of DNA in the samples was removed by TURBO DNase (Invitrogen) treatment. In each sample 0.2 volume of 10 X TURBO DNase buffer and 0.2ul of TURBO DNase was added. The mixture was incubated on 37^{o}C for 30 min. After this 0.2ul of DNase inactivation agents were added to each sample and incubated for 5 min. The tubes are centrifuged for 1.5 min on 10,000g to separate the clean RNA. This was then transferred to a new tube.

2.11 cDNA synthesis

The RNA was then finally used for the synthesis of the first strand of cDNA using super script II reverse transcriptase (Invitrogen). Two reactions of 12ul were set up for each sample in separate tubes. One was +RT while the other was –RT. 1ug of RNA was added in to each tube along with 1ul of dNTPs , 1ul of oligo primers and DEPC water up to 12ul. This mixture was incubated at 65^{o}C for 5 min and than chilled on ice. In each of these tubes 4ul 5X first strand buffer, 2ul 0.1 M DTT and 1ul RNase out was added. This mixture was incubated on at 42^{o}C for 2 min and after this 1ul of superscript II reverse transcriptase was added to them. In the –RT samples, DEPC water added instead. These samples were incubated at 42^{o}C for 50 min and at the end of incubation the reaction was inactivated by the heating at 70^{o}C for 15 min. The remnants of RNA in this mixture were also removed after this. This was performed by the addition of 1ul of RNaseH (2units) and incubating the mixture at 37^{o}C for 20 min. The final product was diluted ten times and used for qPCR.

2.12 Real-time PCR

Quantitative PCR (qPCR) is also known as real-time PCR was used to check the expression of the *tim* transgene. RNA was extracted for this purpose which was used to synthesise cDNA. SYBR Green based qPCR (Agilent technologies) was used for this purpose. The house-keeping gene used in this experiment was RpL 40. For each of the line three independent RNA extractions were carried out using about 50 heads in each. So each line was having three biological replicates and three technical replicates. cDNA was synthesised and analysed by qPCR using 2xSYBR green mix in duplicates along with –RT and blank controls. In each tube 25ul of total reaction was set up with 5ul of cDNA and 0.4uM of specific forward and reverse primers. The cycling conditions were in two steps 95^{o}C for 15 sec and then 60^{o}C for 45 sec, in total 45 cycles. Standard curves were compared. The data was organized in excel and used for the statistical analysis.

Chapter 3:

Natural polymorphism in Drosophila *cryptochrome*

3.1 Introduction

Evolution has played its role it the tuning of all living organisms to adapt them to almost all available niches. They have acquired fins, wings, tails and many other structures to live in different environments. One parameter which has been rather neglected is the regular daily cycles of temperature and light. Almost all organisms are exposed to these environmental changes and have adapted to 24 h rhythm. We have examples from nocturnal, diurnal and crepuscular animals which live together by adapting their activity to different times of the day (Peschel and Helfrich-Förster 2011). Circadian clock genes provide targets for natural selection that drive the selection of newly arising alleles, resulting in molecular adaptations to various environmental conditions (Tauber et al. 2007). It appears that the general clock mechanism has undergone several variations on a theme, with perhaps the same molecules carrying on slightly modified tasks, even in rather close evolutionary lineages (Piccin et al. 2000). Natural selection can be directional, shifting a trait continuously in one direction decreasing the frequency of less fit alleles to a level where they vanish. Balancing selection maintains different alleles in the population.

D. melanogaster has its origin in sub-Saharan Africa and it subsequently expanded its range to the temperate zones of the rest of the world (Stephan and Li 2007). Numerous attempts have been undertaken to study adaptation in this species. Latitudinal clines in the frequency of polymorphism are generally considered a consequence of an adaptive response to different climates that have been analysed for many genes (David and Capy, 1988). The most common example in *Drosophila* are the clines in enzyme polymorphisms like *Adh* (David, et al. 1986).

A comparison of *per* gene sequences in different species of *Drosophila* showed a patchwork of conserved areas interspersed with highly variable regions. One of the variable regions is in the middle of exon 5 and encodes a series of about 20 threonine-glycine (Thr-Gly) repeats in *D. melanogaster* (Citri et al. 1987). The number of repeats was observed to vary in length; both in laboratory and natural populations (Costa et al. 1991; Yu et al. 1987). The most common variants in Europe were the $(Thr\text{-}Gly)_{17}$ and $(Thr\text{-}Gly)_{20}$, alleles (Costa et al. 1991). A robust cline was observed with the $(Thr\text{-}Gly)_{20}$ and $(Thr\text{-}Gly)_{17}$ alleles predominant in northern and southern Europe, respectively (Costa et al. 1991). Theses alleles were observed to be capable of maintaining clock function normally at different temperature but the longer allele gave the best thermostability and was found in the more thermally variable northern Europe, whereas the shorter allele was particularly suited to warmer temperatures, and was found in the Mediterranean area (Sawyer 1997).

Another example of a latitudinal cline in *Drosophila* is that in the circadian gene *timeless* (*tim*), which encodes the light sensitive clock regulator. This gene has two alleles, *ls-tim* and *s-tim* which show a latitudinal/distance cline across Europe (Tauber et al. 2007). A single base insertion/deletion in *tim* generates the two alleles. In one,

ls-tim, two major TIM isoforms are generated, L-TIM and S-TIM which differ by 23 residues at their N-terminal, whereas in *s-tim,* only the truncated S-TIM isoform is produced. The two allelic variants differ in their circadian photo-responsiveness providing a putative substrate for natural selection in the Europe's diverse range of photoperiods (Sandrelli et al. 2007). The *tim* cline appears to be a product of directional selection with the new allele, *ls-tim* spreading from southern Europe where it initially emerged, at most a few thousand years ago (Tauber et al. 2007).

CRYPTOCHROME (CRY) is a blue-light photoreceptor which is well conserved and associated with the circadian system in a broad range of organisms from plants to animals (Chaves et al. 2011). It serves as a core clock protein in mammals where it heterodimerizes with PER (Yagita et al. 2002). In *Drosophila,* CRY is the dedicated circadian photoreceptor. Studies shows that *cry* null mutants fail to synchronise their rhythms with the external light-dark cycle (Dolezelova et al. 2007). A survey was carried by Pegoraro et al (submitted)) to study genetic variation in *cry* alleles from European and North-American populations was followed by investigation of any possible functional role for this polymorphism. In the initial survey a complete protein-coding sequence of 10 natural *cry* alleles from various European wild populations were studied which revealed an extensive amount of genetic variation. The data showed 21 silent single-nucleotide polymorphisms (SNPs) and 7 replacement SNPs, four of which were mapped to the FAD binding domain of the protein. One of these SNPs encoded a radical Leucine-Histidine change (L232H). Pegoraro et al used the recently obtained crystal structure of CRY (Zoltowski et al. 2011) to visualise the possible effect of the SNP variants on the protein (Figure 3.1). L232H (as well as two linked SNPs) was mapped to the protein surface, away from the FAD-binding domain of the protein, and

therefore unlikely to affect the redox status of the co-factor, which is thought to be important for the photoactivation of CRY (Hitomi et al. 2009; Partch and Sancar). However, when they used the Site Directed Mutator (SDM) algorithm (Worth et al. 2011) to test the effect of the L232H polymorphism on protein stability, it was found that the H variant is slightly destabilised compared with the L variant (stability score $\Delta\Delta G$ = -0.70).

In five *cry* alleles from the closely related species, *D. simulans*, only the L variant was present, suggesting that *cry-H* is the derived variant.

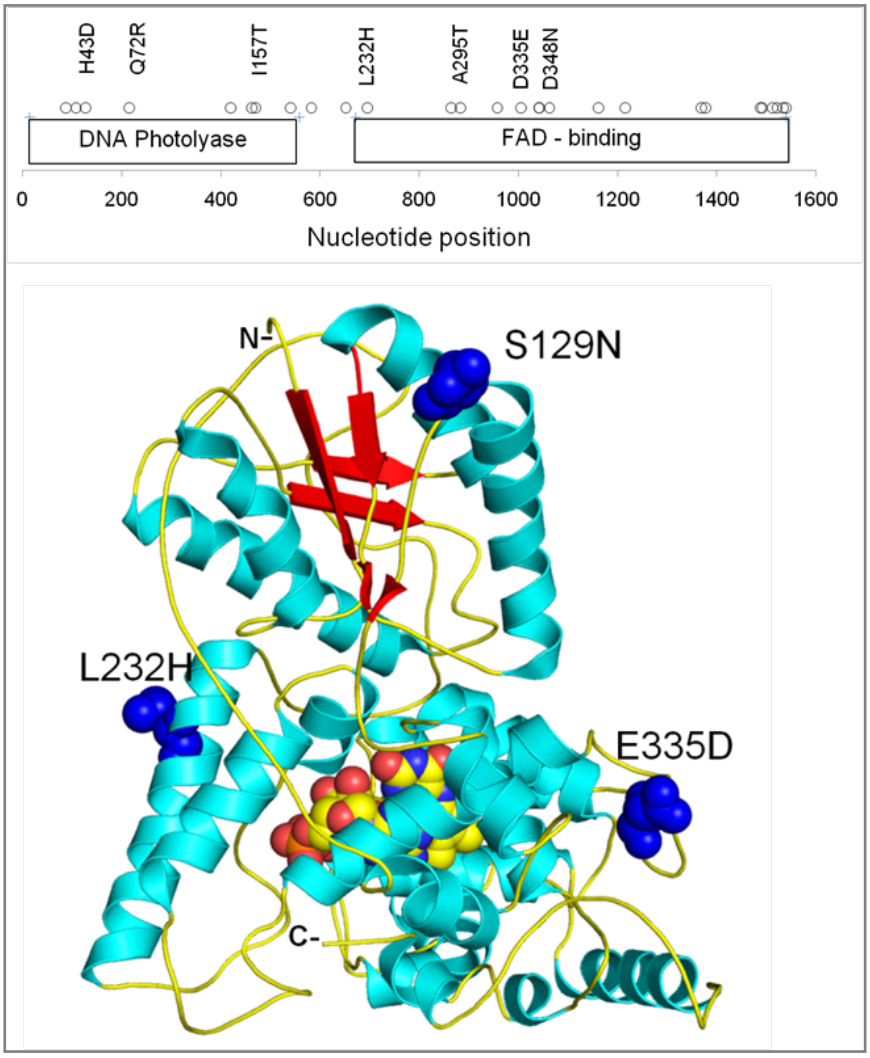


Figure 3. 1: Natural genetic variation in *Drosophila cry.* (Pegoraro et al submitted). polymorphisms found in the coding DNA (CD) of *cry* are indicated (circles) in 10 Old- world alleles. Replacement SNPs are also depicted. Below, the L232H SNP is mapped onto the crystal structure of dCRY together with two other SNPs which are in strong linkage disequilibrium. All SNPs are located on the protein surface, away from the FAD pocket.

Further study

The aims of my study regarding the L232H polymorphism were to check for the presence as well as the spatial distribution of this polymorphism across Europe and to compare its frequency in different populations. The function of the two alleles was also studied by comparing the period, phases of locomotor activity and also their

sensitivity to light pulses in homozygous and heterozygous flies. The role of *cry* in the eclosion rhythms of the flies is also important (Myers et al. 2003) so I also wanted to examine whether there might be a role of L232H polymorphism in this phenotype. After finding the frequency of this SNP in the wild, a number of population cages were set up in the laboratory using two genetic backgrounds of flies in an attempt to mimic the situation in nature.

3.2 Materials and methods

3.2.1 Fly strains

Two genetic backgrounds of flies were used for the study of the L232H *cry* polymorphism, Canton-S and a natural line from Rende Italy. Homozygous strains for cry^H and cry^L as well as the heterozygotes were studied in both of these backgrounds.

3.2.2 Canton-S (CS) line

This is a highly inbred line although inbreeding has been relaxed in our laboratory. This line is originally from America but been kept in the laboratory for more than 80 years. We found that the Canton-S laboratory lines in our laboratory were cry^H homozygotes in the *s-tim* background. A cry^L allele from a natural line from Naturno (Italy; NAT) was repeatedly backcrossed into the Canton-S genetic background for 10 cycles, with the *cry* and *s-tim* alleles identified at each generation by PCR as *s-tim* is more light-sensitive than *ls-tim* (Sandrelli et al. 2007). The *s-tim;* cry^{HL} F1 of this couple were backcrossed 9 times with *s-tim* ; cry^{HH} to make the cry^{HL} genotype stable and reduce genetic differences between the two lines. Each time, a large number of

crosses were set up and male parents were genotyped and only *s-tim; cry^{HL}* were selected for the subsequent cross to *s-tim; cry^{HH}*.

After the emergence of flies in the selected vials, pairs with one parent homozygous i.e. *cry^{LL}* and one *cry^{HL}*, were selected. About half of the flies emerged from selected vials were homozygous *cry^{LL}*, and these were used as parents to generate the *s-tim ; cry^{LL} strain*.

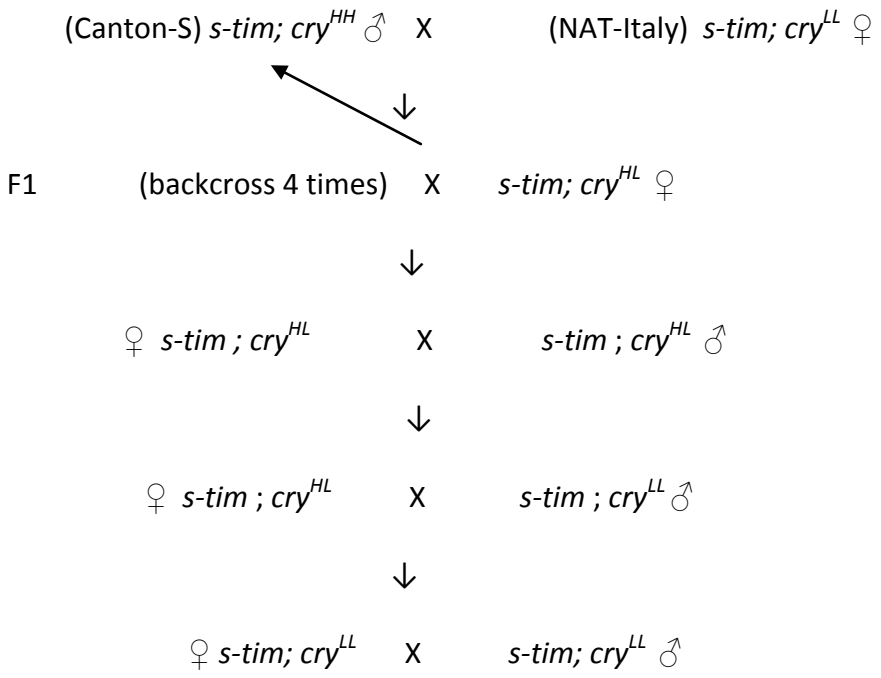


Figure 3.2: Genetic cross for placing *ls-tim/s-tim ; cry^{LL}* in a *s-tim; cry^{LL}* background.

3.2.3 Rende line

This was a natural fly strain from an isofemale line from Rende, southern Italy (REN latitude 39.19 N) collected in October 2006. After virgin collection, a couple of crosses were performed and the parents were genotyped for *cry^{H}* and *cry^{L}* and also for *s-tim* and *ls-tim*. Ultimately one couple was selected which was *s-tim* but heterozygous

for cry^H and cry^L. The flies that emerged in the vial were check by wing PCR and selected for further crosses. After three generations of crosses *s-tim* ; cry^{HH} and *s-tim;* cry^{LL} flies were isolated.

♀ *s-tim ;* cry^{HL} X *s-tim* ; cry^{HL} ♂

↓

♀ *s-tim* ; cry^{HH} and *s-tim* ; cry^{LL} ♂

3.2.4 Genotyping of the flies by Polymerase chain reaction (PCR)

DNA was extracted from the individual fly in a PCR plate according to the protocol described in Chapter 2. The following primers (Table3.1) were used for the amplification of cry^H and cry^L alleles, reverse primers being the same for both. The PCR amplification gave a product of 1.16 Kbp. The protocol used for the thermo cycler was as follows (Table 3.2).

S.No	Primer name	Sequence
1	Forward cry^H	AGAAACACAGGCCTTAGAT
2	Forward cry^L	AGAAACACAGGCCTTAGTT
3	Reverse cry^b	GTACTCCTTCAAACCACCA

Table 3.1: Primers used for the amplification of cry^H and cry^L alleles.

Step	Temperature	Time duration
1	92 ^{o}C	2minutes
2	92 ^{o}C	30 seconds
2	54.5^{o}C	30 seconds
4	72 ^{o}C	1 minute
5	Repeat step 2 for 35 cycles	
6	72 ^{o}C	10 minutes
7	10 ^{o}C	Forever

Table3.2: PCR protocol used for the amplification of *cry^{H}* and *cry^{L}* allele

3.2.5 Population cages

In each of the cages 500 flies were placed in the following genotypic frequencies 90% *s-tim; cry^{HH}* and 10% *s-tim; cry^{LL}* (225 *s-tim; cry^{HH}* virgins, 225 *s-tim*; *cry^{HH}*; males; 25 *s-tim* ; *cry^{LL}* ; virgins and 25 *s-tim* ; *cry^{LL}* ; males) in cage A1 and A2. In cage B1 and B2 the proportion of the two genotypes was reversed i.e. 90% *s-tim; cry^{LL}* and 10% *s-tim; cry^{HH}*.

The experiments were replicated using the Canton-S and Rende backgrounds. The cages were kept on 25^{o}C in LD 12:12. Food was changed at the rate of 6 vials per 10 days. The cage takes 12 vials so no food vial remained for more than 20 days. After one month the old food vials were removed and kept at 25^{o}C. 48 flies (half PCR plate) that emerged in them were sampled and genotyped for the frequency of *cry^{H}* and *cry^{L}*. This procedure was repeated every 2 months.

The first experiment was started in October 2009 for cage A1 and B1 and in November 2009 for A2 and B2 using Canton-S and finished in April 2010. The second

cage experiment performed with Rende was started in July 2010 for the cage A1 and A2 and August 2010 for B1 and B2 and sampled till May 2011.

3.2.6 Fitness experiment

The following cross was performed to compares the fitness of both Canton-S and Rende lines in the cry^H and cry^L background (Figure3.3). The number of homozygotes for each of two alleles obtained in the F2 generation was indirectly compared to each other via the use of balancers as a general measure of pre-adult fitness. The advantage of this method was that it did not involve countless expensive PCRs. 10 maize food vials were set up for each of the backgrounds and in each vial 3 virgin females were placed along with 3 males.

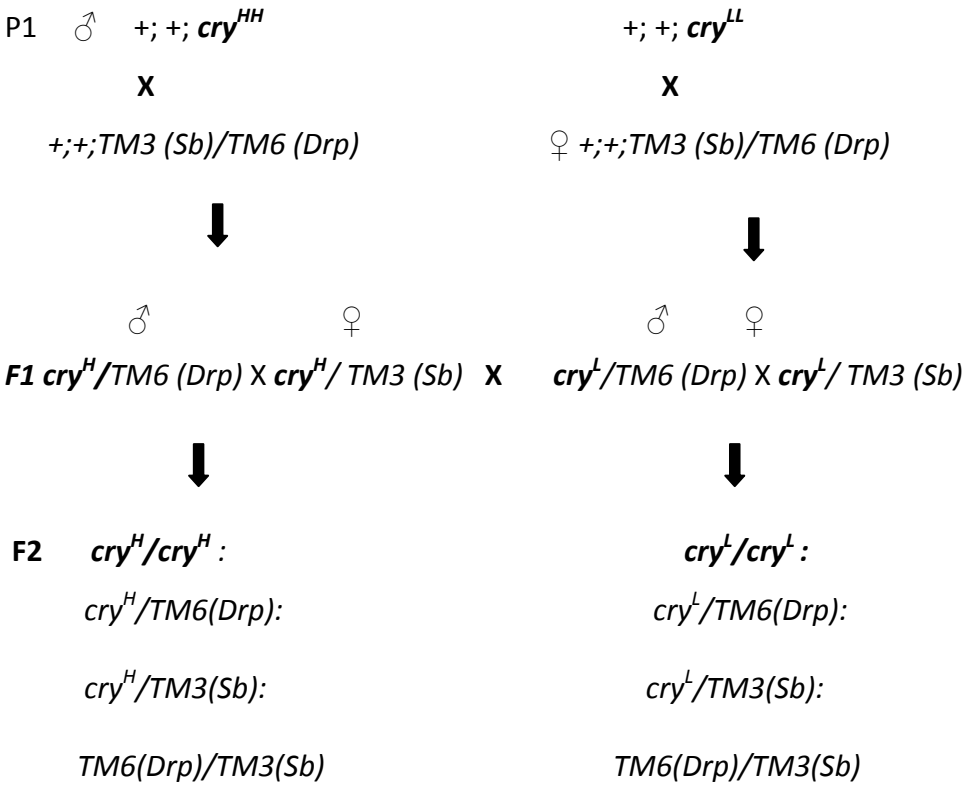


Figure 3.3: Crosses for the fitness experiments (for details see text). The balancers used in this cross had markers on the third chromosome. *Tm3 Sb* had Stubble (short) bristles on the body and *TM3(Drp*) had Drop (elongated) eye phenotype.

3.2.7 Behavioural analysis

The period of locomotor activity was measured by entraining the flies for 3 days in LD 12:12 and then placing in total darkness (DD) for 7 days. The photosensitivity of these lines was also compared. The protocol was as follows: flies were entrained for 4 days in LD 12:12 after which they were placed in DD for 3 days. They were again entrained for 4 days in LD12:12 and a light pulse was applied for 20 minutes at ZT15 to probe for delays and ZT21 for advances. The flies were maintained in DD for three further days (Figure 3.4).

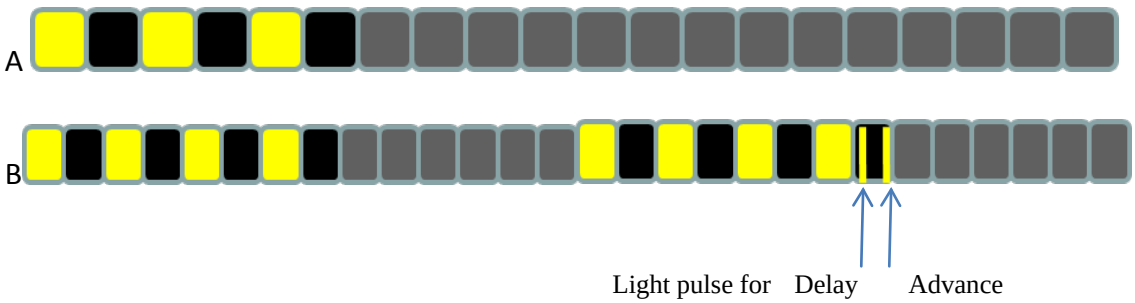


Figure 3.4: the protocol used for studying the period of locomotor activity (A) and light phase response (B). The yellow bars present the light on period while the black and grey bars are the time without light on.

3.2.8 Data analysis

The locomotor activity events were arranged into 30 min bins using DAM file scan. CLEAN, a high resolution spectral analysis was used to obtain the period (see Kyriacou and Hall 1989), as well as autocorrelation, and actograms of individual flies were also inspected. The analysis for the light-induced phase shifts was performed by using a semi-automated method based on a Python script designed by Mauro Zordan (University of Padova). For statistical analysis of phase data "Oriana" was used which is

circular statistics software (Kovach Computing Services, UK). For ANOVA and graphs "R" was used.

3: RESULTS

3.1: *cry^{H} / cry^{L}* polymorphism

A number of European samples were collected from different latitudes and individual flies were genotyped using PCR for the presence of the *cry^{H}* and *cry^{L}* allele, each with 24-50 individuals or in some cases more than 100. Pearson production-moment correlation was performed in "R" to test the correlation between latitude and the frequency of these two alleles. The genotyping result of 13 populations (Table 7.1, Appendices) showed that there was no significant geographical difference ($F_{1,25}$=1.84, p=0.19) for the frequency of these two alleles among these populations (Figure 3.5), with neither a cline in the frequency of *cry^{H}* or *cry^{L}* allele nor in the level of heterozygosity (*cry^{HL}*) along latitude (Figure 3.6). Although the origin of this polymorphism is after the D. *melanogaster/D. simulans* split about 1.17 Mya (Cutter 2008) the allele frequencies were quiet similar in all the population tested which suggest that L232H polymorphism is under balancing selection.

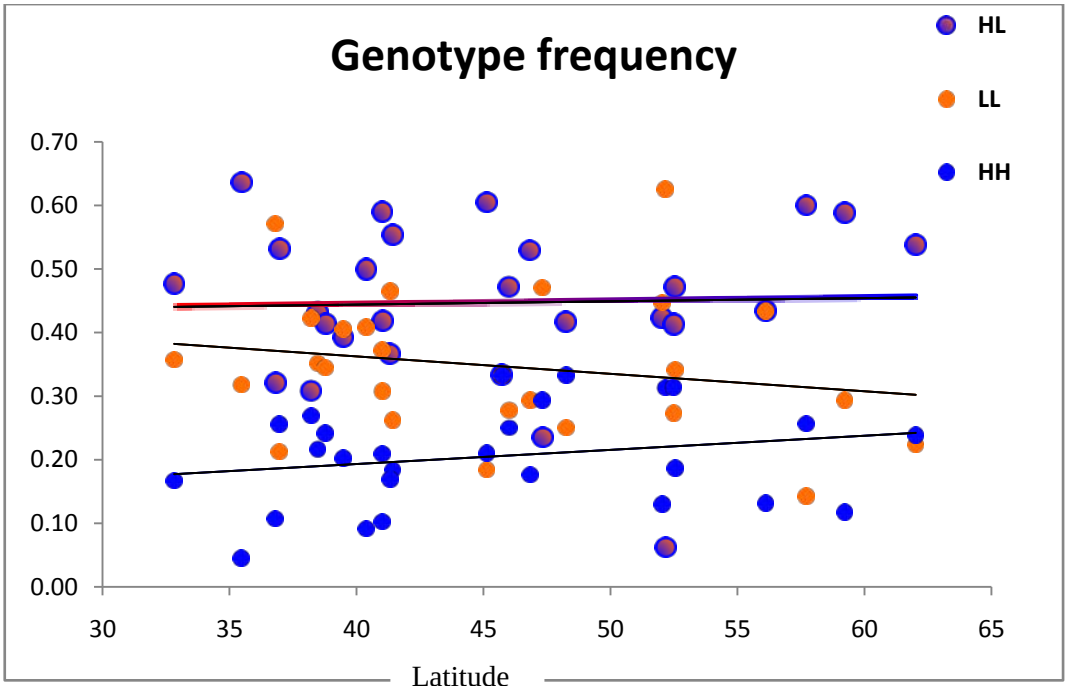


Figure 3.5: Frequency of the *cry*^{HH} (R^2 0.049, p=0.48), *cry*^{LL} (R^2=0.037, p=0.4) and *cry*^{HL} (R^2= 0.001, p=0.78) genotypes along latitude in the 27 populations of flies collected across the Europe. (*cry*^{HH,} *cry*^{LL} and *cry*^{HL}).

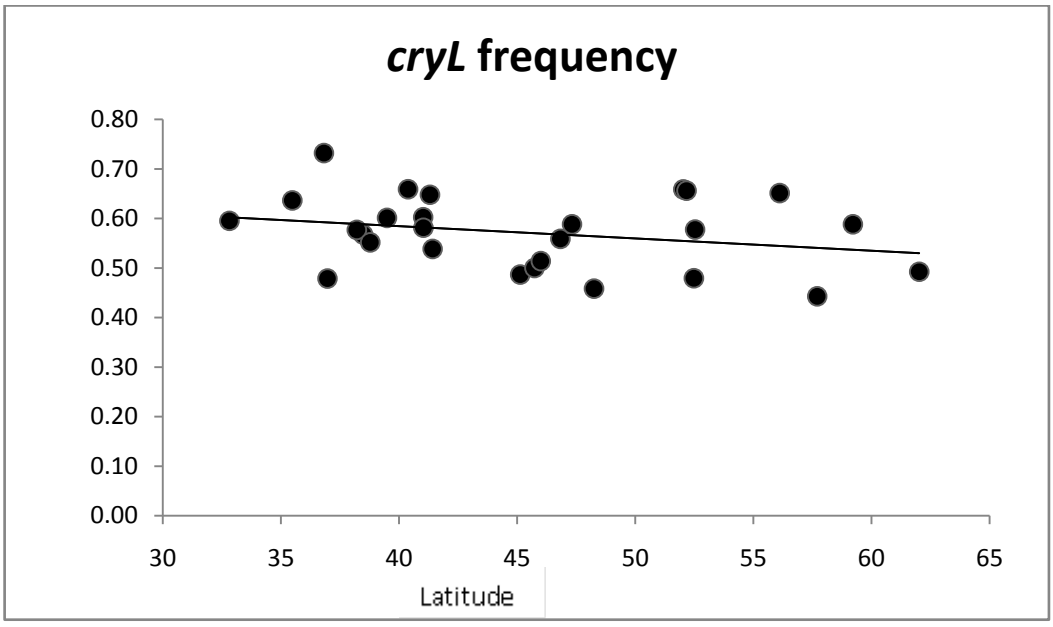


Figure 3.6: Frequency of *cry*^{L} allele against latitude (R^2=0.072, p=0.48).

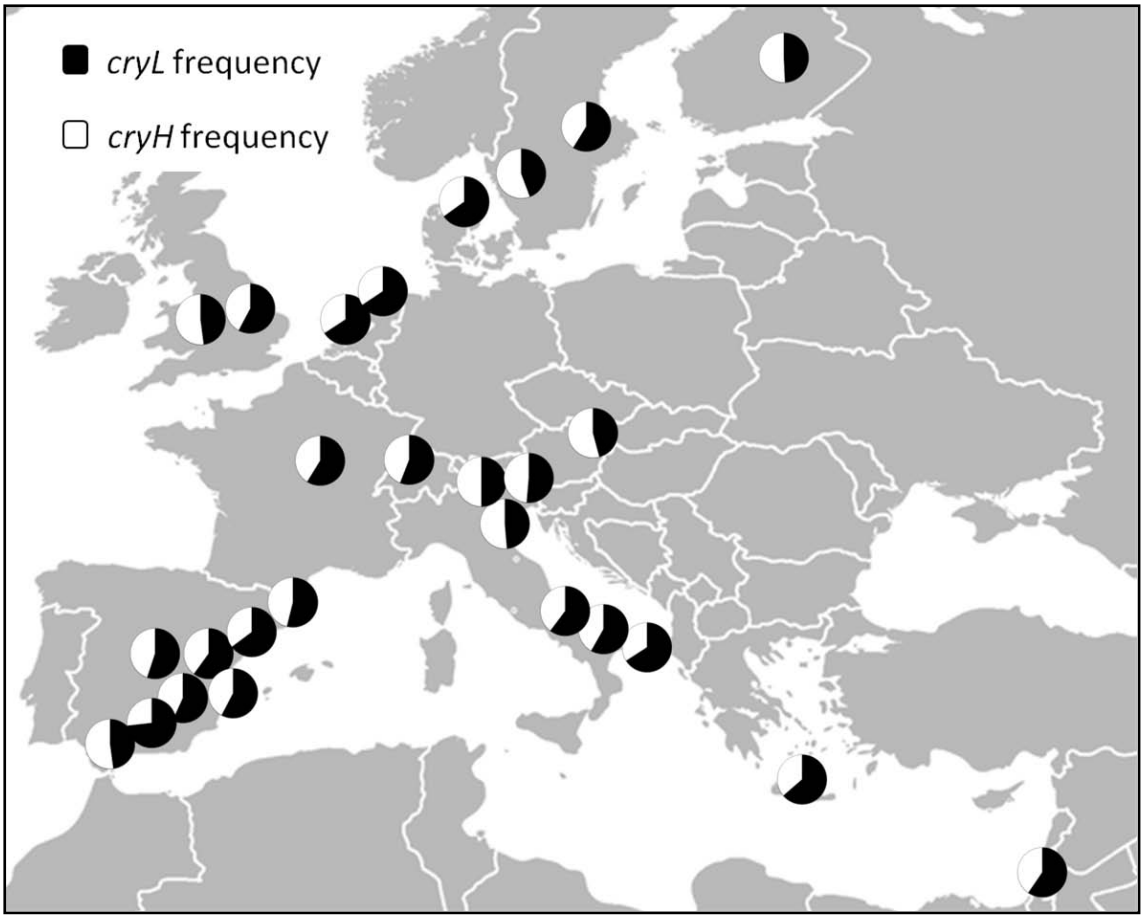


Figure 3.7: Spatial distribution of the L232H *cry* polymorphism (Figure adopted from Pegoraro et al submitted)

3.2 Population cages

The comparison of allele frequencies suggests the presence of a balanced polymorphism based on a character that is not modulated by latitude, perhaps via heterozygote advantage. In an attempt to test how stable these frequencies are, four population cages were initiated. The average number of flies in each cage at any time point was between 800 and 1500. This was estimated by counting the total number of flies that emerged from the two vials collected from each cage at the end of experiment.

3.2.1 Population cages using Canton-S lines

The cages were sampled for the period of about seven months. The final allele frequency in these all the four cages was in the favour of cry^H allele which was observed to climb from 90% to 99% in cage A1 and 97% in cage A2 (Figure 3.8).

The same result was observed in the other two cages where the frequency of cry^H allele increased dramatically from just 10% to about 88%. The cry^{LL} genotype totally disappeared, while the ratios of heterozygotes for these alleles was increased from 0% to 26% in both cages (Table 7.2 Appendices).

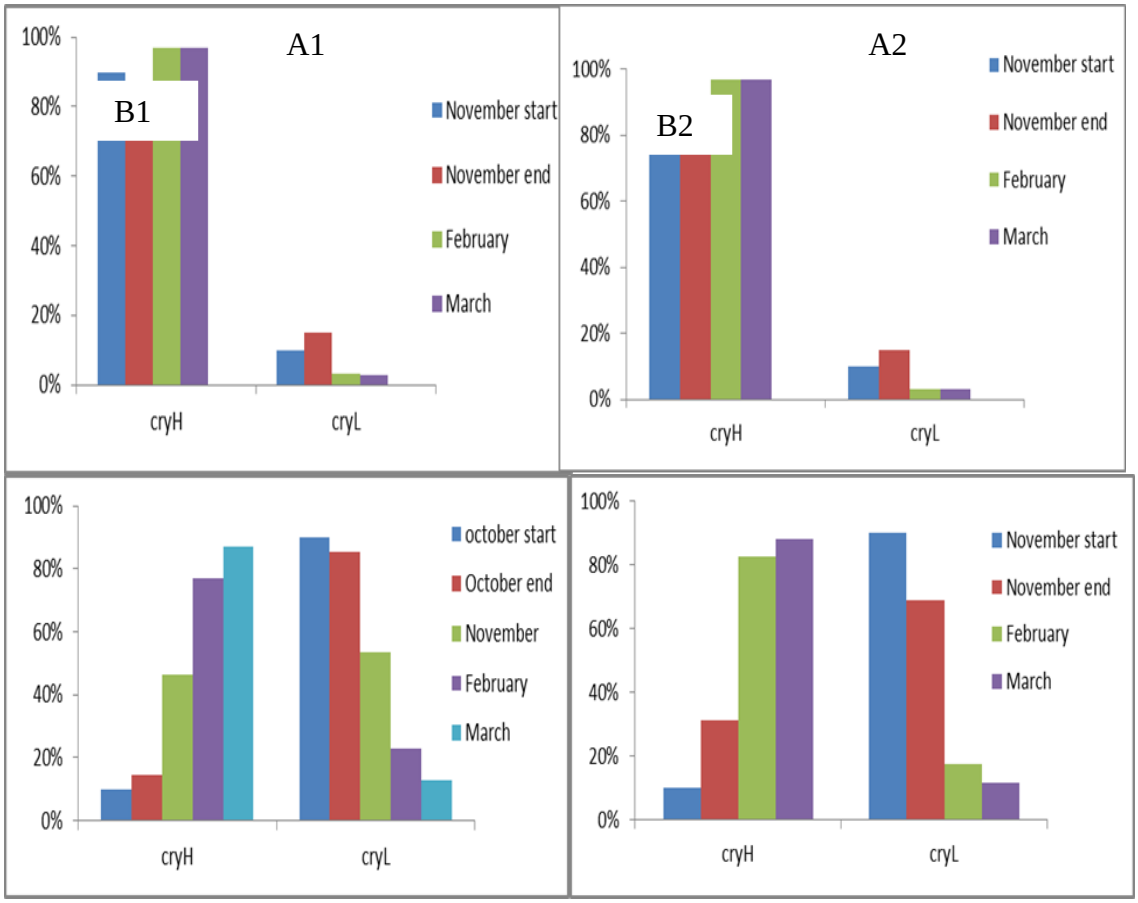


Figure 3. 8: Results for the Canton-S population cages A1 and A2initiated with 0.9H:0.1L. In both of the cages cry^H allele has been favoured and its frequency increased to almost 99%. Results for the Canton-S population cages B1 and B2 initiated with 0.1H: 0.9L. In both of the cages cry^H allele has been favoured and its frequency increased from 10% to 87% and 88% respectively.

3.2.2 Population cage experiment using Rende fly strain

These cages were kept over a period of 22 month and the changing frequency of the two alleles was observed at different times. Somewhat remarkably, cry^H and cry^L allele frequencies converged to an approximately equal frequency after a period of 8 months (~16 generations) recapitulating the situation in the wild (Figure 3.9).

The cages A1 and A2 had initial 90% cry^{HH} ; 10% cry^{LL} frequencies(Table 7.3 Appendices). In cage A1 the frequency of the *H* alleles changed from 90% to 75% in the first two months and then it kept on decreasing unless it reached to 53% in May 2011. The cry^L allele frequency increased to 47% in May 2011. After that it kept on fluctuating 10-20% around the same frequency till it reached around 50%H: 50%L in April 2012.

If we look at the genotype ratios, the percentage of heterozygotes (cry^{HL}) increased dramatically from zero to 49% over the period of two months. The Hardy-Weinberg test also shows that there is a marginal departure from Hardy Weinberg equilibrium. (χ^2 = 4.93, df=1, p=0.02). After September 2011, heterozygotes fluctuated around 50%.

In cage A2 almost the same trend was observed with the percentage of cry^H alleles decreasing from 90% to 79% in September 2010, 63% till February 2011 until it reached to 52% in May 2011. The case with cry^L was also similar to A1 and increased from 10% to 21% in September 2010, 37% in February and 48% in May 2011. After that it kept increasing till it reached 64% in November 2011. This cage was severely infected with fungi so the cage was changed at the end of November 2011 and the flies were transferred to a new cage. In April 2012 the last observation showed 26.6% cry^H and

73.4% cry^L. The genotype ratio of the heterozygotes increased from 0 to 41% in the first two months of the experiments and to 59% by August 2011. Overall the genotype frequency of the population in this cage remained in the Hard-Weinberg equilibrium with no major deviation (Table 7.4 Appendices).

The cages B1 and B2 were started with the reciprocal frequencies of the two alleles. In the cage B1 the ratio of the cry^L was continuously decreasing from 90% to 67% by November 2010, 60% by February 2011 and 38% by June. In August the ratio of the two alleles was 1:1. In April 2012 there were 38% cry^H and 62% $cry^{L.}$. The genotype frequency showed a very rapid increase in the percentage of heterozygotes from 0% to 47% in two months. The population showed a deviation from Hardy-Weinberg equilibrium in the month of February (χ^2 = 7.34, df=1, p=0.006).

The other replicate cage B2 showed the same frequency changes but the increase for cry^L was very slow. The allele frequency of cry^H and cry^L hovered around 50% till April 2011. After that it fluctuated considerably. This cage was also having a very severe fungal infection so the flies were transferred to another new cage in the end of November. The last sample was taken at the start of April 2012 and the allele frequency was balanced around 50%. The genotype frequency of heterozygotes kept on increasing from 0% to 60% in April 2011, it stayed almost same till August but after that it came down to 28% in September. It again increased to 32% in November and observed to be 55% in April 2012.

The overall trend observed was towards an equal frequency of both alleles.

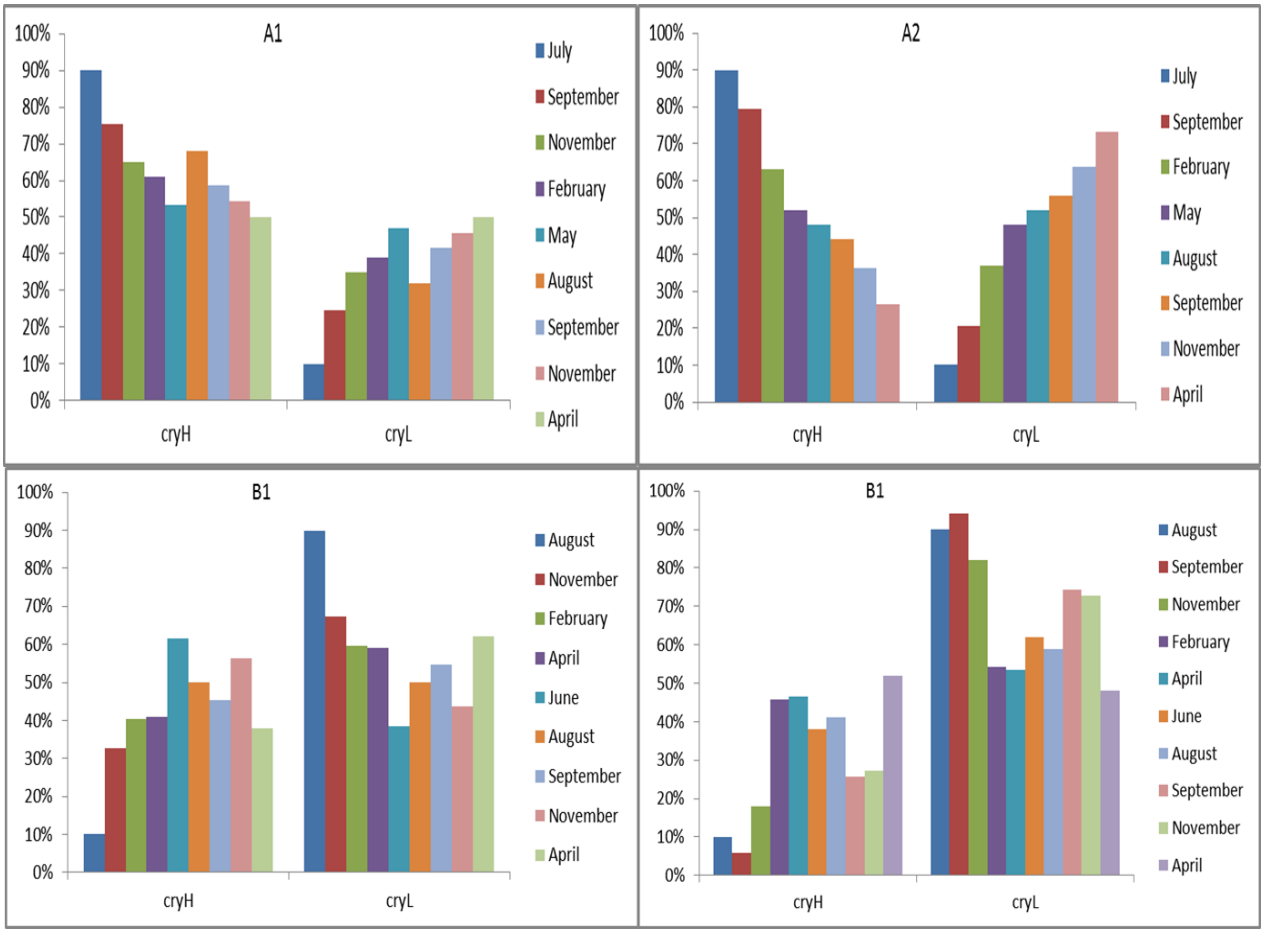


Figure 3.9: Results for the Rende population cages. A1 an A2 started with 0.9H: 0.1L initial frequency. In both of the cages *cry*^{H} allele frequency reduced from 0.9 to 0.5. *cry*^{L} increased from 0.1 to 0.5. B1 and B2 started with 0.9H: 0.1L. In both of the cages *cry*^{H} allele frequency has increased from 0.1 to 0.5 and *cry*^{L} decreased from 0.9 to 0.5.

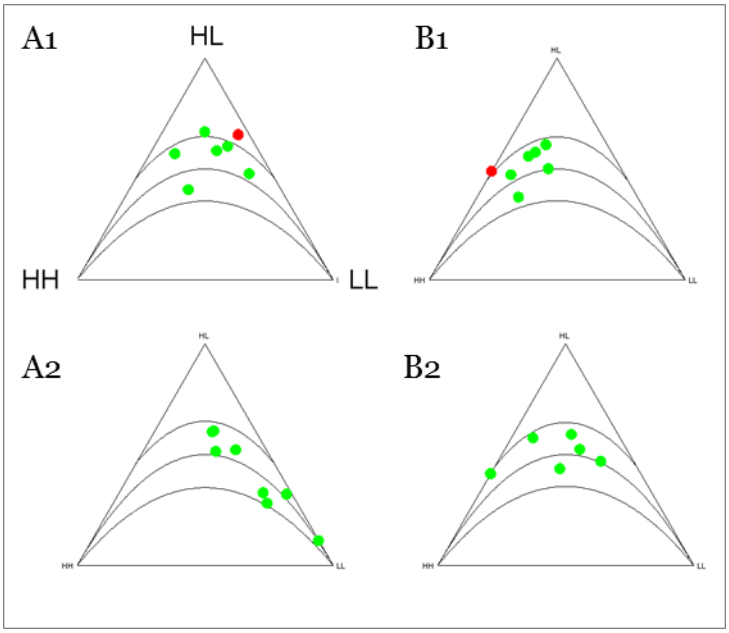


Figure 3.10: Change in genotype frequencies of the L232H SNP in Rende population cages. Ternary plots showing the genotype frequency in each of the four population cages. Populations were genotyped 6 times during the experiment (16 months). Genotype frequencies under HWE are within the 95% limits and shown in green. Significant departures from HWE are shown in red.

3.2.3 Fitness experiments

Fitness of the line carrying either of the two alleles cry^{H} and cry^{L} or both was also compared using Canton-S and Rende backgrounds. They were crossed to the balancers on the 3rd chromosome as shown in the section 3.2.6. The F2 data was used for the analysis. Ration of the homozygous cry^{HH} and cry^{LL} flies among the total flies emerged from the vial was used for the analysis. A t-test was performed for the comparison of mean of the two genotypes using “prism”.

3.2.3.1 Canton-S

Although statistical comparison showed that there was no overall difference in the pre-adult fitness of the cry^{H} and cry^{L} (t=0.46, df=26, p=0.64; Figure 3.11 A) the number of flies that emerged on the first day for cry^{HH} are significantly higher than cry^{LL} (t=2.58, df=26, p=0.015; Figure 3.11B). This might contribute to the situation in the Canton-S cages where the cry^{HH} genotype outcompeted cry^{LL}. Because after emerging earlier these flies would have started egg lying before cry^{LL}.

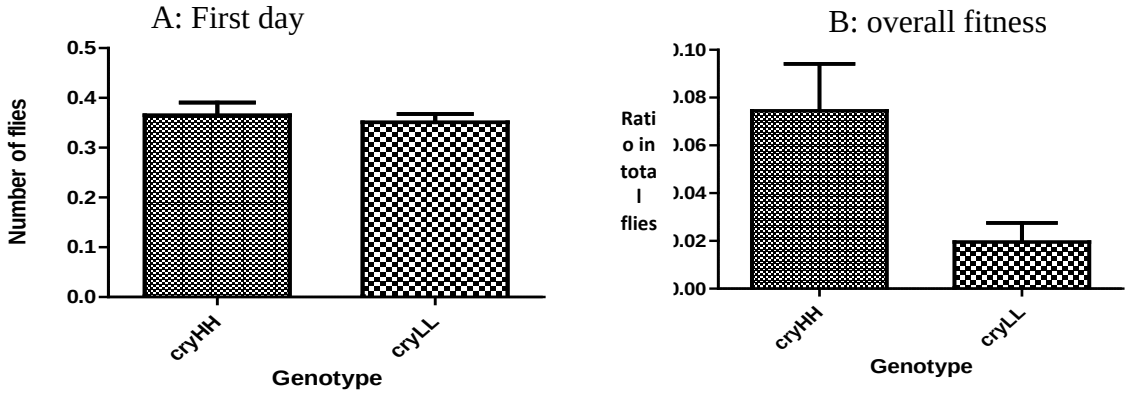


Figure3.11:

(A) Comparison of the Canton-S cry^{HH} and cry^{LL} flies emerged on 1st day (p=0.015). Average number of flies with SEM. (B) Overall comparison in the viability of Canton-S H and L homozygotes (p>0.05).

3.2.3.2 Rende lines

The same fitness experiment was repeated for the Rende lines. Here I did not get any significant difference in the number of flies that emerged on the first day (t=0.49, df=18, p=0.63 Figure 3.12A) but overall the fitness of cry^{LL} flies was observed

to be significantly higher than cry^{HH} flies (t=3.4, df=18, p=0.002; Figure 3.12B). This could explain the general increase in the frequency of cry^{L} alleles in most of the cages.

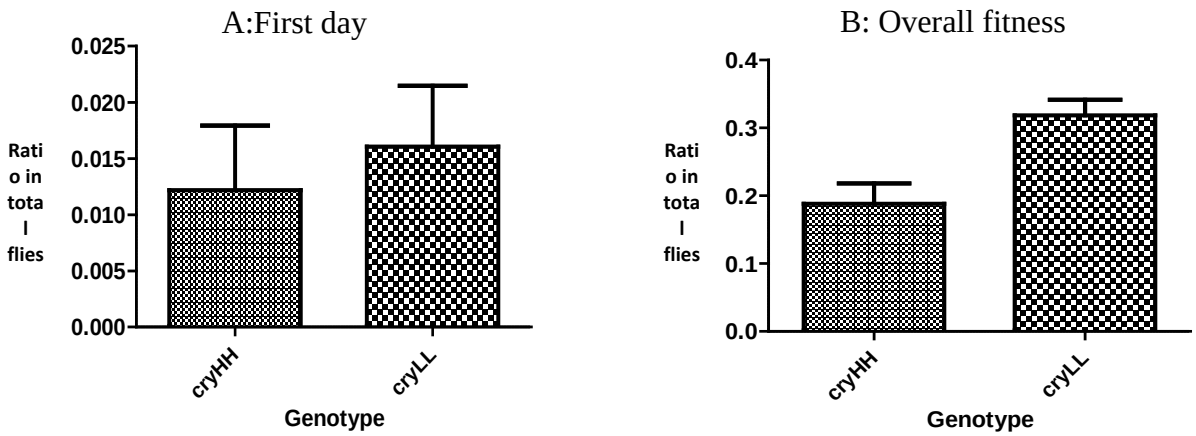


Figure 3.12: (A) Comparison of the Rende cry^{HH} and cry^{LL} flies emerged on 1st day p>0.05). Average number of flies with SEM. (B) Overall comparison in the viability of Rende cry^{HH} and cry^{LL} homozygotes (p=0.002).

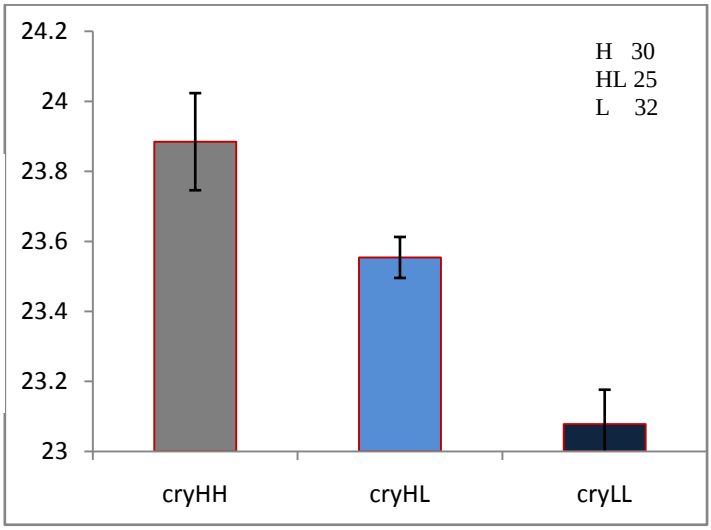
3.2.4 Circadian analysis - Canton-S flies

Flies homozygous for cry^{H} and cry^{L} on both genetic backgrounds, Canton-S and Rende were monitored for 3 days in LD12:12 for entrainment followed by 7 day of DD to find the period and phase. The genotypes were compared through ANOVA and Tukey *post-hoc* test using "Prism".

In the first experiment only males were used for the activity analysis. For Canton-S flies the average period of cry^{HH} males was 23.88±0.14h, cry^{LL} 23.08±0.10 h, cry^{HL} 23.55±0.06 h. An overall significant difference ($F_{(2,67)}$=12.6 , p=0.0001) was

recorded. The average period of cry^{HH} was significantly longer than cry^{LL} and also cry^{LL} was significantly different from cry^{HL} (Figure 3.13 A). The females only experiment showed similar results ($F_{(2,103)}$=6.28, p=0.0027, Figure 3.13B).

A) Males



B) Females

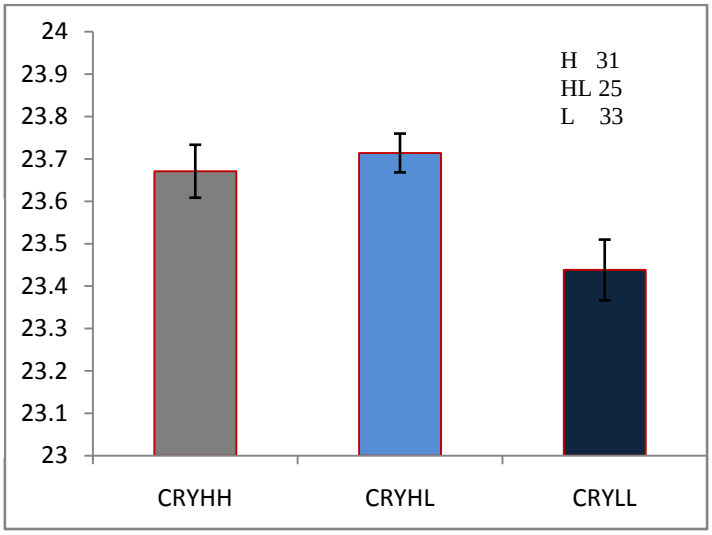


Figure 3.13: Average period of the cry^{HH}, cry^{LL} and cry^{HL} in the Canton-S flies. Average period with SEM. A= males only, B= females only (for details see text).

3.2.4.2 Rende lines

For male ANOVA of the free-running periods of the three genotypes was significant ($F_{(2,67)}$=12.6, p=0.0001). Significant difference was calculated between cry^{HH}

and cry^{LL} and also between cry^{HL} and cry^{LL} flies (Figure A) with cry^{LL} showing the longest period. The female experiment showed the same results ($F_{(2,111)}$=8.91, p=0.003). The Tukey multiple comparison of means also showed similar difference between all the genotypes for females.

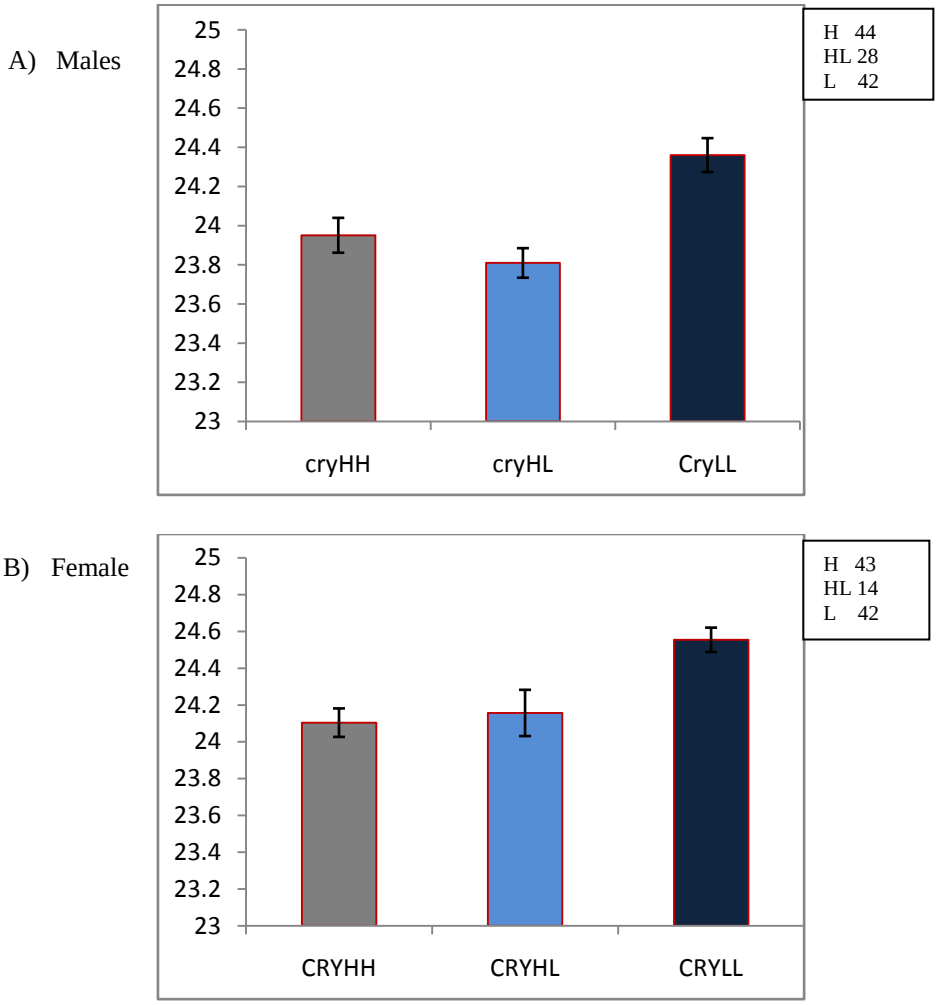


Figure 3.14: Average period of the cry^{HH}, cry^{LL} and cry^{HL} rende lines. Average period with SEM. A=males, B=females

3.2.5 Circadian photoresponsiveness

Light pulse experiments were designed to test circadian photosensitivity and a delay and advance experiment was conducted for each. Watson-Williams F-test was performed using "Oriana". For Canton-S no difference ($F_{(2,96)}$=0.5, p=0.6) was recorded

for the delay phase between the *cry*^{HH} and *cry*^{LL} lines on the third day after the light pulse was administered at ZT15 (Figure 3.15 A & B) nor for the advance zone ($F_{(2,61)}$=0.15, p=0.6, Figure 14). Rende lines also showed the same pattern of photosensitivity with no significant difference either in the delay ($F_{(1,54)}$=1.14, P=0.322) or advance ($F_{(2,99)}$= 1.2, p= 0.23) experiments (Figure 3.15 C&D).

The results of the light pulse experiments (delay and advance) support the idea that *cry*^{HH} and *cry*^{LL} alleles do not differentially affect photic phase shifting

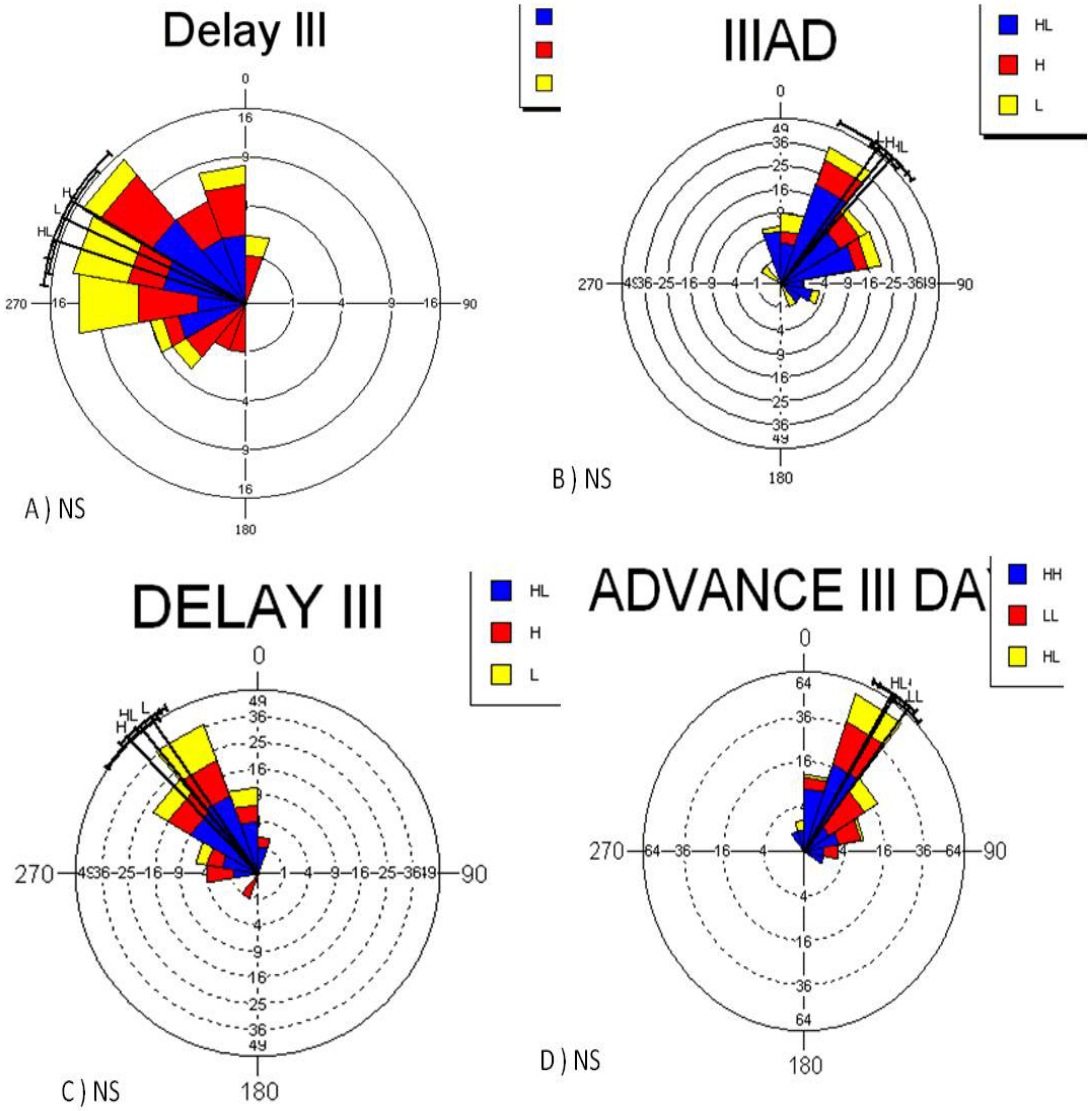


Figure 3.15: No differential phase shifts between *cry* alleles recorded on third day (III) in DD. No significant difference was recorded on third day (delay III) for the Delay (A) and advance

(B) for Canton-S flies using circular statistics. Similar results were obtained with the Rende flies (C and D).

3.2.6 Phase analysis

3.2.6.1 Canton-S lines

Detailed phase analysis was performed using the available locomotor data for LD cycles 12:12 at 25^{o}C, using our semi-automated Python algorithms. The 4th day of the initial entrainment was used for the phase analysis. The bins of activity for each phase were selected by hand picking. Flies observe a bimodal activity profiles under LD 12:12 in laboratory. The least bin of activity was selected for the morning and evening onset of activity. After this the highest peak was selected for the peak of activity both for morning and evening however the peaks caused by the sudden light on or off (masking effect) were avoided. The locomotor activity again reduces after the morning and evening peaks so the least bin was selected for the comparison. A total of 98 flies were analysed. Morning Onset (MON), Morning Peak (MP) Morning Offset (MOFF), Evening Onset (EON), Evening Peak (EP) and Evening Offset (EOFF) were recorded (Figure 3.16).The comparison was performed through Watson-Williams F-test using "Oriana".

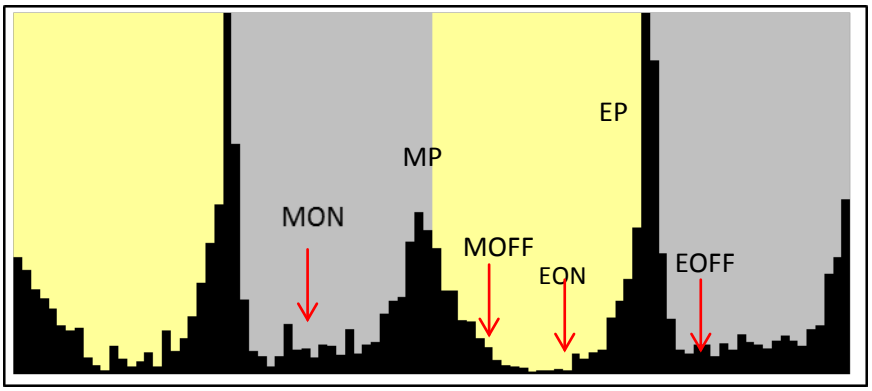


Figure 3.16: Bins of activity during the lights on and off period. Phases of activity used for the comparison of the *cry*^{HH}, *cry*^{LL} and *cry*^{HL} flies locomotor activity profile in LD 12:12.

An overall comparison showed a significant difference ($F_{(2,270)}$=14.8, p=7.87E-07) in the MON of activities on 3rd day (Figure 3.17 a) in LD. *Post hoc* comparison of means revealed that *cry*^{HH} flies have an earlier MON than *cry*^{LL} ($F_{(1,218)}$=17.44, p=4.27e-05, Figure 3.16 A) with a difference of about 26.4 min. The time difference of MON was also significant between *cry*^{HH} and *cry*^{HL} lines ($F_{(1,149)}$= 0.79, p=3.76E-01), and between *cry*^{LL} and *cry*^{HL} ($F_{(1\text{-}173)}$=35, p=1.59E-08). Overall analysis showed that *cry*^{HL} flies are active the earliest. The overall comparison of time interval between these lines, for attaining the morning peak (MP) was also found to be significant ($F_{(2,268)}$=4.26, p= 0.015, Figure 3.17B). The *post hoc* test showed that *cry*^{HH} flies reached their peak of activity 18.7 min earlier than *cry*^{LL} flies ($F_{(1,216)}$=11.9, p=6.70e-04) (Figure 3.16 B). However no significant difference could be recorded between *cry*^{HH} and *cry*^{HL} lines ($F_{(1,147)}$=0.134, p=0.715) nor between *cry*^{HL} and *cry*^{LL} flies ($F_{(1,173)}$=3.25, p=0.07). The data shows that all these flies ended their phase of morning activity (MOFF) almost together so no significant differences ($F_{(2,270)}$=0.5, p=0.6) were detected.

The analysis for the virgin females showed no significant difference among the three genotypes in MON ($F_{(2,111)}$=1.6, p=0.19), MP ($F_{(2,89)}$=1.8, p=0.16) and MOFF ($F_{(2,103)}$=1.7, p=0.184).

A significant difference ($F_{(2,272)}$=26.4, p=3.26E-11) was detected in the overall comparison of EON for male of these three lines (Figure 3.17D). cry^{HH} flies initiated their evening activity about 30 minutes earlier than cry^{LL} flies. Same was the observation with female EON phase ($F_{(2,102)}$ =8.02, p=5.78E-04, Figure 3.18). No overall significant difference was recorded in the male flies for the evening peak (EP) ($F_{(2,270)}$=2.2, p=0.113) and Evening offset of activity (EOFF) ($F_{(2,272)}$=1.928, p=0.147) between the three genotypes. However for Canton-s female EOFF was significantly different ($F_{(2,102)}$=3.50, p=0.034). The *post hoc* test showed In the evening cry^{HH} finished its activity earlier than cry^{LL} ($F_{(1,64)}$=8.446, p=0.005, Figure 3.18) .

A summary of all these observation are given in the in the Table 3.3. Slight differences were detected in the phases of the activty for these three genotypes. For morning activity, males and female cry^{HH} homozygotes were observed to be starting their activity earlier. The difference in Evening onset of the activity was also in the same direction for both males and females and cry^{HH}started their activity earlier than cry^{LL} .

	Phases	Mean phase of each Genotype in hours					
		cry^{HH}	cry^{LL}	cry^{HL}	F	P-value	Earliest
Males	MON	0.02	0.46	23.89	$14.8_{(2,270)}$	7.87E-07	Cry^{HL} and then Cry^{HH}
	MP	1.02	1.33	1.09	4.2(2,268)	0.015	Cry^{HH}
	MOFF	3.49	3.50	3.70	$0.50_{(2,270)}$	0.607	
	EON	8.44	9.19	7.83	$26.4_{(2,272)}$	3.26E-11	Cry^{HL} and then Cry^{HH}
	EP	11.82	11.95	11.65	$2.20_{(2,270)}$	0.113	
	EOFF	13.37	13.15	13.11	$1.92_{(2,272)}$	0.147	
Females	MON	0.26	0.45	0.41	$1.68_{(2,111)}$	0.19	Cry^{HH}
	MP	1.76	1.79	1.48	$1.8_{(2,89)}$	0.167	
	MOFF	4.57	5.26	4.83	$1.71_{(2,103)}$	0.184	
	EON	7.71	8.27	8.92	$8.02_{(2,102)}$	0.0005	Cry^{HH}
	EP	10.91	11.42	11.32	$1.96_{(2,102)}$	0.145	
	EOFF	12.69	13.03	12.94	$3.49_{(2,102)}$	0.034	Cry^{HH}

Table 3.3: Summary of the phase of activity in the Canton-s cry^{HH}, cry^{LL} and cry^{HL} male and female flies. Table shows mean phase of activity for each genotype during LD 12:12 under 25°C. The significant difference is shown is the red. The genotype starting their activity the earliest at a particular phase is also mentioned.

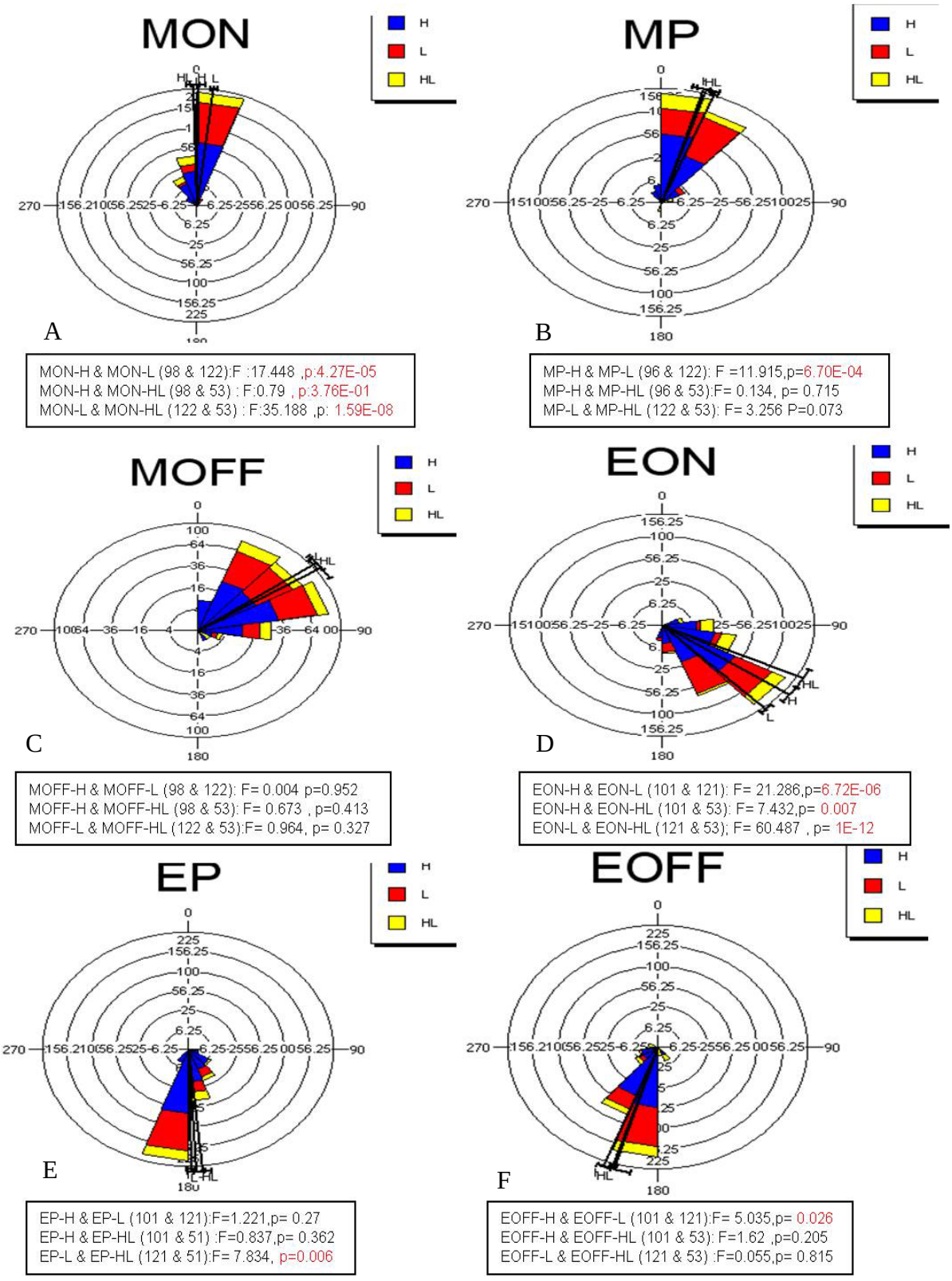


Figure 3.17: Phases of activity in Canton-S male *cry*^{HH}, *cry*^{LL} and *cry*^{HL} lines. The activity on Morning On Set (MON), Morning Peak (MP), Morning Off set (MOFF), Evening On Set (EON), Evening Peak (EP), Evening Off (EOFF) are compared. The significant differences are in red.

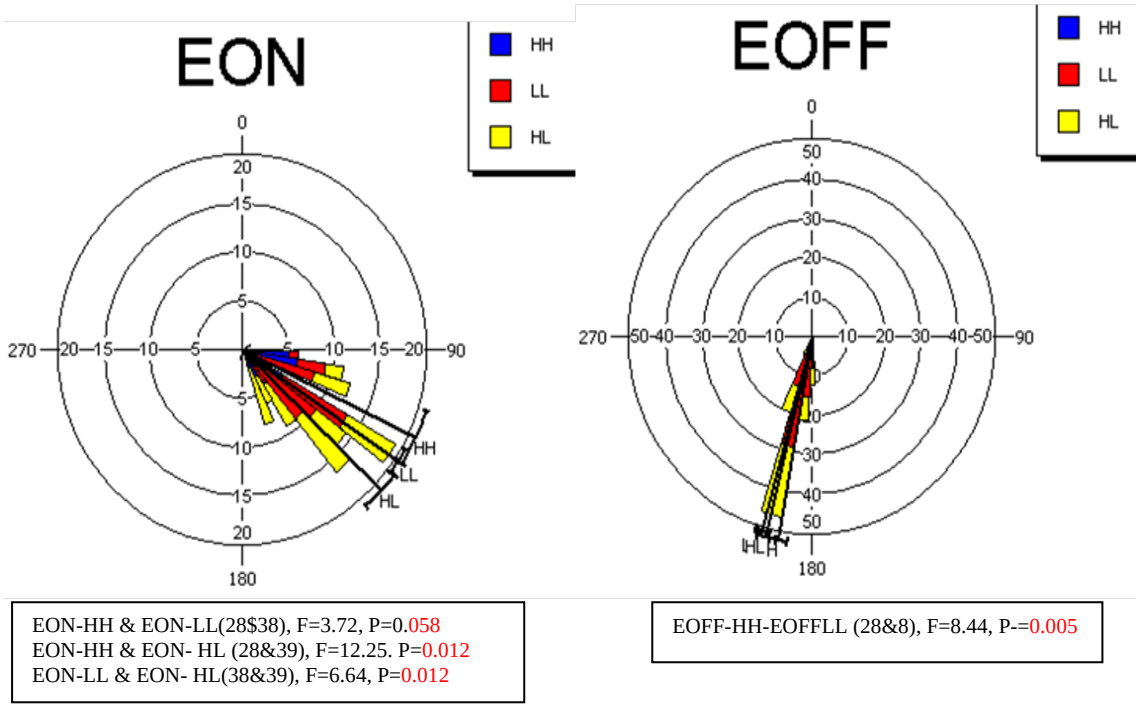


Figure 3.18: Phases of activity in Canton-S female *cry^{HH} cry^{LL} and cry^{HL}* lines. The activity of Evening Onset (EON), Evening Off (EOFF) were found to be significantly different.

3.2.6.2 Phase analysis for Rende lines

MON in the Rende male flies was earlier in *cry^{LL}* than *cry^{HH}* ($F_{(1,43)}$= 6.3, p=0.016, Figure 3.19A). While in the case of virgin females the MON of activity was opposite in respect of genotype and *cry^{HH}* was observed to start earlier than *cry^{LL}* flies ($F_{(1,75)}$= 6.5, p=0.013, Figure 3.19B). No difference was recorded in the MP and MOFF of males and females (Table 3.4). In the evening when the flies start their activity again; *cry^{LL}* males started their activity much earlier than *cry^{HH}* males ($F_{(1,42)}$=10.7, p=0.002). The time difference was about 53 minutes (Figure 3.19C). For the females there was also a significant difference ($F_{(1,180)}$=10.99, p=0.001) between these two lines in the onset of evening activity but was in the opposite direction, so *cry^{HH}* females started their activity early than *cry^{LL}* females.

There was a significant difference ($F_{(1,180)}$=4.3, p=0.04) in the evening peak of activity in the Rende females. It was observed that cry^{HH} females reached the peak of their activity early than cry^{LL} females (Figure 3.19D). The difference was not significant in the males only experiment ($F_{(2,62)}$=0.034, p=0.93) but still in the same direction as that of the females (Table 3.4).

	Phases	**Mean phase of each Genotype in hours**					
		cry^{HH}	cry^{LL}	cry^{HL}	F	P-value	Waking Earliest
Males	MON	23.96	21.97	23.88	$17.09_{(2,58)}$	1.51e-06	cry^{LL}
	MP	2.63	2.13	2.34	$0.58_{(2,49)}$	0.563	
	MOFF	4.95	4.35	4.15	$0.64_{(2,33)}$	0.531	
	EON	9.56	8.67	9.18	$3.72_{(2,61)}$	0.03	cry^{LL}
	EP	11.22	11.35	11.35	$0.07_{(2,65)}$	0.93	
	EOFF	12.66	12.56	12.58	$0.11_{(2,64)}$	0.89	
Females	MON	23.89	0.29	23.80	$4.2_{(2,100)}$	0.017	Cry^{HH}
	MP	1.88	1.55	1.78	$1.4_{(2,104)}$	0.241	
	MOFF	4.64	4.42	4.10	$1.1_{(2,106)}$	0.322	
	EON	7.84	8.76	8.80	$5.7_{(2,106)}$	0.004	Cry^{HH}
	EP	10.59	11.12	11.40	$5.1_{(2,106)}$	0.007	Cry^{HH}
	EOFF	12.04	12.27	12.89	$2.8_{(0,106)}$	0.06	Cry^{HH}

Table 3.4: Summary of the phase of activity in the rende cry^{HH}, cry^{LL} and cry^{HL} male and female flies. Table shows mean phase of activity for each genotype during LD 12:12 under 25oC. The significant difference is shown is the red. The genotype starting their activity the earliest at a particular phase is also mentioned.

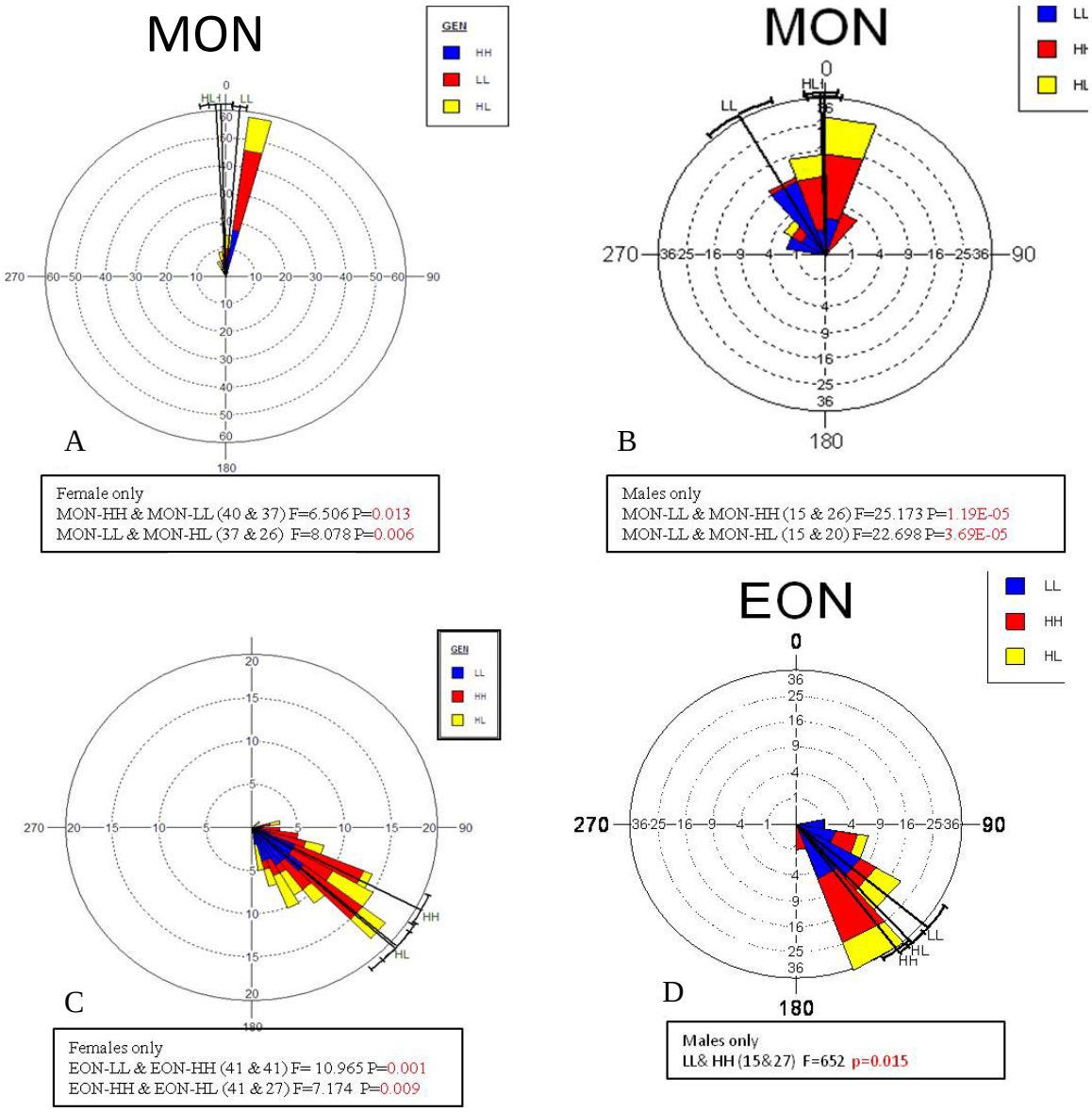


Figure 3.19: Comparison of morning onset (MON) and evening onset (EON) of activity in the Rende *cry*^{HH}, *cry*^{LL} and *cry*^{HL} females (A &C) and males (B&D).

So overall comparison of the males and females for Rende line (Table 3.4) shows that the initiation of the morning and evening bouts of activity for *cry*^{HH} and *cry*^{LL} are in the opposite direction. *cry*^{LL} males start their activity earlier than *cry*^{HH} males both in the morning and evening. For females, *cry*^{HH} were observed to be starting their activity earlier than *cry*^{LL} both in the morning as well as in the evening.

These results for this phase difference were followed by the comparison of the total amount of activity by males and females of these two genotypes. The amount of activity for cry^{HH} *f*emales was highest while for cry^{HH} males was the lowest in the overall comparison. Which shows that during the day the cry^{HH} females are more active than the cry^{HH} males (Figure 3.20)

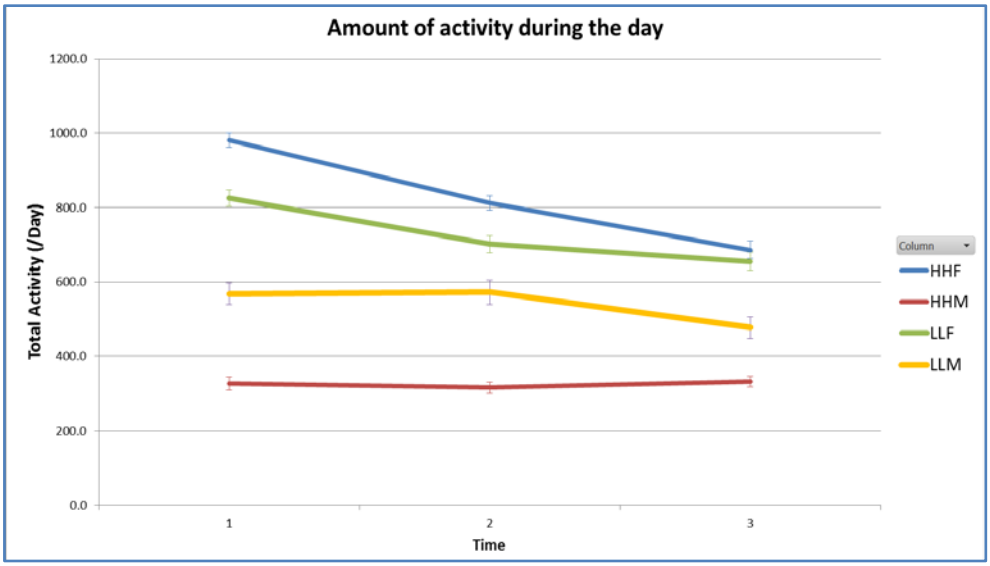


Figure 3.20: Comparison of the total amount of activity during the day for cry^{HH} and cry^{LL} males and females. cry^{HH} females were observed to be most activity while cry^{HH} males were the least active.

3.2.7 Flies under constant light

This experiment was performed to compare the light sensitivity of the three different genotypes. Males were exposed to continuous light (LL) for 7 days after being entrained for 3 days in LD12:12 at 25°C. Under LL, wild-type flies become arrhythmic (Konopka et al. 1989). For Canton-S lines the percentage of rhythmic flies was observed to be higher in the cry^{LL} flies as compared to the other two genotype

(Table 11). The rhythms observed were multiple rhythms in most of the flies. It shows that at least for this background cry^{LL} flies are less light sensitive than cry^{HH} and cry^{HL} flies. The same experiment was repeated using Rende flies as well but all flies of the three genotypes showed arrhythmic behaviour under continuous light suggesting they are not different in their light sensitivity.

Genotype	*No.*	Arrhythmic	%Arrhythmic	Rhythmic	Single peak	Multiple peaks	Period	SEM
cry^{HH}	32	23	85.19	4.00	0	4	21.16	0.86
cry^{LL}	32	17	53.13	15.00	2	13	24.65	0.52
cry^{HL}	32	28	93.33	2.00	0	2	25.07	1.31

Table 3.5: Canton-S flies observed under constant light (LL).

3.2.8 Eclosion phenotypes for L232H polymorphism

The two genetic backgrounds containing three different genotypes for L232H SNP were compared for the timing of adult eclosion. The comparison was performed using the Watson-Williams F-test in "Oriana". For both Canton-S (F=1.62, p=0.2) as well as Rende strains (F=1.24, p=0.29) there was no significant difference in the eclosion rhythms among the three male genotypes (Figure 3.21 b&d). The comparison of females revealed significant difference among the three genotypes. In the case of Canton-S cry^{HH} homozygous females were recorded to be eclosing 3.85 h earlier than cry^{LL} flies (F=9.2, p=0.004, Figure 3.21a). The eclosion phase for the heterozygotes was intermediate. Similar results were obtained with the Rende line (Figure 3.22 a). For these flies the difference was 2.5 h with the cry^{HH} females emerging earlier than cry^{LL} (F=4.55, p= 0.03).

We also compared the eclosion phases between the males and females for these three genotypes. It was observed that in case of cry^{LL} the male flies are eclosing 4 h earlier for Canton-S (F=11.03, p=0.002) and 2.4 h earlier for Rende (F=7.465, p=0.008) than females (Figure 3.22). No difference was recorded for the other two genotypes.

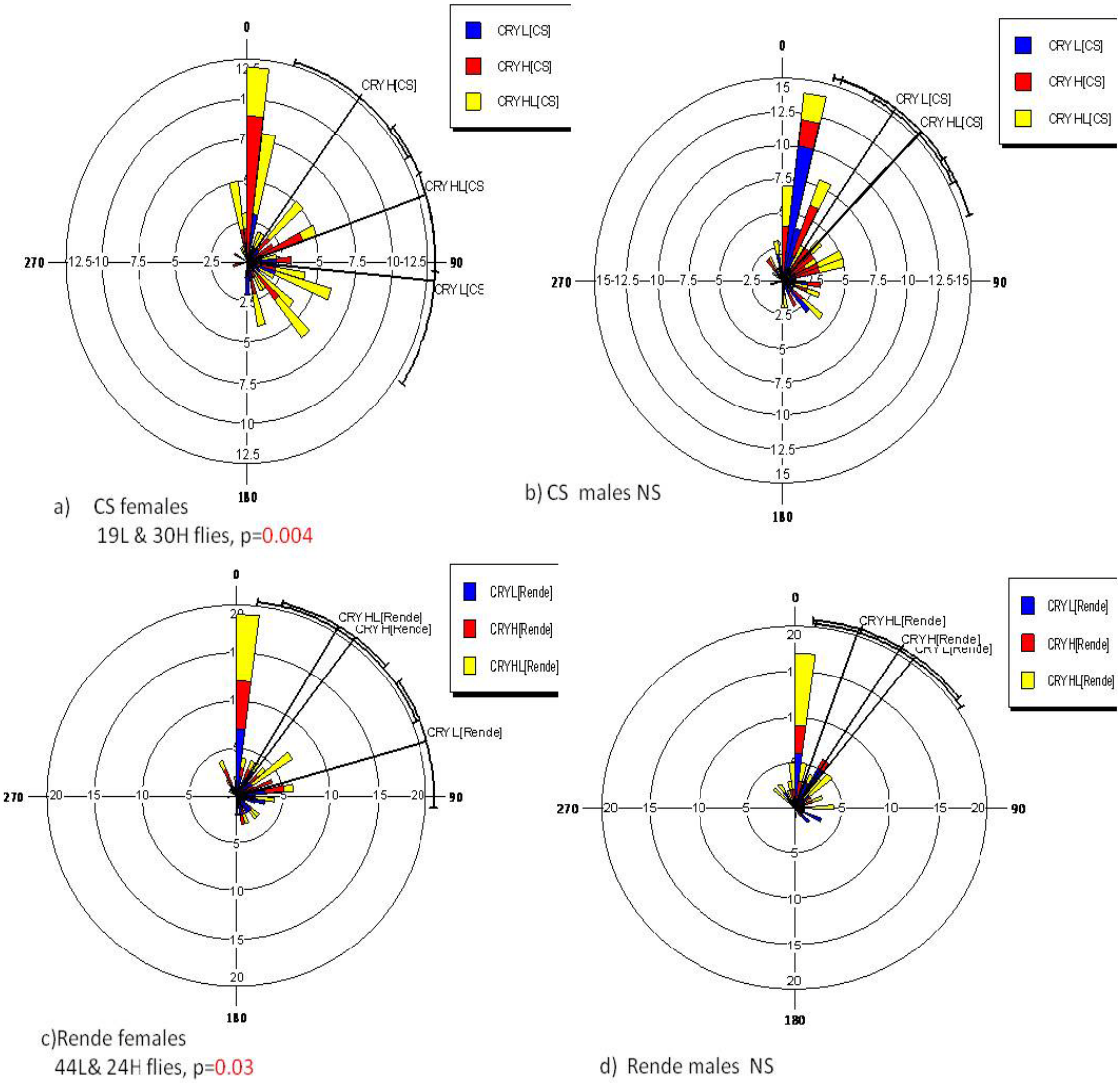


Figure 3.21: Eclosion phase of female and males for Canton-S (a&b) and Rende (c&d). For both of the fly strains cry^{HH} flies are emerging significantly earlier than cry^{LL} in case of females. While no significant difference is obtained while observing male flies.

a) Rende flies p=0.008 b) Canton-S flies p=0.002

Figure 3.22: Comparison of the phase of eclosion between cry^{LL} male and female flies showed a significant difference for both Rende (a) and Canton-S (b). Males are emerging earlier than females.

3.4 Discussion

The L232H polymorphism has uniform spatial distribution along the latitudinal gradient throughout Europe which seems to be maintained by balancing selection. This intermediate frequency of the two alleles also suggests that the role of this SNP is neither temperature nor photoperiod related otherwise we could expect to see a cline in its distribution. Our observation of similar circadian responses by the flies harbouring the two alleles to the light pulses also supports this view. However Pegoraro et al. (submitted) observed some differences in the level of expression of CRY in the two variants by western blot. In both Canton-S and Rende background, the level of expression of cry^{L} was higher than cry^{H}. By examining the structure of CRY, this difference was expected to affect its stability but as stated earlier, my light pulse experiments do not show any difference in the phase shifts of the two variants to the

light pulse. Pegoraro et al also performed a yeast two-hybrid assay to test the binding of the CRY variants to the TIM but found no difference in the strength of their dimerization. However this difference in the level of expression in Canton-S flies is supported by examining them under constant light where cry^{LL} flies showed comparatively less photosensitivity (and were quite rhythmic under LL) suggesting the presence of less degraded CRY. We could not see any difference with the flies in the Rende background under LL. However by repeating these experiment under reduced light intensities might give a greater probability of uncovering a subtle difference in the phenotypes of the flies harbouring these two alleles.

Canton-S gave very contrasting phenotypes in the population cage experiment where cry^{HH} appeared to be at an extreme competitive advantage to cry^{LL}. In the fitness experiment cry^{HH} flies emerged earlier and it is conceivable that they might lay their eggs earlier giving them a potential advantage over cry^{LL}. This observation was later confirmed with the eclosion experiment where cry^{HH} females eclosed significantly earlier than cry^{LL} females. The phase analysis of these flies in LD showed that cry^{HH} start their activity earlier than cry^{LL} in the morning. This phase difference between the genotypes was significant for male but not for females. In the evening both males and female cry^{HH} homozygotes again started their activity significantly earlier than cry^{LL} homozygotes. There seems to be a general phase advance of cry^{HH} over cry^{LL} in several phenotypes on this background.

My experiments with population cages using the Rende background yielded more interesting results and the frequency of the two alleles converges to almost 1:1. The Rende lines showed that the frequency for L232H polymorphism which we see in wild can also be recapitulated in the laboratory. The mechanism maintaining the

equal frequency might be heterozygote advantage or assortative mating or perhaps some other mechanism. No consistent deviation from HWE was observed that might favour heterozygote advantage. All these observations suggest that the frequency of the L232H polymorphism seems to be maintained by negative frequency dependent selection, where the frequency of the given alleles is decreased as its frequency increases (Ayala and Dobzhansky 1974; Fitzpatrick et al. 2007) this mechanism was suggested to be responsible for maintaining the natural polymorphism in the *foraging* gene of Drosophila (Fitzpatrick et al. 2007).

The behavioural analysis of the Rende lines showed some interesting activity profiles for each of the genotypes. The cry^{LL} flies had a marginally longer period than cry^{HH} flies. When these strains were compared for their fitness, cry^{LL} showed higher viability than cry^{HH}. All the analyses were performed under 25^{o}C temperature conditions which are relatively warm. Also the observation of comparatively high frequency of cry^{L} (average 57%) in the lower latitude supports the idea that cry^{LL} might be more temperature tolerant or it is favoured in the warmer conditions.

The phase variation encoded by different clock alleles may serve as a first step towards temporal speciation, resulting in the pre and post zygotic barriers. This can be seen in the case of *per* where transgenic *D. melanogaster* flies carrying a heterospecific *per* transgene from *D. pseudoobscura*, showed circadian locomotor and mating phases similar to the parental *D. pseudoobscura* (Petersen et al. 1988; Tauber et al. 2003). These mating phase differences led to assortative mating so *per* potentially contributes to the temporal speciation (Tauber et al. 2003). Kolaczkowski et al. (2011) performed a study on the genomic differentiation between the temperate and tropical Australian population of *D. melanogaster*. They showed extremely high genetic differentiation in

cry among the Australian populations of *D. melanogaster.* In the melon fly *Bactrocera cucurbitae*, variation in the *cry* gene was suggested to be responsible for the difference in the mating times between different populations. This led to pre-mating reproductive isolation (Fuchikawa et al. 2010).

The variation observed in the phases of activity in the *cry* variants of *D melanogaster* might also be important in this regard, as we saw that in the case of Rende flies, male cry^{LL} started their activity early both in the morning as well as in the evening, while for the females the phases were totally opposite to this i.e cry^{HH} females started their activity earliest in morning and evening. The eclosion data also confirmed this phase difference in the case of cry^{LL} where male flies eclose earlier than females. This observation was further supported by the comparison of the total amount of activity of the males and females of the two genotypes during the day. Which showed that female cry^{HH} flies are most active among all others in comparison while cry^{HH} males were the least activity. This could lead to more interaction between the flies of the opposite sex carrying the two variants (cry^{HH} female flies and cry^{LL} males) and disassortative mating. This might explain the almost equal frequency of the two alleles in wild as well as in population cages. It suggests that when cry^{HH} females start their day, they find cry^{LL} males around and both of them mature almost together and have more chances of mating with each other than their own genotypes hence lead to production of more hetrozygotes. This detailed analysis of the phenotypes and fitness of each of the *cry* variants reveal some differences in behaviour that could potentially lead to the balanced polymorphism that we see in nature and in some laboratory backgrounds. However there is a possibility that some other genes located close to *cry* also contribute to these effects we see in these lines especially with the

contrasting results obtained using canton-S flies. The reason behind the failure of Canton-S population cages to mimic the wild, even after backcrossing might also be that a deleterious mutation very close to the *cry* locus must have been crossed inadvertently into cry^{LL} or cry^{HH} .

Chapter 4:

PER :TIM Coevolution

4.1 Introduction

The molecular basis of the circadian clock has been extensively studied in many animals and has led to the idea that the general mechanism that underlies the clock is conserved. It consists of interlocked autoregulatory feedback loops that function through the transcription/translation of the positive and negative elements (Bell-Pedersen et al. 2005). In Drosophila the model for rhythms generation in the pacemaker cells involves several core genes. The interaction of the protein products of these genes with associated kinases and phosphatases leads to pace-setting of the clock by regulating the timing of nuclear entry and inter-molecular interactions (Zheng and Sehgal 2008).

The study of circadian oscillators in different organisms so far shows an astonishing degree of complexity and diversity in the underlying mechanisms (Brown et al. 2012). The evidence shows that multiple clock mechanisms are in operation at the same time (Brown et al. 2012). For example in cyanobacteria, the transcriptional translational feedback loop (TTFL) oscillation of Kai operon can exist independently of cyclic phosphorylation of KaiC under certain conditions (Kitayama et al. 2008). These studies as well as the phylogenetic analysis of the clock protein sequences shows that

clock in the different organisms use different genes (Brown et al. 2012). Even in organisms that share a close evolutionary relationship, these circadian molecules have diverged as have their circadian phenotypes (Piccin et al. 2000). This divergence among species has been used to suggest that PER and TIM coevolve at their inter-molecular binding regions (Piccin et al. 2000 see Chapter 1). The idea of the PAS mediated interaction between *Musca* PER and *melanogaster* TIM (Piccin et al 2000), can be further extended to other *melanogaster* clock proteins such as dCLK and dCYC. In addition, Peixoto et al. (1993; 1998) using *D. melanogaster-D. pseudoobscura* chimeric genes revealed that *per* itself undergoes intramolecular coevolution around its Thr-Gly repeat region.

Phylogenetic analysis of *tim* from *D. virilis* and *D. hydei* revealed that TIM, is more conserved than PER (Ousley et al. 1998). Ousley et al. (1998) also reported the first robust rescue of the tim^{01} mutant using a *tim* transgene with an average period of 24h. Nishinokubi et al (2003) showed that *D. ananassae-tim* transgene was also able to rescue behavioural rhythms of *D. melanogaster* tim^{01} mutants. In another study the same group induced the *D. ananassae* TIM protein through heat shock and examined the behaviour of the *D. melanogaster* tim^{01} flies (Nishinokubi et al. 2006). The level of TIM protein was increased initially by the application of heat shock and decreased after some time. Their results demonstrated that by applying this heat shock at different time of the day, these transgenic *D. melanogaster* tim^{01} flies became nocturnal like wild *D. annanasae*. They also analysed the mating activity rhythms of these transgenic lines and found that the profiles of mating activity were different from both *D. melanogaster* and *D. ananassae.* They inferred from their observations

that species-specific mating activity rhythms are controlled by many factors and their pathways are different from those which control the locomotor activity rhythms. Their data showed that like *per, tim* might also play a role as a speciation gene. These examples provide further implicit support for the molecular coevolution between PER and TIM, as these heterospecific *tim* transgenes can provide some level of rescue.

This chapter aims at studying the intergenic coevolution between PER and its partner TIM. This will be performed by transforming the *D. melanogaster* flies simultaneously with *D. pseudoobscura per* and *tim* in the double mutant background per^{01}; tim^{01}. The level of rescue of these transgenes and the periods obtained will be compared with the *D. melanogaster* control lines which have *D. melanogaster* transgenes in the mutant background. This analysis will be performed for the transgenes individually and in combination to see the level of rescue and period of locomotor cycles. I will also investigate if I can switch the *D. melanogaster* hosts behavior to that of *D. pseudoobscura* and thereby map species-specific behavior to these candidate genes (Tauber et al. 2003).

4.2 Materials and Methods

4.2.1 The transgenics

I initially re-examined stocks that had been maintained in the laboratory for many years in order to confirm that they had not been contaminated. Initially 6 strains of flies were obtained for this purpose, some containing the *D. pseudoobscura per* transgene (Peixoto et al. 1998) and other generated by Eran Tauber (pers comm) carrying the *D. pseudoobscura tim* gene These lines were genotyped using PCR (see

Chapter 2), for the presence of tim^{01}, tim^{+}, per^{+}, per^{01} and also for the transgenes of *pseudoobscura-tim* and *pseudoobscura-per*. The results obtained were compiled in Table 4.1.

The next step was to find out the location of each transgene. The location of the *pseudoobscura per* inserts was already known from Peixoto et al (1998). The *tim* transgenes are marked with w^{+}. In order to locate the chromosomal position of the *pseudoobscura tim* inserts, flies were crossed to double autosome balancer virgin females *w; CyO/Sco; TM6b/MKRS*. Among the F1, *CyO* and *MKRS* male flies with red eyes were selected and crossed to *w;* tim^{01} virgins. If all the *CyO* flies in the F2 were white- eyed, this revealed that the transgene is located on chromosome II. Similarly, if all *MKRS* flies were white-eyed this reveals a chromosome 3 insert. An X chromosome insertion was indicated when all the flies in the F1 are white-eyed.

4.2.2 Fly strains

A total of 12 *D. melanogaster* transgenic lines were used for the study of coevolution. The results of PCR and genetic crosses with double balancers revealed the position of the transgenes for the following lines (Table 4.1)

1	***w; tim219(19)***	*per^{+};tim^{01},pseudoobscura-tim*	II	
2	***w; tim219(21)***	*per^{+}; tim^{01}, pseudoobscura-tim*	II	
3	***w; tim35(14)/CyO***	*per^{+}; tim^{+}tim^{01},pseudoobscura-tim*	II	Homozygous lethal
4	***170B***	*per^{+}; tim^{+}; pseudoobscura-tim*	III	
5	***187/CyO***	*per^{+},pseudoobscura-tim;tim^{+}tim^{01};*	II	Homozygous lethal
6	***223 tim^{0}/ CyO***	*per^{+}; tim^{+} tim^{01};*	No insert	
7	***I20/CyO***	*per^{01}; tim^{+}, pseudoobscura-per*	II	Homozygous lethal
8	***I26***	*per^{01}; tim^{+}; pseudoobscura-per*	III	

Table 4.1: PCR and crossing results for all tranformants initially selected for the study showing the presence or absence of the transgene and also its chromosomal position.

4.2.3 Genotyping

Genotyping for each line was performed using PCR. The list of primers and their annealing temperatures is given in Table 4.2. In the case of *per^{01}* and *per^{+}* flies the distinction required an initial PCR product of 199 bp followed by a restriction of this fragment into two fragments of 107 and 92bp in the case of *per^{01}* flies. The composition of the mixture per sample for this process is as follows

XBAI= 1ul: Buffer2 or 4= 3ul BSA= 0.3ul: DNA from the PCR mixture= 10ul: PCR Water= 15.7ul

Total mixture= 30ul. Incubate on 37C for 1-3 h and then run on 2% agarose gel

1	*DmTim0 F*	*GCTCATCGCTTTTCATATGTT*	57
2	*DmTim R*	*AGGATGTGATTGGTAACCAC*	57
3	*DmPer0 F*	*TACCACCACGAGGACCTCTC*	57
4	*DmPer R*	*GATGGTGTCCGACGACAAAT*	57
5	*Pseudoobscura per F*	*ACCACCACGATGACCTCCCC*	59
6	*Pseudoobscura per R*	*TTGTTCTGCAACTCCTCCGCG*	59
7	*Pseudoobscura tim F*	*ACATACCGGAAACGCACGGG*	59
8	*Pseudoobscura tim R*	*CTTGTAGATCAGCGCGATCAAC*	59

Table 4.2: The table list all the primers used for the genotyping of the transgenic flies. Their names, sequence and annealing temperature is given.

4.2.4 Crosses to bring the two *D. pseudoobscura* transgenes together

The two transgenes; *psuedoobscura-per* and *pseudoobscura-tim* were brought together by following a series of crosses to obtain the homozygotes per^{01}; tim^{01} , tim^{ps}; per^{ps}. (See Figures 4.2, 4.3, 4.4, 4.5)

P ♀ *w/w;* tim^{01},tim^{ps}/ tim^{01}, tim^{ps};+/+ (Orange eye *pseudoobscura –tim* transgenics) X ♂ *w/Y; CyO/Sco; TM6b/MKRS* (*white* eye double balancers)

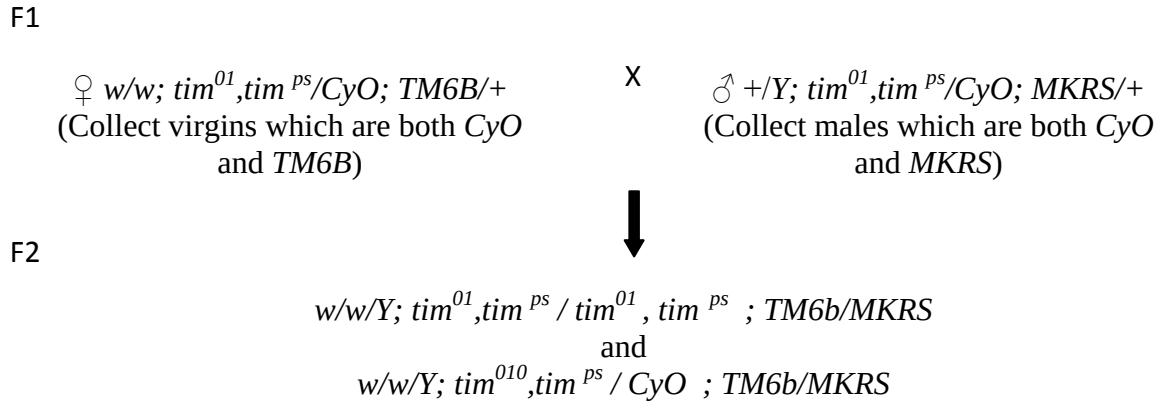


Figure 4.2: Genetic cross using *pseudoobscura-tim*

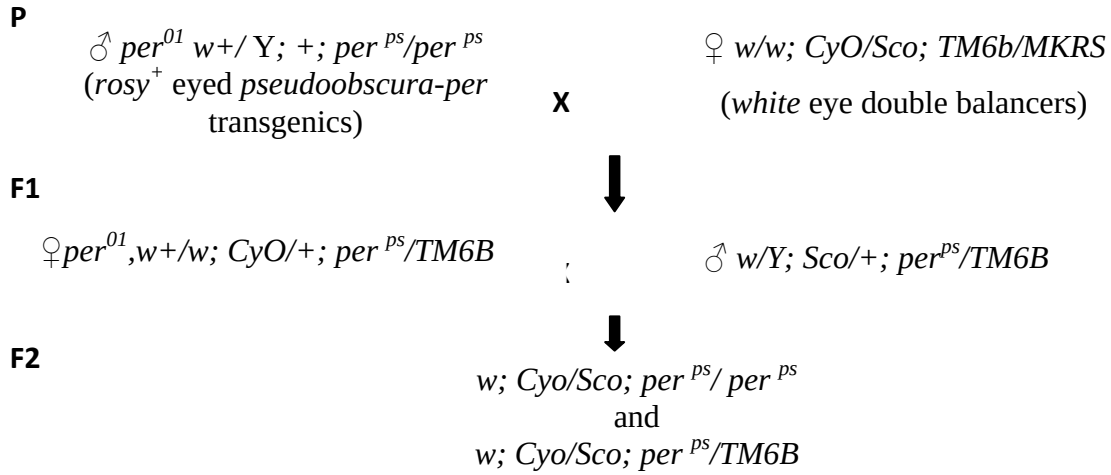
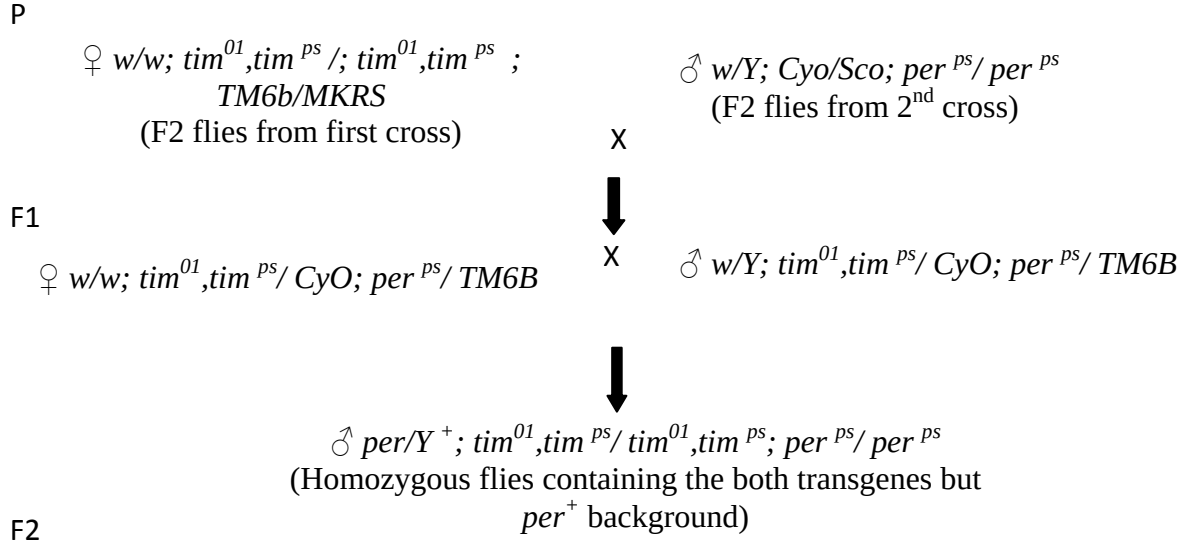


Figure 4.3: Genetic crosses with *pseudoobscura-per* transgene



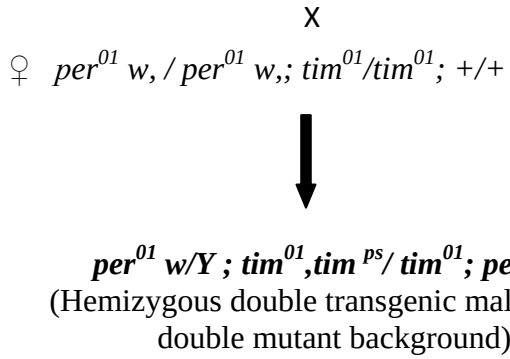


Figure 4.4: Crosses to obtain one copy of each transgene in the double mutant background.

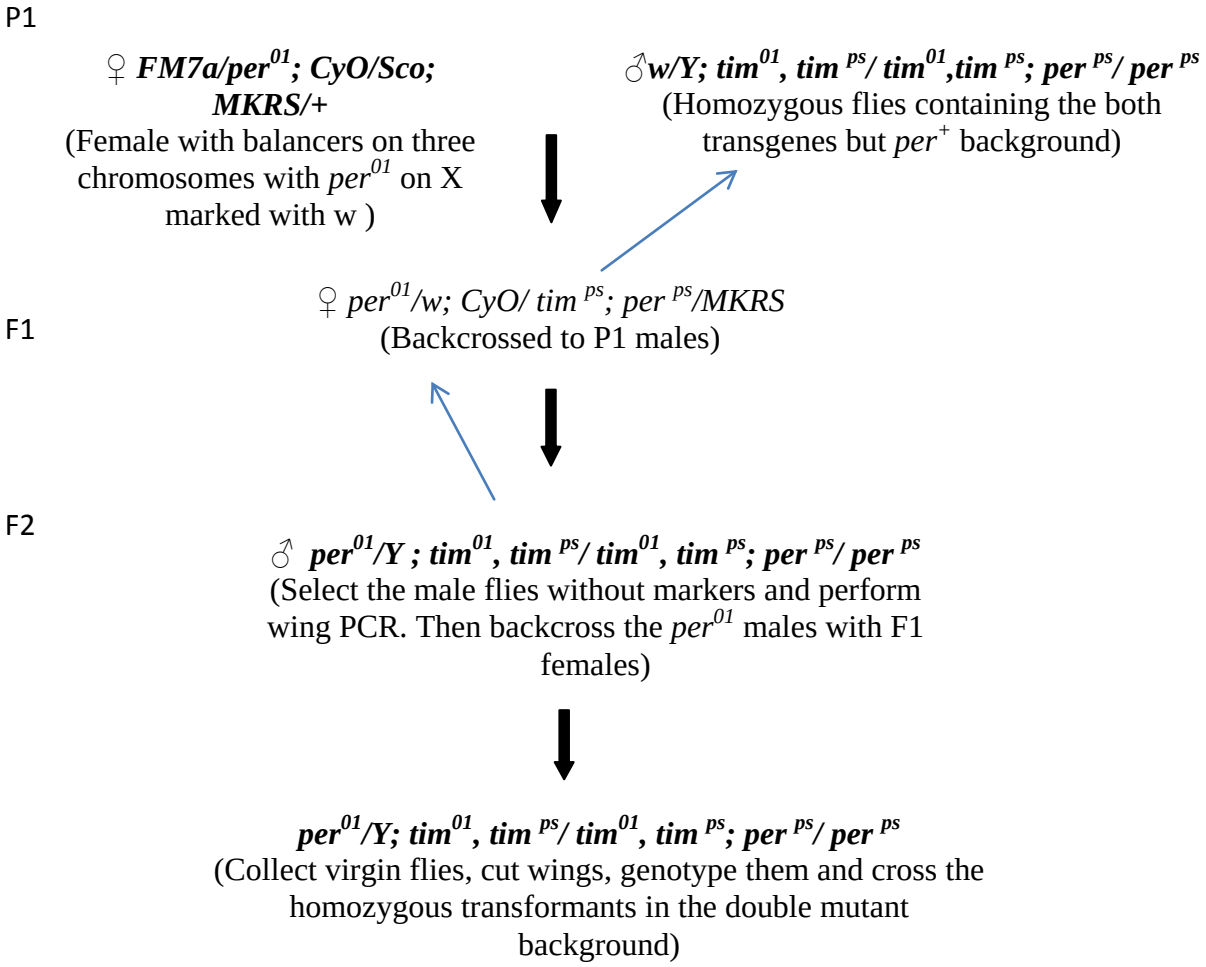


Figure 4.5: Final cross to obtain the homozygous transformant flies having two copies of *pseudoobscura-tim* and *pseudoobscura-per* in the *per^0; tim^0* background.

4.2.5 Crosses with strain I20

The PCR and genetic crosses show the *pseudoobscura-per* gene and *melanogaster-tim* both located on the second chromosome. So before starting the crosses the first task was to remove tim^{+} and put the inserts into a tim^{01} mutant background by recombination. With crosses and wing PCRs flies which were tim^{01}/tim^{01}, per^{ps} /+ were selected. During these cross the per^{01} background was lost and the resulting flies ended up as per^{+}/per^{+}; tim^{01}/tim^{01}/ $per^{ps/}$ /+. These flies were further crossed to per^{+}/per^{+}; tim^{ps}, tim^{01} (strain 19 and 21). After obtaining flies which have one copy each of both *D. pseudoobscura-per* and *tim* in the tim^{01} but per^{+} background, the behavioural analysis was performed

4.2.6 Behavioural analysis

The behaviour of the flies was recorded at 25°C and 18°C inside an incubator. The analysis was performed as described in the chapter 2. Flies were entrained in LD 12:12 for 3-4 days and then left in DD for 7-10 days. Phases of the peak of morning activity as well as evening activity were compared among the transgenic lines under LD 12:12 at the two different temperatures. Statistical analysis was performed using R, Statistics 5 and Oriana.

4.2.7 Western blots

Western blots were performed for PER proteins. Flies were entrained under LD 12:12 on either 25°C or 18°C and collected in liquid nitrogen at different time interval. They were kept at -80°C if not being used. Protein was extracted from the heads of

about 50 flies according to the protocol described in Chapter 2. Polyacrylamide gels were prepared and proteins were loaded.

4.2.8 Quantitative Real-Time PCR

qPCR was performed to check the expression of *tim*. Flies were entrained under LD 12:12 at 25^{o}C for 3 days than collected in liquid nitrogen at ZT12. RNA was extracted from the heads and qPCR was performed according to the protocol described in Chapter 2. The following primer was used for the amplification of cDNA. They were designed against the variable regions between the two species genes.

1	*GATCTGCTGGGATGGACGAT*	FTIMpseud1
2	*GCCACCTCGTTGTCACACTC*	RTIMpseud1

Table 4.3: Primer sequences used for the amplification of *pseudoobscura-tim* in qPCR

4.3 Results

4.3.1 Protein Sequence comparison of *D. melanogaster* and *D. pseudoobscura* clock genes

Before performing the practical behavioural comparison of the transgenes I compared the protein sequences for all the important clock genes *per*, *tim, dClock, dCyc* and *cry* from available insects focusing particularly on *D. melanogaster* and *D. pseudoobscura*. The protein sequence of PER has already been compared between *D. melanogaster* and *D. pseudoobscura* and a phylogenetic analysis has been performed

(Colot et al. 1988; Piccin et al. 2000). The protein sequence alignment showed 62% similarity between *D. melanogaster* and *D. pseudoobscura* PER. Sequences were retrieved from NCBI and by using Clustlaw2 (Clustalw 2.1 multiple sequence alignments) they were aligned against each other. The species studied were *D. persimilis, D. simulans, D. yakuba, D. annanasea, D. willistoni,* and *D. virilis.* Sequences from other biological clock model organism such as *Mus musculus, Nasonia vitripennis, Musca domestica, Anopheles gambiae* and *Antheraea pernnyi* were also used where available. All these sequences were compared by phylogenetic trees using "Geneious R6.1" software.

4.3.1.1 TIM

Protein sequence of the TIM was aligned from the previously mentioned species and the level of similarity was obtained (Figure 4.7). A phylogenetic tree was constructed by using the Neighbour Joining method (Figure 4.6). For *D. melanogaster* the highest similarity was observed with *D. simulans* (98%) and *D. yakuba* (95%). The similarity between *D. melanogaster tim* and *D. pseudoobscura tim* was 75%. For *D. pseudoobscura* the highest level of similarity was observed with the sibling species *D. persimilis* (89%). Of note is that the *D. pseudoobscura/persimilis* cluster is further from *D. melanogaster* than *D. virilis,* which is believed to have had a common ancestor with *D. melanogaster* further back in the past than *D. pseudoobscura/persimilis* (63 million years for *D. virilis* and 25-30million years for *D. pseudoobscura* (Tamura et al. 2004; Russo et al 1995; Schlotterer et al 1994). The phylogenetic trees were constructed both using the full length protein sequences as well as with only PER interaction domains PER1 and PER2. Given the switching of positions of TIM interacting domains in

PER (Piccin et al. 2000) we might expect something similar in the PER interacting domains of TIM. However, in both cases the position of *pseudoobscura* in relation to the *melanogaster* and other species remained the same (Fig 4.6).The bootstrap values were above 90% for most of the clades. *D. pseudoobscura* clusters away from the other Drosophila species and is placed in same clade with *D. persimilis.* The closest TIM sequence to dTIM among the other model organism was that of the *M. domestica.*

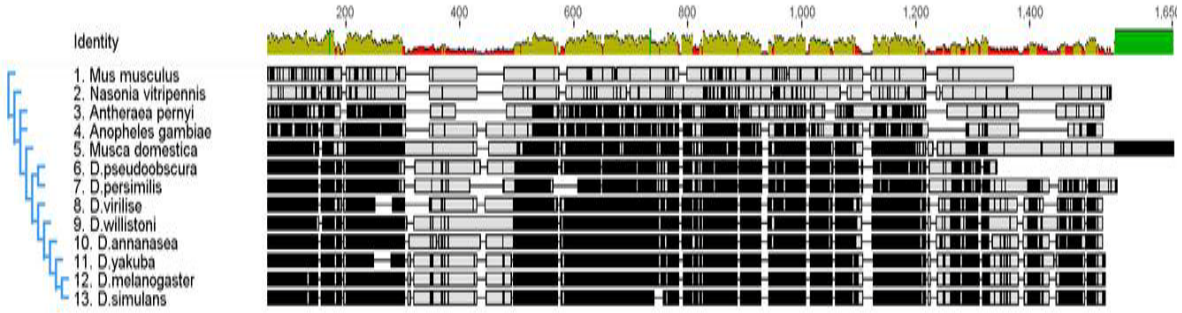


Figure 4.6: Sequence alignments for TIM sequences showing the conserved domains as well the phylogenetic position. The black bars are the conserved regions while grey represent the less/non conserved regions.

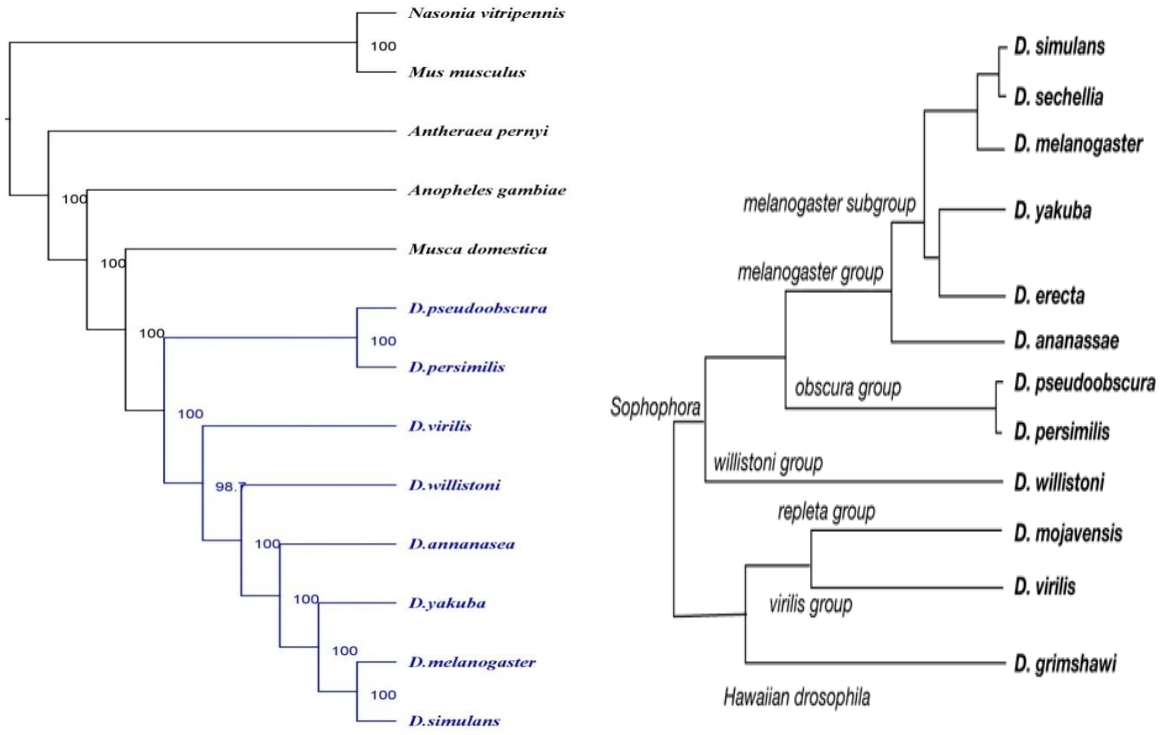


Figure 4.7: (A) Phylogenetic tree of full length TIM sequences in selected species of insects and *Mus musculus.* Numbers refer to bootstrap values (%). *Mus musculus* and *Nasonia vitripennis* act as out group as they have a paralogue of the *tim* i.e. *tim2*. (B)Reference phylogram from 12 Drosphila genome project created using pair wise genomic mutation distances.

4.3.1.2 CLK

PER-TIM also interact with CLK-BMAL1/CYC. Consequently CLK protein sequences from several species of Drosophila as well as *Mus musculus, Nasonia vitripennis, Anopheles gambiae* and *Antheraea pernyii* were also aligned and a phylogenetic tree was constructed (Figure 4.8). CLK sequences from *M. domestica* were not available from the above mentioned resources so could not be included. The results were the same for dCLK in relation to other species as that of dTIM. Again, the *D. pseudoobscura* clade clusters further away from *D. melanogaster* than *D. virilis. D.*

virilis and *D. willinstoni* CLK were in the same clade. The CLK sequence of *N. vitripennis* (hymenoptera) was placed closer to the Diptera than *A. gambiae* in the same clade as the Drosophila group. The position of *D. pseudoobscura* in relation to the *D. melanogaster* was the same for CLK as for TIM. CLK sequence comparison for these two species show 54.5% similarity.

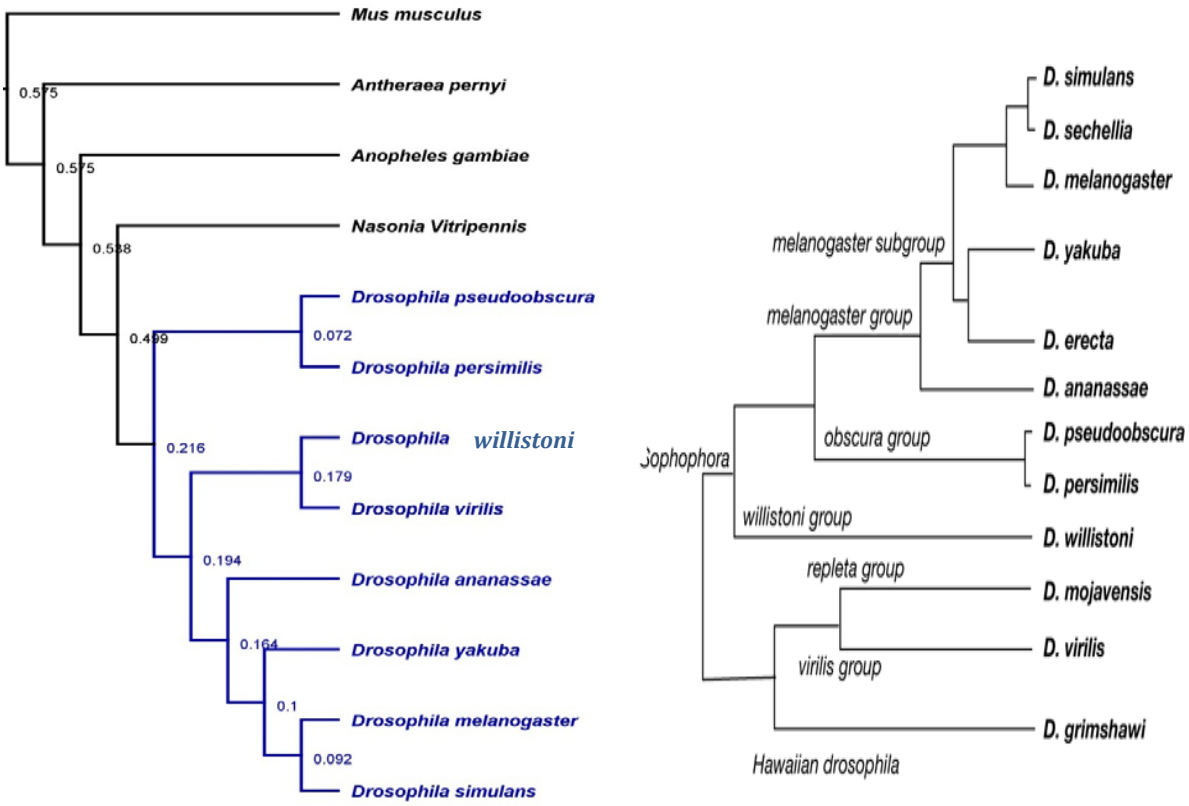


Figure 4.8: Phylogenetic tree for full length CLK protein sequence. Sequences are included from selected insects and also from *Mus musculus* (Numbers shows divergence). (B)Reference phylogram from 12 Drosphila genome project created using pair wise genomic mutation distances.

4.3.1.3 CYC/BMAL1

Very surprisingly, the phylogenetic analysis showed more similarity between *D. melanogaster* and *D.yakuba* CYC than *D. simulans* (Figure 4.9). The position of *D.*

pseudoobscura was closer to *D. melanogaster* than *D. virilise*.The CYC protein sequence similarity was 70% between *D. melanogaster* and *D. pseudoobscura*.

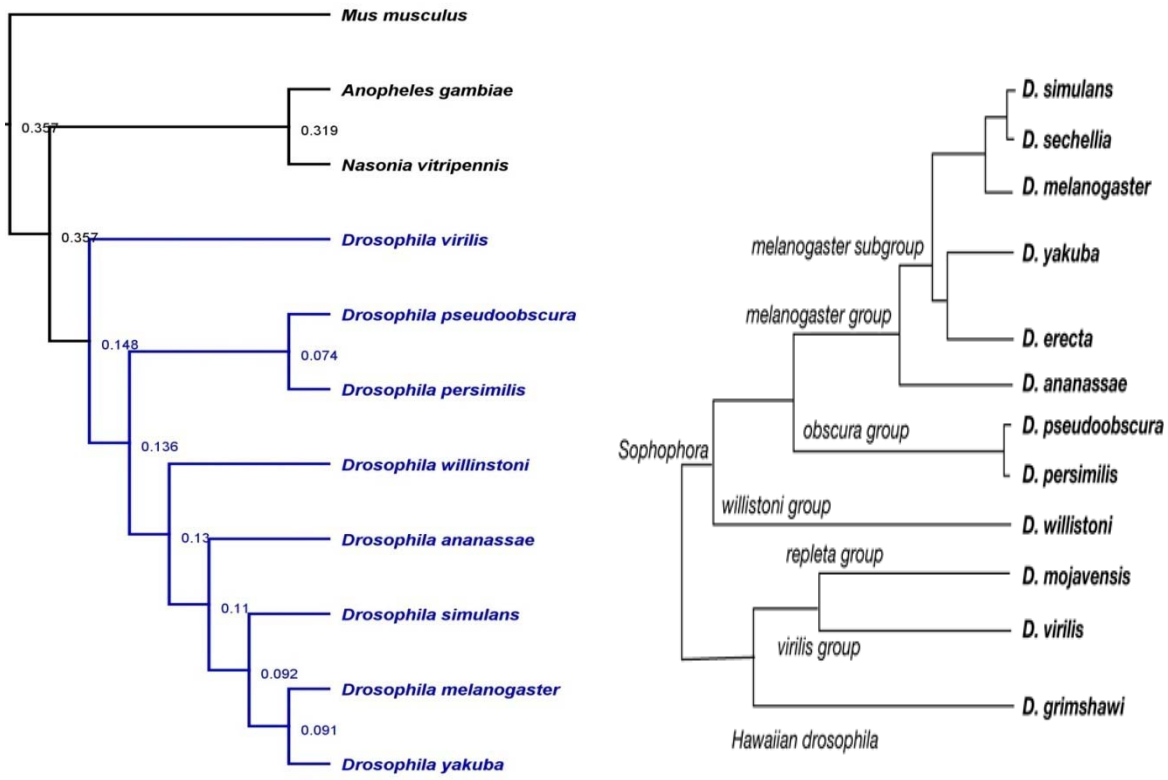


Figure 4.9: Phylogenetic trees for CYC/BMAL1 full length protein (numbers represent divergence). (B) Reference phylogram from 12 Drosphila genome project created using pair wise genomic mutation distances.

4.3.1.4 CRY

The sequence similarity for CRY was found to be 81% between *D. melanogaster* and *D. pseudoobscura* but the topology of the tree is rather different to those of the previous clock proteins. In particular, *D. pseudoobscura* and *D. persimilis* CRY is relatively closer here to *D. melanogaster than D. virilis* and *D. willistoni* (Figure 4.10). Of all the trees, this one resonates with the accepted phylogenetic positions of the different species.

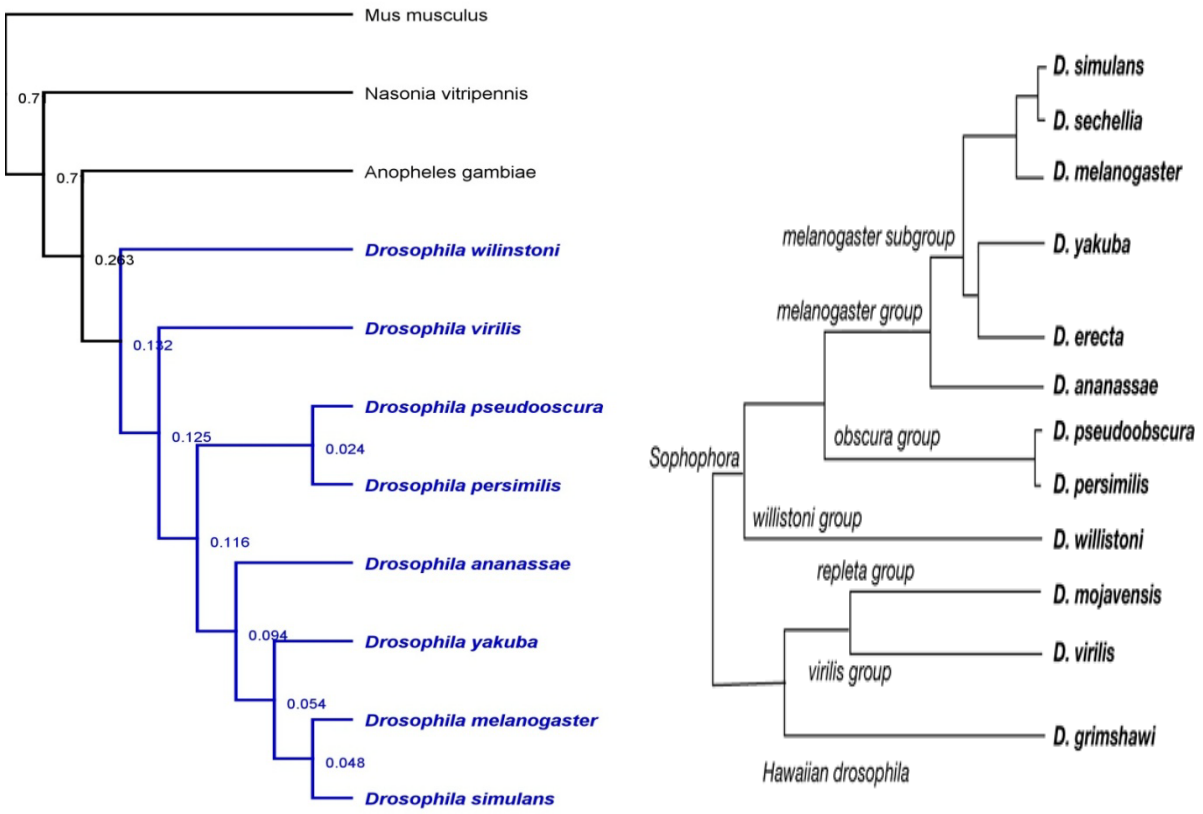


Figure 4.10: Phylogenetic tree for CRYPTOCHROME full length protein (numbers represents divergence). (B)Reference phylogram from 12 Drosphila genome project created using pair wise genomic mutation distances.

Considering the evolutionary distance between all Drosophila species, the position of the *D. pseudoobscura-persimilis* clade is anomalous compared to *D virilise* for both CLK and TIM but not for CRY and CYC.

4.3.2 Locomotor activity rhythms

Behavioural analysis was performed on all the transgenic (hemizygous and homozygous) and control lines at 25^{o}C and 18^{o}C. Flies were entrained under LD12:12 for 3-4 days and then placed in DD for 7-10 days. The flies were classified as rhythmic, weakly rhythmic and arrhythmic according to the criteria in the Chapter 2. A summary

of all the results for the hemizygous flies are shown in Table 4.4 and homozygous flies in Table 4.5.

4.3.2.1 The single transgenics

The *melanogaster tim (w/Y; tim^{01} tim^{mel} / tim^{01} t28s*) and *per* (*per^{01},w/Y ; per^{mel} /+ 2A*) transgenics could rescue the behaviour of the flies which were mutant for these genes at levels of up 100% under 25°C (Table 4.4, Figure 4.11).

A total of 5 *D. melanogaster* mutant lines carrying *D. pseudoobscura* transgenes were used. Two carried *D. pseudoobscura per* (lines *I20* and *I26*) in *per^{01}* background, while three *D. pseudoobscura tim* in *tim^{01}* background (Lines *tim19, tim 21,* and *tim35*). The hemizygous single *pseudoobscura-per* transgenic *per^{01} /Y; tim^{+}; per ^{ps} /+ (I26)* showed rhythmicity of 68 to 96% at 25 and 18°C respectively. *per^{01}/Y; tim^{+}, per ^{ps}/+* (*I20*) also showed 66-88% rhythmicity these temperatures (Table 4.4, Figure 4.11).

The hemizygous *pseudoobscura-tim* transgenics *w/Y; tim^{01}, tim ^{ps} / tim^{01}* (*tim19*) showed 53% rhythmicity at 25°C which was increased to 74% at 18°C. The *w/Y; tim^{01}, tim ^{ps} / tim^{01} (tim21)* had 73% and 64% of the flies rhythmic at 25°C and 18°C respectively. The level of rhythmicity was also similar for these two lines when tested in the homozygous condition (Table 4.5). Hemizygous *w/Y; tim^{01}, tim ^{ps} / tim^{01}(tim35)* showed 54% rhythmicity at 25°C (Figure 4.11).

4.3.2.2 Coevolution between *per* and *tim*

After studying the effect of each transgene individually in all these lines a number of crosses were performed (4.2 Materials and methods) in an effort to

combine the *per* and *tim* transgenes of *D. pseudoobscura* together in the *D. melanogaster* double mutant background ($per^{01}; tim^{01}$).

An overall comparison of levels of rhythmicity in the hemizygous single transgenic $per^{01}/Y; tim^{+}; per^{ps}/+ (I26)$, and $w; tim^{01}, tim^{ps}/tim^{01} (tim19)$ with the hemizygous double mutant $per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ (19)$ flies showed that there was no significant improvement in the level of rescue at 25°C (χ^2 = 3.4, df = 2, p = 0.17, Figure 4.11).

The hemizygous $per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ (21)$ which were produced after crossing $per^{01}/Y; tim^{+}; per^{ps}/+ (I26)$ and $w; tim^{01}, tim^{ps}/tim^{01} (tim21)$ also gave the same results. The overall comparison for the three lines showed no significant difference (χ^2 =3.4, df=2, p=0.18) in the number of rhythmic flies at 25°C (Figure 4.11). However for both of these lines, the rhythmicity of the double transgenics becomes very low at 18°C (Table 4.5, Figure 4.12). The homozygous flies $per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}\ tim^{ps}; per^{ps}/per^{ps}$ showed almost total arrhythmic behaviour with only few flies observing weak rhythms at both of the temperature conditions (Table 4.5).

The corresponding *tim35*-derived lines also showed no improvement in rhythms after obtaining both *pseudoobscura* transgenes. The hemizygous double transgenic $per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+$ flies were 77.6% arrhythmic at 25°C .

In order to further investigate this interaction between *pseudoobscura-per* and *tim* in the double transgenic flies I crossed the *pseudoobscura-tim* transgenic lines with the $per^{+}/Y; tim^{01}, per^{ps}/+$ (*I20*) created from $per^{01}/Y; tim^{+}, per^{ps}/+$ (*I20*) line (see Materials and method). The resulting $per^{+}/Y; tim^{01}, tim^{ps}/tim^{01}, per^{ps}$ flies gave higher rescue than $w/Y; tim^{01}, tim^{ps}/tim^{01}$ (lines *19 21* and *35*). Up to 96% of the flies were observed to be rhythmic under 18°C (Table 4.4).

The results of the per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; $per^{ps}/+$ shows no improvement in rhythmicity but, not surprisingly, the level of rescue in a per^{+} background (per^{+}/Y; tim^{01}, tim^{ps}/tim^{01}; $per^{ps}/+$) is enhanced.

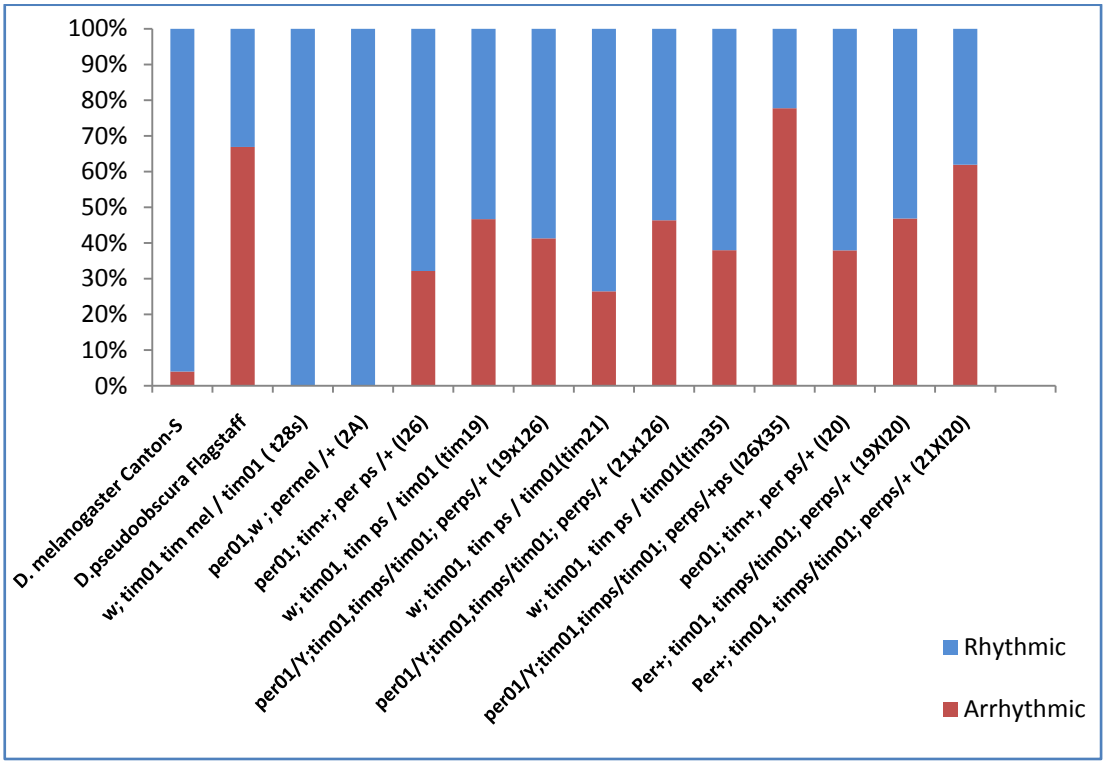
	25°C						18°C							
Genotype	N	Narr	Nr	%r	Period	SEM	N	Narr	Nr	%r	Period	SEM	Chi sq.	**Pref**
***D. melanogaster* Canton-S**	**25**	**1**	**24**	**96**	**23.95**	**0.13**	**22**	**11**	**11**	**50.00**	**23.64**	**0.08**	χ^2 **= 10.7** **p= 0.001**	**25°C**
***D.pseudoobscura* Flagstaff**	**30**	**20**	**10**	**33**	**23.70**	**0.16**	**31**	**4**	**27**	**87.00**	**22.67**	**0.05**	χ^2 **= 16.2** **p= 5.458e-05**	**18°C**
***w/Y; tim^{01} tim ^{mel} / tim^{01}* (t28s)**	**23**	**0**	**23**	**100**	**23.20**	**0.16**	**28**	**6**	**22**	**80.00**	**23.00**	**0.16**	χ^2 **= 2.8** **p= 0.08**	
***per^{01}/Y,w ; per^{mel}* /+ (2A)**	**30**	**0**	**30**	**100**	**24.87**	**0.06**	**11**	**0**	**11**	**100.00**	**24.01**	**0.07**	χ^2 **= 0.008** **p= 0.92**	**25°C**
per^{01}/Y; tim^{+}; per ^{ps} /+ (I26)	**28**	**9**	**19**	**67.85**	**31.23**	**0.46**	**30**	**1**	**29**	**96.60**	**26.77**	**0.07**	χ^2 **= 6.5** **p= 0.01**	**18°C**
***w/Y; tim^{01}, tim ^{ps} / tim^{01}* (tim19)**	**30**	**14**	**16**	**53.3**	**20.82**	**0.39**	**35**	**9**	**26**	**74.20**	**21.09**	**0.21**	χ^2 **= 2.2** **p= 0.13**	**18°C**
per^{01}/Y;tim^{01},tim^{ps}/tim^{01}; per^{ps}/+ (19x126)	**46**	**19**	**27**	**58.69**	**24.51**	**0.79**	**32**	**28**	**4**	**12.95**	**24.89**	**0.23**	χ^2=14.9 **p = 0.0001**	**25°C**
w/Y; tim^{01}, tim ^{ps} / tim^{01}(tim21)	**34**	**9**	**25**	**73.5**	**20.09**	**0.83**	**55**	**22**	**33**	**63.93**	**21.10**	**0.15**	χ^2**= 0.5** **p= 0.4**	**18°C**
per^{01}/Y;tim^{01},tim^{ps}/tim^{01}; per^{ps}/+ (21x126)	**41**	**19**	**22**	**53.65**	**26.54**	**0.63**	**63**	**43**	**20**	**34.60**	**24.77**	**0.15**	χ^2**= 4.08** **p= 0.04**	**25°C**
per^{01}/Y;tim^{01},tim^{ps}/tim^{01}; per^{ps}/+^{ps} (I26X35)	**27**	**21**	**6**	**22.22**	**23.99**	**0.18**	**26**	**17**	**9**	**34.60**	**25.16**	**0.43**	χ^2**= 0.48** **p= 0.48**	
w/Y; tim^{01}, tim ^{ps} / tim^{01}(tim35)	**32**	**12**	**20**	**61.2**	**22.32**	**0.15**								
***per^{01}/Y; tim^{+}, per ^{ps}/+* (I20)**	**29**	**11**	**18**	**62.06**	**30.03**	**0.53**	**25**	**3**	**22**	**88**	**27.16**	**0.12**	χ^2**= 10.7** **p= 0.001**	**18°C**
per^{+}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ (19XI20)	**na**			**53.12**	**21.21**	**0.39**	**25**	**1**	**24**	**96**	**24.00**	**0.29**	χ^2**= 14.8** **p = 0.0001**	**18°C**
per^{+}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ (21XI20)	**21**	**13**	**8**	**38.09**	**25.49**	**1.02**	**24**	**1**	**23**	**95.13**	**23.40**	**0.29**	χ^2**= 14.8** **p = 0.0001**	**18°C**

Table 4.4: Results of the activity analysis for the hemizygous transgenic and control flies used in this study under 25°C and 18°C. Chi-square test was performed to compare the rhythmicity of the flies under two temperature conditions. (N=total number of flies, Narr=No. of arrhythmic flies, Nr= No. of rhythmic flies, %r= percentage of rhythmic flies, SEM=Standard Error of Mean, Pref.= preference for low or high temperature).

	25°C						18°C							
Genotype	**N**	**Narr**	**Nr**	**% r**	**Period**	**SEM**	**N**	**Narr**	**Nr**	**%r**	**Period**	**SEM**	**Chi sq test**	**Pref.**
per^{01}/Y;+;per^{ps}/per^{ps}(I26)	**26**	**6**	**20**	**76.92**	**29.60**	**0.48**	**34**	**4**	**30**	**88.20**	**26.08**	**0.09**	**χ^2= 1.26 p= 0.26**	
w/Y ;tim^{01},tim^{ps} /tim^{o1}, tim^{ps}(19)	**28**	**17**	**11**	**39.2**	**26.16**	**0.23**	**31**	**19**	**12**	**38.70**	**24.39**	**0.38**	**χ^2= 0.001 p= 0.96**	**18°C**
per^{01}/Y;tim^{ps}tim^{o1}/ tim^{ps}tim^{o1};per^{ps} /per^{ps} (19x126)	**47**	**46**	**1**		**0.00**		**21**	**19**	**2**	**9.50**	**22.68**	**0.17**	**na**	
w/Y;tim^{01},tim^{ps} /timo(21)	**53**	**30**	**23**	**4.35**	**25.79**	**0.50**	**29**	**17**	**12**	**41.30**	**22.75**	**0.13**	**χ^2= 0.01 p = 0.8**	**18°C**
per^{01}/Y; tim^{ps}tim^{o1}/ tim^{ps}tim^{o1};per^{ps} /per^{ps} (21x126)	**31**	**26**	**5**	**16.1**	**21.00**	**0.72**	**29**	**17**	**12**	**11.40**	**22.39**	**0.12**	**na**	

Table 4.5: Results of the activity analysis for the homozygous transgenic and control flies used in this study under 25°C and 18°C. Chi-square test was performed to compare the rhythmicity of the flies under two temperature conditions. (N=total number of flies, Narr=No of arrhythmic flies, Nr= No. of rhythmic flies, %r= percentage of rhythmic flies, SEM=Standard Error of Mean, Pref.= preference for low or high temperature).

A



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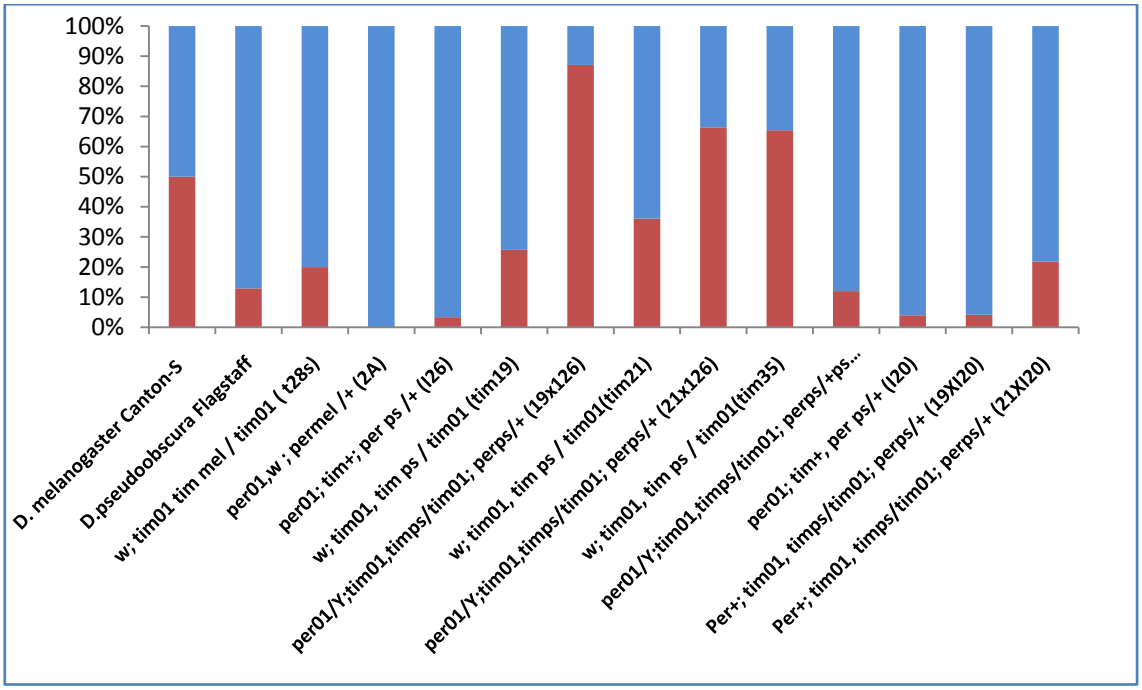
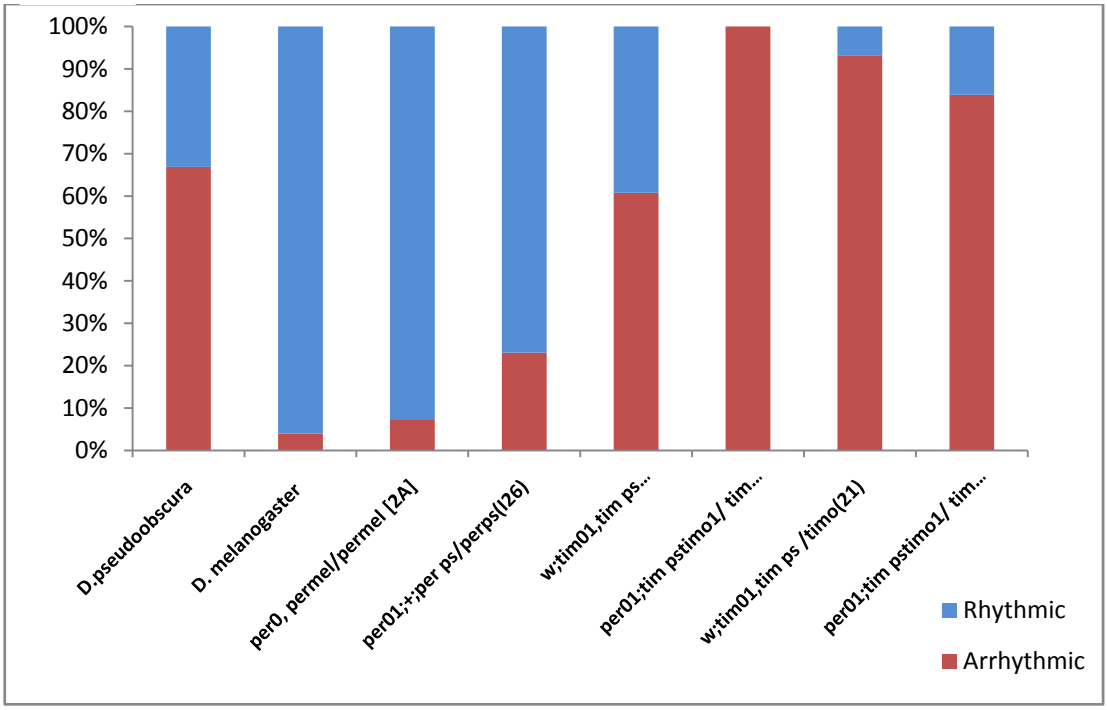


Figure 4 .11: Locomotor rhythms in hemizygous transgenic flies. A= 18°C, B=25°C.

Comparison in percentage of rhythmic versus arrhythmic flies.

A



B

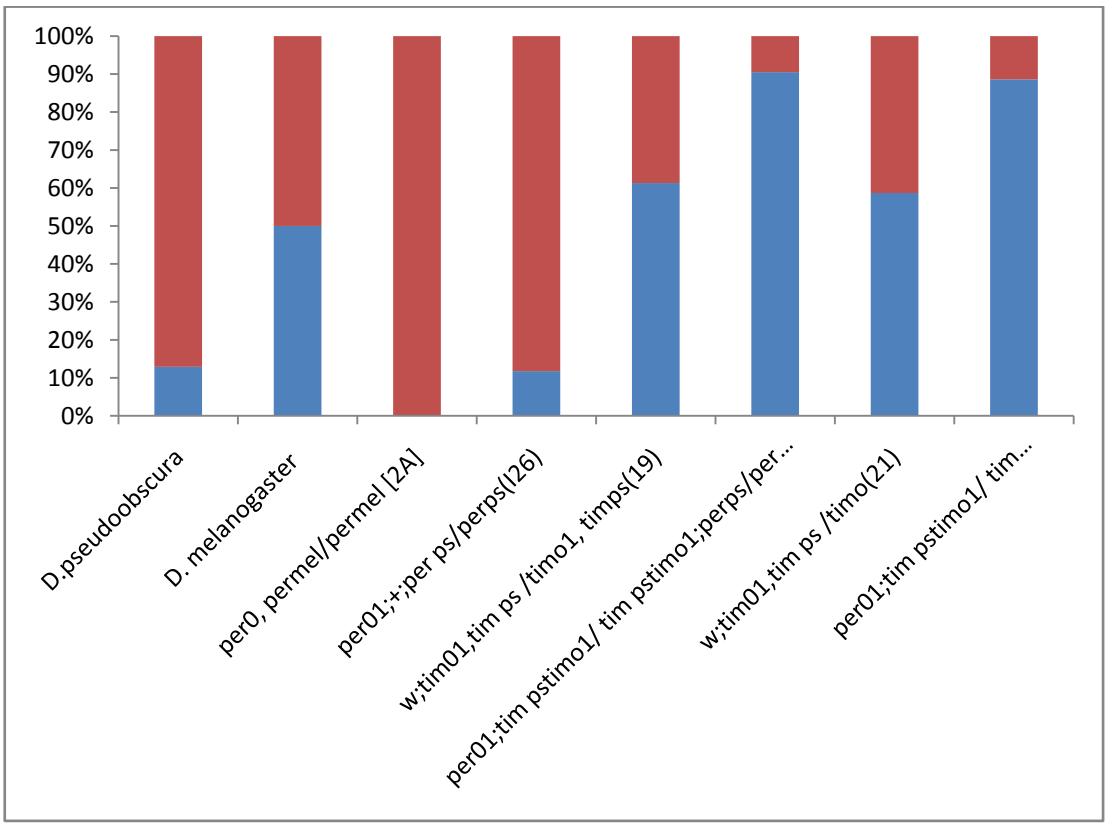


Figure 4.12: Locomotor rhythms in homozygous transgenic flies. A= 18°C, B=25°C. Percentage of homozygous rhythmic, versus arrhythmic flies.

4.3.2.3 The effect of temperature on host species and transgenics.

The behavioural analysis in DD revealed different phenotypes for *D. pseudoobscura* at the two temperatures. At 25°C, only 33.3% of flies were rhythmic and the rhythms were very weak. When tested under 18°C, 87% showed strong rhythms (χ^2 = 16.2, df = 1, p = 5.458e-05). For *D. melanogaster* Canton-S flies the observations were opposite to this with 96% rhythmicity at 25°C and 50% under 18°C (χ^2 = 10.43, df = 1, p= 0.001).

Like *D. pseudoobscura* wild flies, the hemizygous transgenic flies carrying *pseudoobscura-per* are significantly more rhythmic at lower temperature. For example, *per^{01}/Y; tim^{+}; per ^{ps} /+ (I26)* flies were 67.8% at 25°C and 96.6% rhythmic at 18°C. (χ^2 =6.5, df=1, p=0.01) Homozygous *per^{01}/Y; tim^{+}; per ^{ps} /per^{ps}* flies also revealed higher rhythmicity 18°C but the difference was not significant (Table 4.5, Figure 4.12) The *per^{01}/Y; tim^{+}, per ^{ps}/+* (*I20*) transgenics showed 62% rhythmicity at 25°C which was increased to 88% at 18°C (χ^2 = 10.7, df =1, p=0.001). In general, *pseudoobscura-per* imparts the cold temperature preference of the donor species.

tim transgenes only sporadically show the *D. pseudoobscura* temperature preference limited to hemizygous line *w/Y; tim^{01}, tim ^{ps} / tim^{01} (tim19*), and homozygous *w/Y; tim^{01}, tim ^{ps} / tim^{01}, tim ^{ps}* flies for both *tim19* and *tim21* lines (Table 4.5, Figure 4.12 B).

Somewhat oddly, hemizygous double transgenics, *per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+* flies show an overall preference for high temperature for lines *19* and *21* (Table 5.4). The corresponding *per^{+}* background flies, *per^{+}/Y; tim^{01}, tim^{ps}/tim$^{01;}$per^{ps}* /+ lines

19 and *21)* flies showed a strong preference for lower temperature (χ^2 = 14.83, df = 1, p=0.0001, Table 4.4), revealing a 'dominant' effect for *pseudoobscura-per* that is more effective when normal clock function is restored (with *per*$^{+}$*).*

4.3.3 Coevolution of PER and TIM through period

The period of locomotor activity was tested both under 25^{o}C and 18^{o}C performing F-test in "Oriana" (Table 7.4 Appendices). *D. pseudoobscura* flies had ~ one hour shorter average period at 18^{o}C ($F_{(1,26)}$=26.73, p=2.15e-05) than 25^{o}C (Table4.5, Figure 4.13). The *melanogaster* transgenics *w/Y; tim*^{01} *tim* ^{mel} */ tim*^{01} (*t28s*) and *per*^{01}*,w /Y; per*^{mel} /+ *(2A*) had a mean period closer to 24h at 25^{o}C (Table 4.4, Figure 4.13). The *pseudoobscura* single transgenic strains for *per* (*per*^{01}*/Y; tim*$^{+}$*, per* ^{ps}*/+* (*I20*) and *per*^{01}*/Y; tim*$^{+}$*; per* ^{ps} */+ (I26*) and *tim* (*w/Y; tim*^{01}*, tim* ^{ps} */ tim*^{01} (lines *tim19, tim21* and *tim35*) show large differences in their average period. The average range of period of all *w/Y; tim*^{01}*, tim* ^{ps} */ tim*^{01} lines was 20-22h while that for *per*^{01}*/Y; tim*$^{+}$*, per* ^{ps}*/+* (*I26* and *I20*) was 27-31h (Table 4.4, Figure 4.13).

The most important lines to analyse for the period were the double transgenics *per*^{01}*/Y; tim*^{01}*, tim*^{ps}*/tim*^{01}*; per*^{ps}*/+* (lines *19 , 21* and *35*) which showed similar periods to each other of ~24-25 h (Table 4.4). The average period of the *per*^{01}*/Y; tim*^{01}*, tim*^{ps}*/tim*^{01}*; per*^{ps}*/+* (*19)* hemizygous flies was 24.48±1.45h for *per*^{01}*/Y; tim*^{01}*, tim*^{ps}*/tim*^{01}*; per*^{ps}*/+* (*21*) was 25.03±0.9 and *per*^{01}*/Y; tim*^{01}*, tim*^{ps}*/tim*^{01}*; per*^{ps}*/+ (35)* was 24±0.45h at 25^{o}C. These flies also showed a good level of temperature compensation for the period (Figure 4.14). This shows that although there was no improvement in the rhythmicity of the flies when the two transgenes of *D. pseudoobscura per* and *tim*

were brought together in the double mutant background, there was a rescue in the period of locomotor activity (Figure 4.13).

The double transgenes *per^{+}/Y; tim^{01}, tim ^{ps}/per ^{ps} ,tim^{01}* (lines *19* and *21*) flies gave a high rescue in the average period under 18^{o}C. The length of their spectral period was observed to be similar to Canton-S flies i.e. ~24h (Table 4.4).

4.3.3.2 Temperature compensation in period

The overall analysis showed that there is a significant effect of temperature on the period of activity for *D. pseudoobscura,* as well as for the *pseudoobscura-per* transgenic lines *(I26* and *I20)*. Average period of the *D. pseudoobscura* flies was recorded to be 23.7±0.28h at 25^{o}C. While at lower temperature (18^{o}C) a comparatively shorter period of 22.55±0.05h was recorded ($F_{(1,26)}$=26.73, p=2.15e-05). A two way-ANOVA comparing the hemizygous single and double transgenics showed a strong interaction of period with temperature ($F_{(2,68)}$=9.28, p=0.00027). Hemizygous *per^{01}/Y; tim^{+}; per ^{ps} /+ (I26)* flies showed a long period of 30.91±0.77h at 25^{o}C. When tested under 18^{o}C their average period was reduced to 26.75±0.07h ($F_{(1,35)}$=68.86, p=8.79e-10). The average period of *per^{01}/Y; tim^{+}, per ^{ps}/+* (*I20*) flies was also reduced from 29.79±0.6h at 25^{o}C to 27.11±0.13h at 18^{o}C ($F_{(1,29)}$=25.96, p=1.95e-05). The two *pseudoobscura-tim* transgenics (*w/Y; tim^{01}, tim ^{ps} / tim^{01} tim19* and *tim21*) showed no significant difference in their average period under two temperature conditions (Table 4.4, Figure 4.13). When these flies were crossed to the *pseudoobscura-per* transgenics, the double transgenic *per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+* showed high level of temperature compensation (Figure 4.13, Table 4.4).

These results show that by combining the two transgenes from *pseudoobscura* not only gives an improvement in the average period of the flies but also shows enhanced temperature compensation.

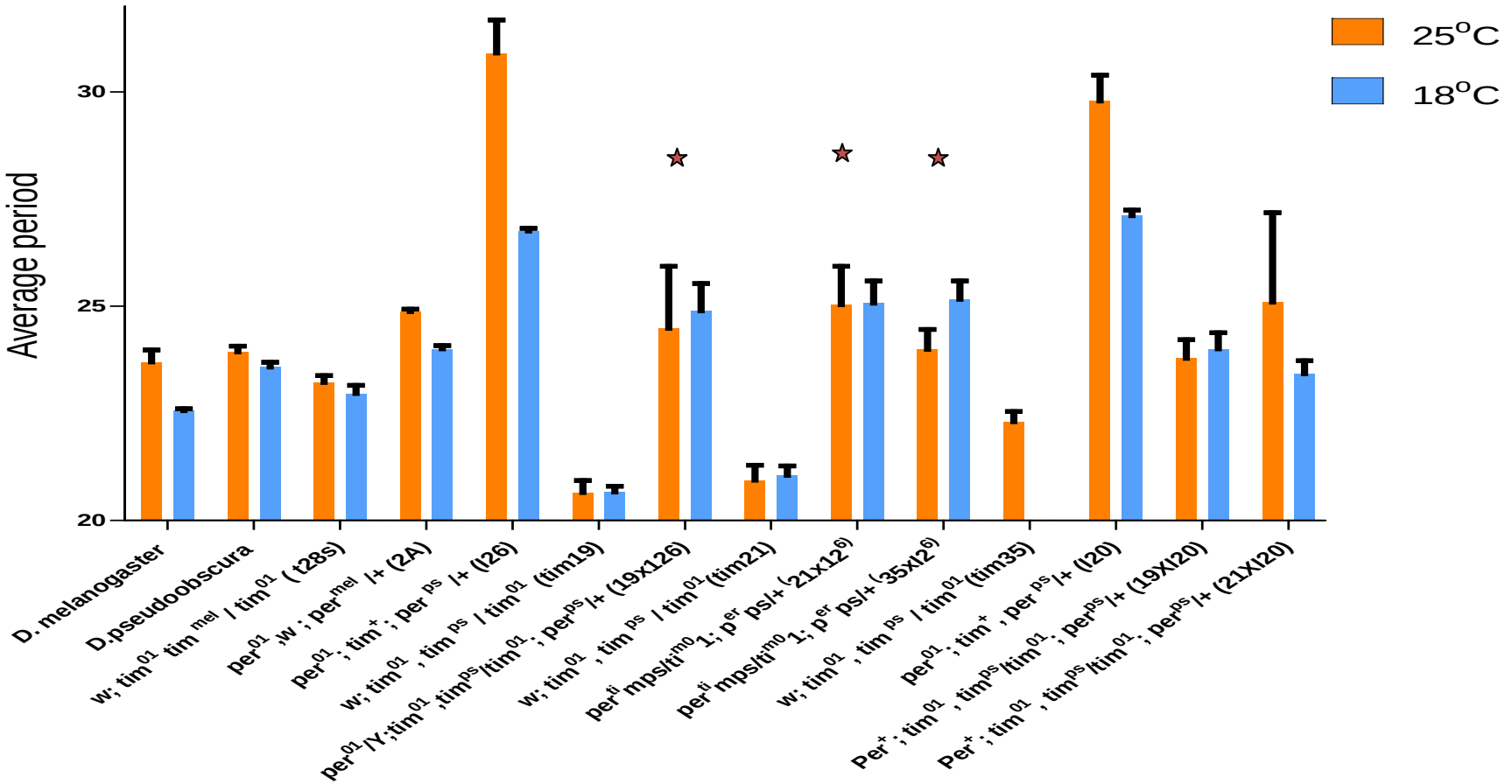


Figure 4.13: Mean period (+/-sem)of locomotor activity of the different fly lines under 25 and 18°C.The lines indicated with stars are the hemizygous double transgenics (carrying *per* and *tim* transgenes from *pseudoobscura)* whose period reverts back to wild-type.

4.3.4 Locomotor activity profiles under LD 12:12 conditions

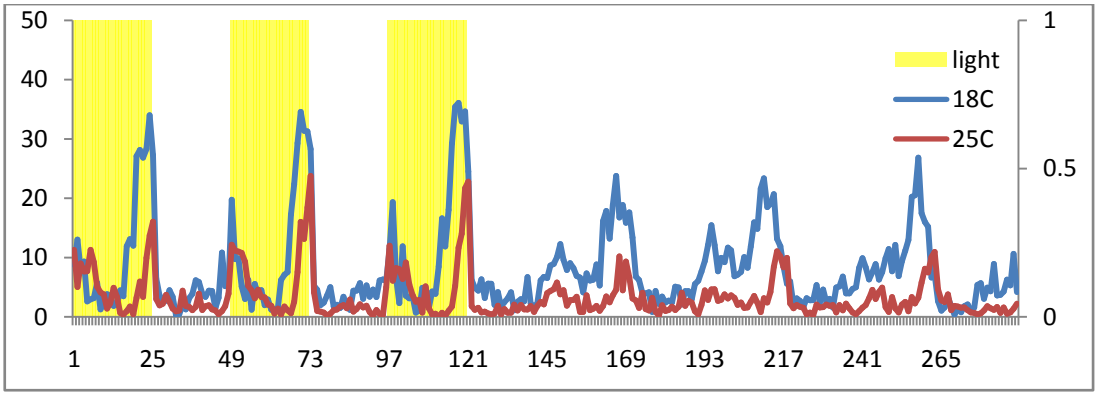
The activity profiles of the transgenic flies were compared different temperature as well as in comparison to each other for the morning and evening components of activity. Bins for the particular phase of activity were picked and statistical analysis was performed using the circular statistics package "Oriana" which implements the Watson-Williams F-Test.

4.3.4.1 *D. pseudoobscura* and *D. melanogaster*

D. pseudoobscura shows a very small amount of activity before lights on in the morning at 18^{o}C but under 25^{o}C morning anticipation (M) was absent. Flies under 18^{o}C shows an earlier evening peak (E) of activity which was advanced by 79 min compared to 25^{o}C ($F_{(1,59)}$=17.98, p=7.9e-05, Figure 4.14 A).

The analysis of the E component of Canton-S flies both at 25^{o}C and 18^{o}C showed no significant effect of temperature ($F_{(1,50)}$=0.25, p=0.618) in the time of evening peak activity (Figure 4.14 B). The E of these two species was compared with each other at 25^{o}C and 18^{o}C (Figure 4.17). It showed a very marginal (15 minutes) phase advance by *D. pseudoobscura* ($F_{(1,59)}$=4.14, p=0.04) at 25^{o}C which became larger (2.1h) at 18^{o}C ($F_{(1,50)}$=25, p=7.43e-06).

A



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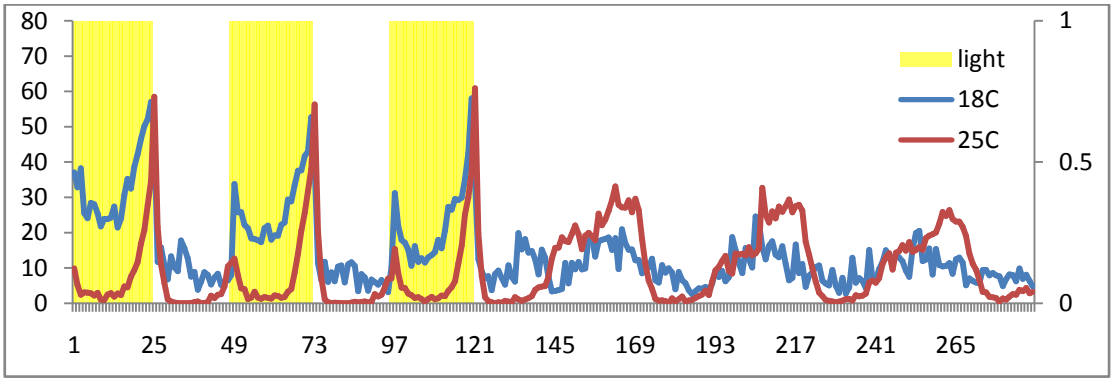


Figure 4.14: Locomotor activity profile of *D. pseudoobscura* (Flagstaff) (A) and *D. melanogaster* Canton-S (B) under 25°C and 18°C in 3LD + 3DD (yellow boxes shows photoperiod in LD 12:12). Amount of activity (Y-axis) against bins of activity (x-axis).

4.3.4.2 Transgenics under LD12:12

The activity profile of hemizygous *w/Y; tim^{01}, tim ^{ps} / tim^{01}* (*tim19*) was observed under LD 12:12 at (Figure 4.15 A). An overall comparison of the morning peak shows a very early morning peak for single *tim* transgenics as compared to the *melanogaster* controls. It was much before lights on which can be clearly seen in the figure 4.17 where the zero degree represent the light on. These flies showed a significantly ($F_{(1,73)}$=6.3, p=0.01) earlier morning peak (MP) of activity before lights at 25°C which was 93 min earlier than at 18°C (Figure 4.18 A). Similar observations were obtained with *w/Y; tim^{01}, tim ^{ps} / tim^{01}(tim21)* (Figure 4.15B). The MP for 25°C was

76min earlier than at 18°C ($(F_{(1,54)}$=11.02, p=0.002), Figure 4.17B). The *pseudoobscura-per* transgenic did not show any anticipation of morning activity (Figure 4.15C).

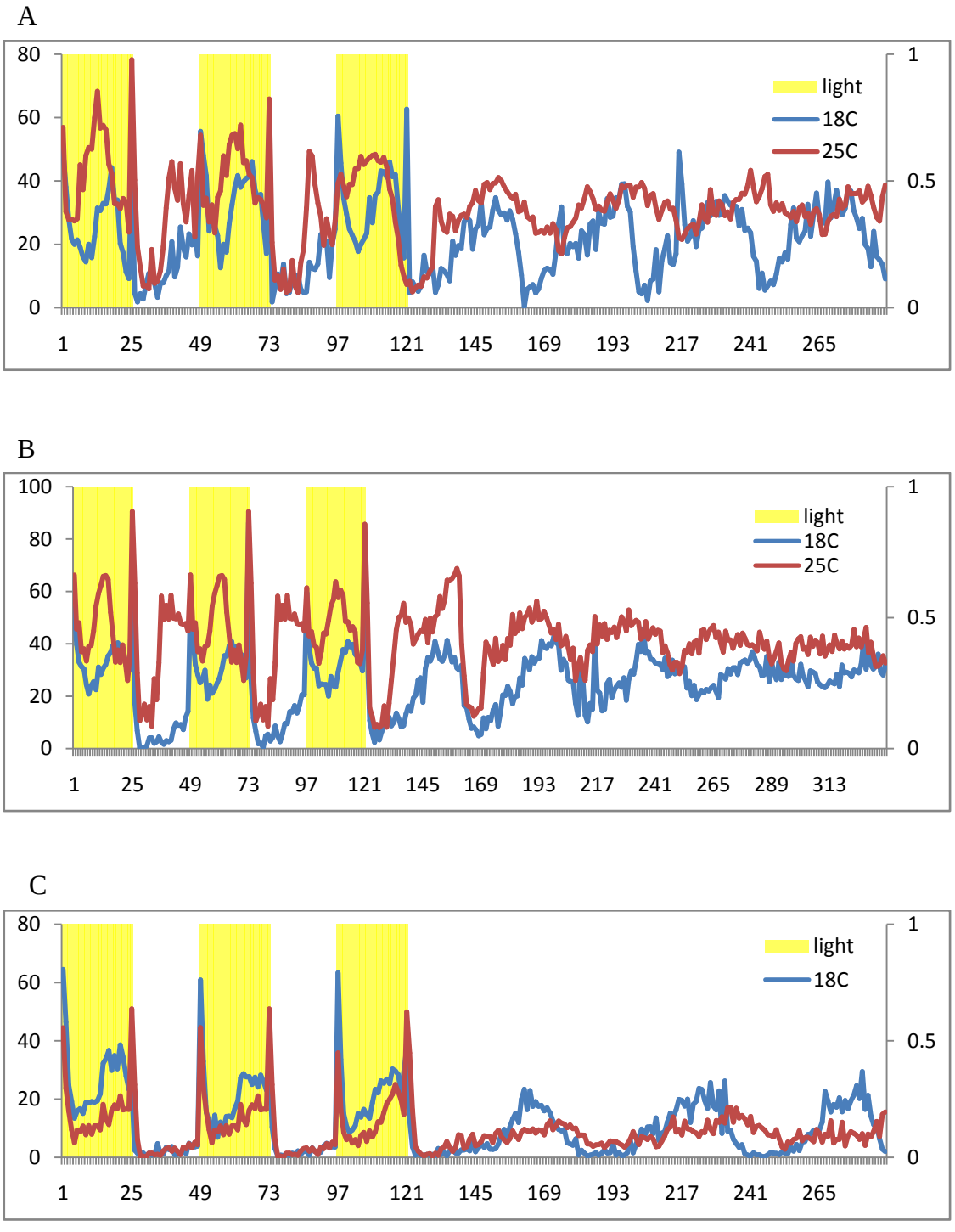


Figure 4.15: Locomotor activity profile of single transgenic lines. A= *w; tim^{01}, tim ^{ps} / tim^{01} (tim19); B= w; tim^{01}, tim ^{ps} / tim^{01}(tim21); C= per^{01}; tim^{+}; per ^{ps} /+ (I26)*.

When these two transgenic lines were crossed together, the double transgenics *per*^{01}*/Y; tim*^{01}*, tim*^{ps}*/tim*^{01}*; per*^{ps}*/+* did not show any anticipation of the morning activity under either temperatures (Figure 4.16 A and B). This was similar to the activity profile of the *D. pseudoobscura* flies.

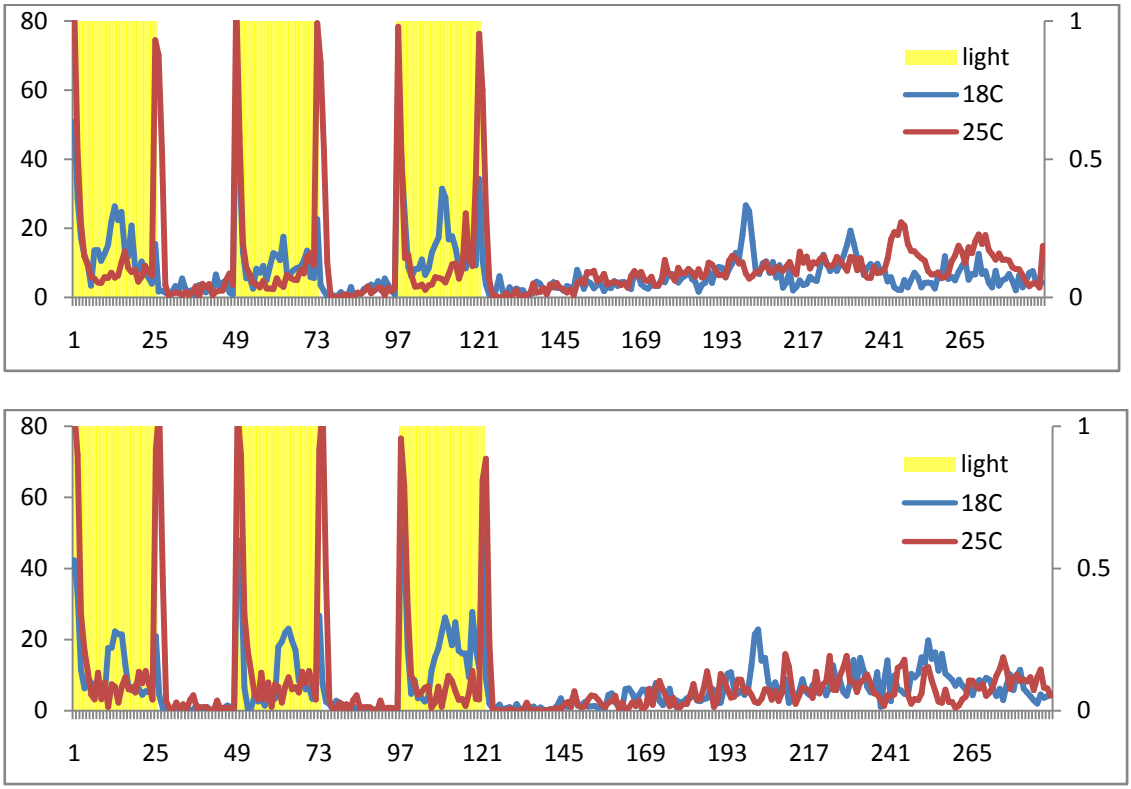
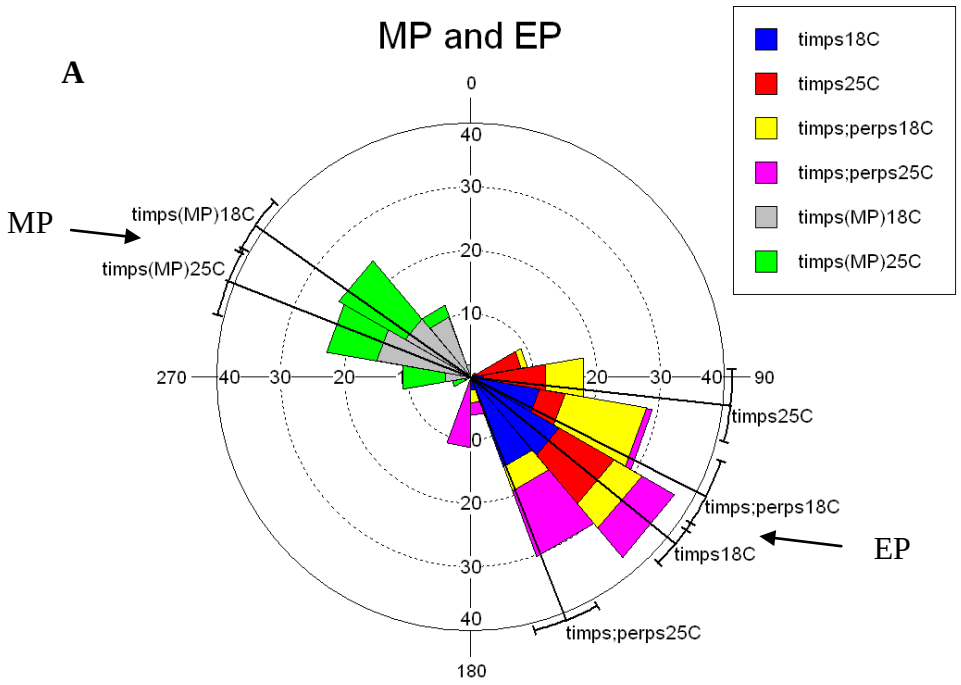


Figure 4.16: Locomotor activity profile of double transgenic flies. A= *per*^{01}*/Y; tim*^{01}*, tim*^{ps}*/tim*^{01}*; per*^{ps}*/+(19); B=per*^{01}*/Y; tim*^{01}*, tim*^{ps}*/tim*^{01}*; per*^{ps}*/+(21).*

The evening peak (EP) of activity of single as well as double *pseudoobscura* transgenics was much early in comparison to the *melanogaster* transgene (Table 7.6 Appendices). The *pseudoobscura-per* transgenics did not show any effect of temperature on the EP of activity. However for the *pseudoobscura-tim* transgenics and the double transgenics, the analysis of the evening peak of activity showed very interesting results. EP showed a significant difference ($F_{(1,77)}$=45.29, p=2.6e-09) for *w/Y; tim*^{01}*, tim* ^{ps} */ tim*^{01} *(tim19)* with the E peak 2.2 h earlier at 25^{o}C than at 18^{o}C

(Figure 4.17). The *w/Y; tim^{01}, tim ^{ps} / tim^{01}(tim21)* flies showed the same activity profile for the EP (Figure 4.17B). However the double transgenic *per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ (19)* showed a later evening peak 25^{o}C. The average difference was 2.7 h and found to be statistically significant ($F_{(1,60)}$=51.22, p=1.3e-09). These flies reached the EP earlier when entrained under 18^{o}C (Figure 4.17B). The *per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ (21)* also showed an earlier peak at 18^{o}C (Figure 4.17 B). The time difference was calculated as 1.05 h ($F_{(1,50)}$=4.8, p=0.03).

This analysis shows an effect of *per* transgene on the species-specific morning anticipation of activity. We also see that the *tim* transgene cannot control the temperature response alone. These flies start their activity in the morning very early under high temperature but cannot adjust the evening peak. However when they also carry *pseudoobscura-per* they show the appropriately delayed late evening activity profile at high temperatures.



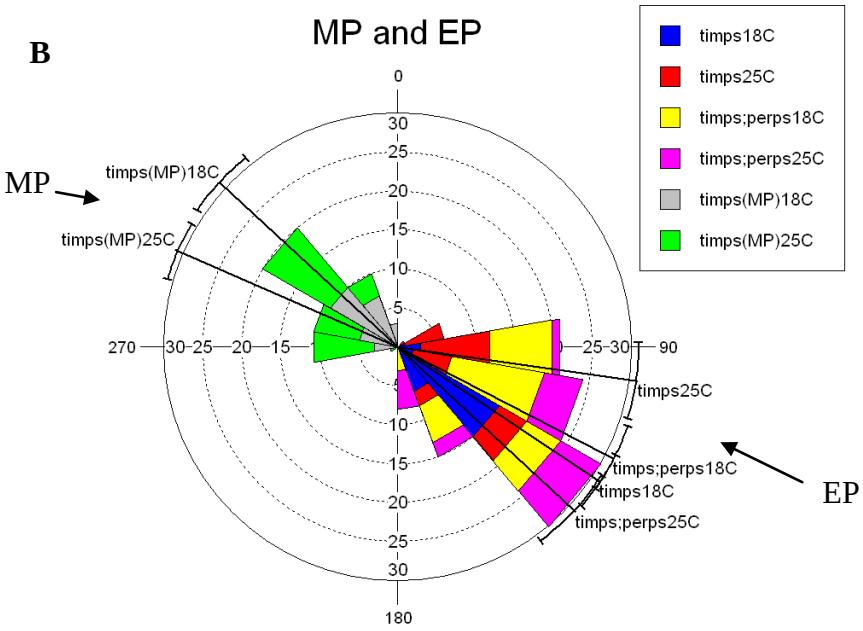


Figure 4.17: A comparison of the Morning peak (MP), before light on (zero degree) and evening peak (EP, after lights on) in the transgenic flies measured both at 25°C and 18°C. A= *w/Y; tim^{01}, tim ^{ps} / tim^{01}* (tim19) (timps) and *per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+(19)* (timps:perps); B= *w; tim^{01}, tim ^{ps} / tim^{01}(tim21)* (timps) and *per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+(21)* (timps:perps)

4.3.5 Western blot

Expression of PER in the different transgenic lines was compared by using western blotting. The results of these experiments could be obtained only with PER antibodies as the *Drosophila* TIM antibodies did not recognise *D. pseudoobscura* TIM (Figure 4.34). Comparison was performed between the single *per* transgenic *per^{01}/Y; tim^{+}; per ^{ps} /+ (I26)* line and the double transgenic *per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+(19)*. *per^{01}*, *w* flies was used as negative control (Figure 4.18). Only one western blot for each genotype could be performed which showed very similar cycling pattern with peaks of expression around ZT24/0 (Figure 4.18A).

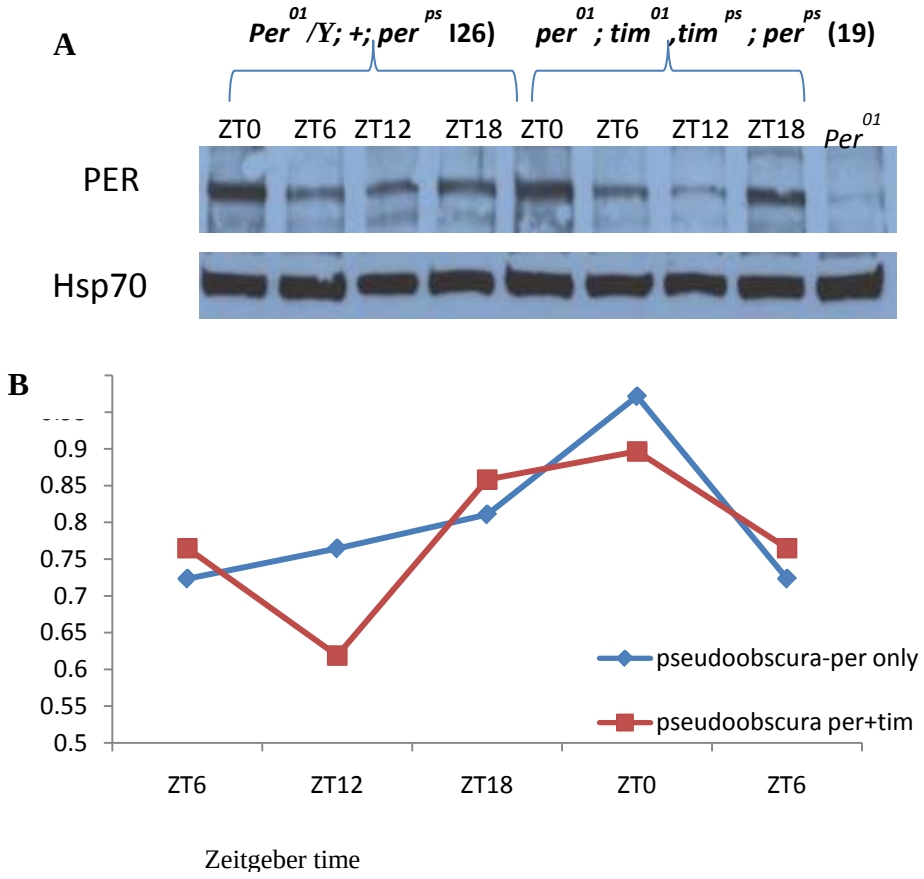


Figure 4.18 : Comparison of PER protein oscillation (PER/HSP70) in *per^{01}/Y; tim^{+}; per ^{ps} /+ (I26)* line and *per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+(19)* lines . A=Western blot (Hsp70 was used as a loading control), B=Graph (level of protein plotted against the time of sample collection).

4.3.6 Real-Time Polymerase Chain Reaction

As the TIM antibodies were not recognising the *D. pseudoobscura* protein the expression of this *tim* transgene was checked by using Real-Time PCR performed according to the protocol described in the Chapter 2. Single *tim* transgenics *w; tim^{01}, tim ^{ps} / tim^{01} (*lines *19* and *21)* the double transgenics *per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ (*lines *19* and *21*). *D. pseudoobscura* and *D. melanogaster* lines were used. Collection of flies was at ZT12 at 25^{o}C. Three samples of each fly line were collected at each time

point and three technical replicates were used for each. The fly line *w; tim*^{01} was used as the negative control.

The analysis showed that the amount of *D. pseudoobscura tim* RNA is far higher than all the other lines (Figure 4.19 A). A statistical analysis was performed by excluding *D. pseudoobscura* and only *w/Y; tim*^{01} *tim* ^{mel} */ tim*^{01} (t28s) showed significantly higher levels of *tim* RNA compared to all other transgenes($F_{(5,47)}$=3.51, p=0.009, Figure 4.19 B). However the very low relative levels of *tim* mRNA are clearly sufficient to drive circadian rhythmicity, both in wild-type and transgenics.

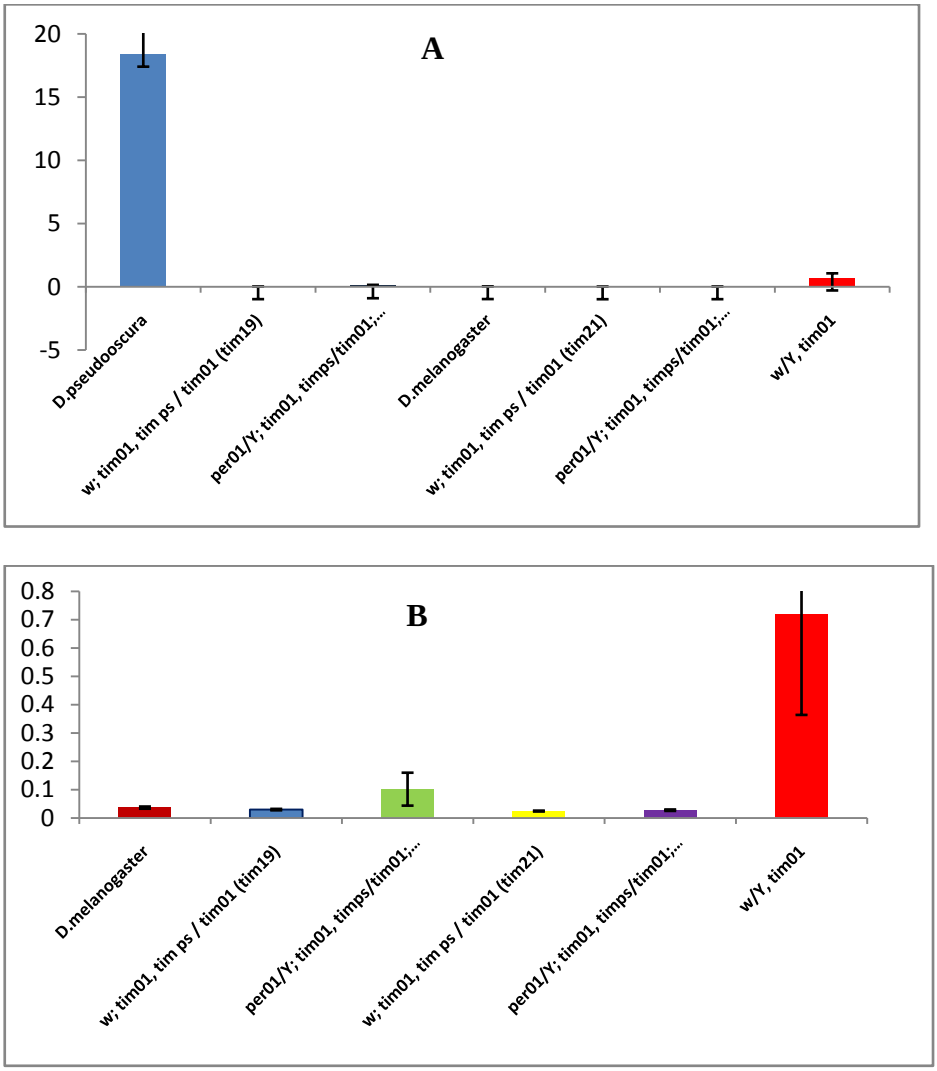


Figure 4.20: Comparison of mean *tim* mRNA level (+/-sem) among the different fly lines (A) with and (B) without *D. pseudoobscura*.

4.3.7 PER levels in *D. pseudoobscura* and *D. melanogaster*

These two species showed a very contrasting behaviour to each other when tested under 25^{o}C and 18^{o}C (Figure 4.20). The comparison of the absolute mean activity profiles shows a high amount of activity for *D. melanogaster* flies at 25^{o}C and low levels for *D. pseudoobscura* (Figure 4.20A). These activity profiles were reversed at 18^{o}C (Figure 4.21B).

To further investigate if this difference was reflected in PER dynamics, western blotting was performed at 25^{o}C. No statistics could be performed on the data as I had only had two replicates. While the absolute levels of PER appear higher in *D. melanogaster* this is to be expected given the antibody is generated against *D. melanogaster* PER. However it does appear that the *D. pseudoobscura* profile is somewhat flatter than that of *D. melanogaster*. Unfortunately I could not perform the corresponding blots at 18^{o}C where we might expect to see this situation reversed.

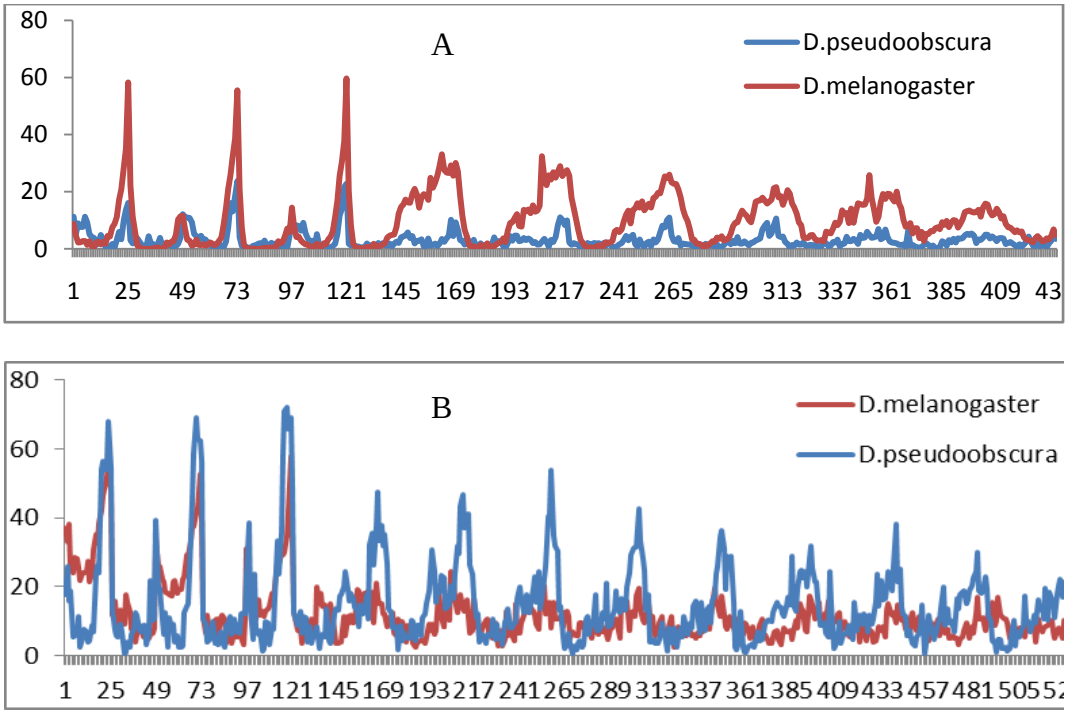
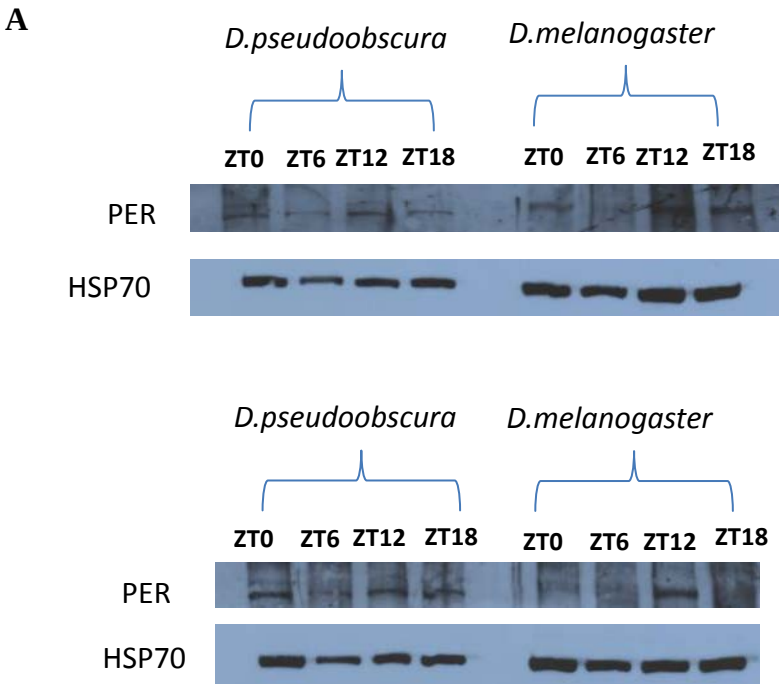
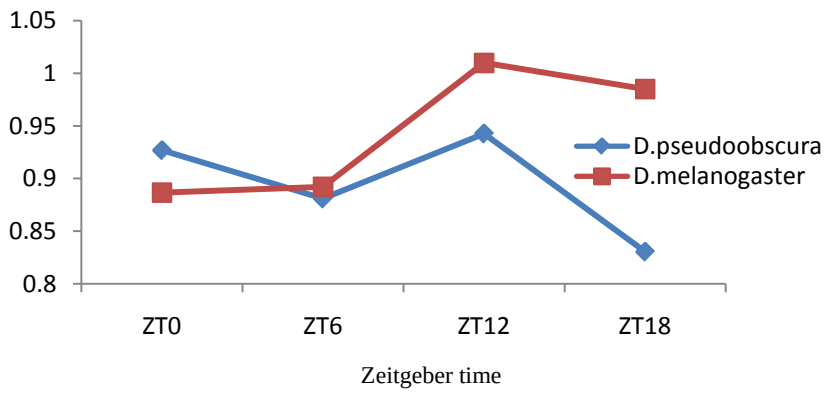


Figure 4.20: Activity profile of *D. pseudoobscura* and *D. melanogaster* at 25°C (A) and 18°C (B).



B



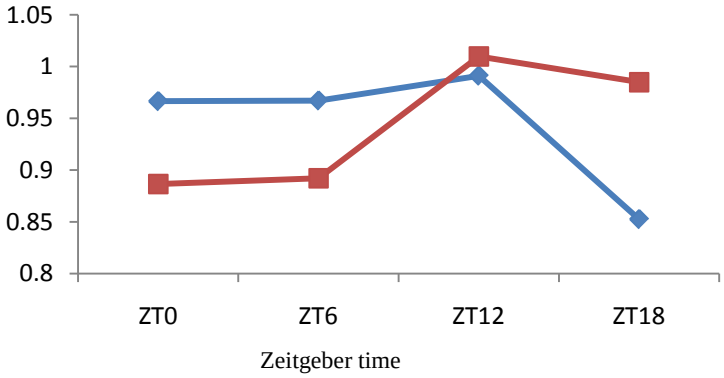


Figure 4.21: A comparison of PER protein collected at four time points (ZT0-ZT18) in LD12:12 cycle in *D. pseudoobscura* and *D. melanogaster* at 25°C . Two replicates are shown. A=Western Blot, B=Graph (Y axis represents relative PER levels normalised to HSP70).

4.4 Discussion

4.4.1 Protein sequence alignment

The phylogenetic positions of *D. virilis* were observed to be anomalous for the clock proteins TIM and CLK. This species had common ancestors with *D. melanogaster* further back in the past than *D. pseudoobscura/persimilis* (63 million years (Tamura et al. 2004). *D. pseudoobscura* separated from the *melanogaster* group between 25 (Russo et al. 1995) and 30mya (Schlötterer et al. 1994). However for both CLK and TIM, the position of the *D. pseudoobscura-persimilis* clade is further away than the *D. virilis* which is placed near to *D. melanogaster*. This may suggest some kind of non-neutral

evolution of these proteins, and provides an additional rational for further exploring the coevolutionary idea.

For *D. pseudoobscura* the highest level of similarity was obtained as expected with its sibling North American species *D. persimilis*. The overall protein sequence of TIM showed more similarity as expected between *D. pseudoobscura* and *D. melanogaster* than with TIM of *M. domestica.* Two PER interaction domains have been identified on TIM , PER1 and PER2 (Saez and Young 1996). Piccin et al (2000) reported the same phylogenetic positions when using the full PER protein sequence but by comparing only the PAS interaction domains of PER, *M. domestica* was grouped closer to *D. melanogaster* than *D. pseudoobscura*. This corresponded with the enhanced rescue of *Musca* PER compared to *D. pseudoobscura* PER. The same was observed with the comparison of the PAS region of *Ceratitis capitata* PER (Mazzotta et al 2005). PAS is the protein-protein interaction domain important for signalling and sensory function (Gu et al 2000). In *Drosophila* its function is to promote the interactions of PER with TIM, and CLK with CYC (Mazzotta et al. 2005). However on PER there is special Clock Cycle Interaction Domain (CCID) for interaction with CLK (Chang and Reppert 2003).

The unusual phylogenetic position of PAS may be due to the amino acids in this region being under selective constraints so it does not evolve independently. It may coevolve in concert with the dimerization domains of its conspecific molecular partners (Mazzotta et al. 2005). Perhaps the PER interaction domain of TIM should also reflect the unusual phylogeny of the PAS domain of its partner PER. However results of the phylogenetic analysis using the amino acid sequence of only PER1 and PER2 interacting domains in TIM gave the same position for *D. pseudoobscura* and *M.*

domestica as that of the full TIM sequence. Interestingly, the protein sequence of *Nasonia vitropennis* TIM was clustered with *tim2* the mammalian *tim*; which plays a role in light resetting of the clock in the fly (Benna et al 2010), suggesting it is a paralogue of *tim*.

As far as TIM is concerned, no phylogenetic evidence for a coevolutionary scenario, which would require *Musca* sequences to be more closely related to *melanogaster* than *pseudoobscura* was obtained at this level of resolution.

4.4.2 Coevolution

Coevolution is an evolutionary process in which a heritable change in one entity establishes selective pressure for a change in another entity. These entities can range from nucleotides to amino acids to protein to entire organism and perhaps the whole ecosystem. The most well studied example of this coevolution involves physically interacting proteins in which precise, complementary structural conformations of interacting partners are needed to maintain a functional interaction (Fraser et al. 2004). The basic aim of my study was to investigate if heterospecific PER and TIM together make a coevolved module that can interact more efficiently with other clock proteins compared to the situation where only one of these negative regulators was heterospecific. For this purpose transgenes of *per* and *tim* from *D. pseudoobscura* were introduced into *D. melanogaster* host.

The comparison of the level of rescue in the rhythmic behaviour of hemizygous/homozygous transgenics with single *per* or *tim* or both was performed. Previous studies using *mps1* transformants (per^{01}/Y; tim^{+}; per^{ps} /+ (I26) and per^{01}/Y; tim^{+}, per^{ps}/+ (I20)) showed that *pseudoobscura-per* cannot rescue rhythmic behaviour

efficiently. It has a low penetrance (30%) and flies have very long period of 26-31h (Peterson et al. 1988; Peixoto et al. 1998). The same observations were confirmed in the present study where these two lines were tested under two temperature conditions. The level of rescue for the *pseudoobscura-tim* transgenic flies was also less than 50% under 25^{o}C but increased at 18^{o}C although many flies were arrhythmic. The period of these lines was short (21-22h). These results suggested that the two *pseudoobscura* proteins are not able to interact fully with their *melanogaster* counterparts.

When both heterospecific transgenes were studied simultaneously in double mutant hosts, no significant increase in levels of rhythmicity was obtained. However a significant and dramatic improvement in the average period of the hemizygous double transgenics was observed. The average period ranged from 24-25h for all three lines (*19, 21* and *35*), all well within wild-type ranges. Thus the simultaneous introduction of the two heterospecific proteins appeared to support a coevolutionary scenario. The western blots and qPCR also confirmed that the transgenes of PER and TIM from *D. pseudoobscura* are being expressed.

The genetic composition of the *per*$^{+}$ double transgenic (*per*$^{+}$*/Y; tim*^{01}*,tim*^{ps}*/tim*^{01}*; per*^{ps}*/+(19)*) was similar to the single *tim* transgenics except it had an extra *pseudoobscura-per*. These fly had a very high level of rhythmicity (96%) at 18^{o}C. The average period was also ~24 h under 18^{o}C. This shows the TIM and PER *pseudoobscura* protein were interacting efficiently with the other circadian clock components because the rescue was enhanced over that of the *tim* transgenes alone. The double transgenic with two copies of the *pseudoobscura per* and *tim* transgenes showed almost totally arrhythmic behaviour under DD, suggesting that these flies

cannot tolerate the high doses of *pseudoobscura* PER and TIM. However in LD they were rhythmic in terms of entrainment with similar activity profiles to those of the rhythmic hemizygous double transgenics.

4.4.3 Species-specific role of PER

The activity profiles of the transgenic flies shows some aspects of the species specific behaviour controlled by *per*. There were few major differences noticed in the activity patterns of *D. melanogaster* and *D. pseudoobscura*. One of them is the cold temperature preference for *D. pseudoobscura* and warm preference for *D. melanogaster*. Absence of morning anticipation at 25^{o}C and early evening peak of activity are also specific to *D. pseudoobscura* (Hennessy 1999; Petersen et al. 1988). The per^{01} transformants lines carrying the hemizygous *D. melanogaster per* transgene had *melanogaster* like activity profiles in LD under 25^{o}C while those which had *D. pseudoobscura* transgene (per^{01}*/Y; +; per* ^{ps}*/+ I26* and *I20*) displayed *pseudoobscura* like activity profile (Tauber et al. 2003).

Previous studies show that in the per^{S} and per^{L} mutants, only the evening component of activity shows corresponding advances and delays and the morning peak remains rather unaffected. Their results suggest that *per* is more important for the E peak and that the M component is rather independent of *per* (Hamblen-Coyle et al. 1992). My observations show that both *pseudoobscura-per* single transgenic lines as well as the double transgenics have lost morning anticipation as in wild *D. pseudoobscura* flies. However they are present in the *pseudoobscura-tim* transgenics at both temperatures. More interesting observation was obtained when I crossed the double transgenics into a per^{+} background; they regain the morning anticipation

suggesting a dominant role for *melanogaster per+* in this phenotype. Indeed removing PER from the s-LNvs (the PDF expressing neurons or M neurons) results in an absence of the M component, which shows that clearly PER is involved (Stoleru et al. 2004). That per^{s} and per^{L} mutants do not shift the M behaviour does not mean it is not under PER control, as both of these mutant proteins nevertheless express PER.

The comparison of the E peak of activity between *D. melanogaster* and *D. pseudoobscura* showed a difference of up to 1.1h under 18°C. Tauber et al (2003) showed that the activity profiles as well as the mating rhythms of the *mps1* (per^{01}*/Y;* tim^{+}*; per* ^{ps} */+)* transformants crossed into the wild-type per^{+} background was also clearly like *D. pseudoobscura*. My study also reported early evening peaks in the single *pseudoobscura-per* and double *pseudoobscura* transgenics and confirms that *per* carries the species-specific information for circadian locomotor and activity rhythms (Tauber et al. 2003). However the *pseudoobscura-tim* transformants also show an early peak of evening activity when compared to *melanogaster* transformants under both temperature conditions. This suggests that *tim* also has a role in the expression of the species-specific evening peak of activity.

4.4.4 Effect of temperature

Transcription levels of clock genes *per, tim, vrille, Pdp1ε* and *Clk* all are under temperature control with their levels changing according to temperature (Currie et al. 2009, Yoshii et al. 2007). In *D. melanogaster* the locomotor peak of activity is temperature modulated so that with a rise in temperature the E peak of activity moves later in the day, generating a mid-day siesta allowing the fly to avoid the desiccating effect of the hottest part of the day (Majercak et al. 1999, Collins et al. 2004, Low et al.

2008). The analysis of locomotor activity of the different fly strains under two different temperature conditions gave some interesting results.

D. pseudoobscura clearly favoured colder temperatures for expressing rhythmic behaviour while *D. melanogaster* flies were more rhythmic under high temperature conditions. Hennessy (1999) also reported similar observation with *D. melanogaster* transgenes and *pseudoobscura-per* transgenes favouring warmer and colder conditions respectively. In my study, the *D. melanogaster-tim* transgenic *w/Y; tim^{01} tim^{mel} / tim^{01}* (*t28s*) behaves like *D. melanogaster* Canton-S flies for temperature preference in the level of rhythmicity. However the *pseudoobscura-tim* transgenics *tim19* (*w/Y ; tim^{0},tim^{ps})* were in *a per^{+}* background but they still favoured colder condition like wild *D. pseudoobscura.* This suggested that restoring wild-type clock function, the *pseudoobscura-tim* transgene takes on a dominant effect. Such dominant effects of *pseudoobscura* transgenes in mating rhythms have been seen before with *pseudoobscura-per* in *per^{+}* backgrounds (Petersen et al. 1988; Tauber et al. 2003). These observations reveal that restoring TIM from *pseudoobscura* in tim-null mutants appears to generate *pseudoobscura*-like colder temperature characteristics. The situation with *per^{+}/Y; tim^{01}, tim^{ps}/ tim^{01} per^{ps} ,* flies was also similar where high rhythmicity was shown under colder temperature by both of the lines (*per^{+}/Y; tim^{01}, tim^{ps}/per^{ps} , tim^{01}* 19 and 21). These observation further confirmed the species specific role of *per* in circadian temperature preferences.

Temperature influences the splicing of the 3' untranslated region of *per* leading to the changed in the dynamics of the upswing in *per* mRNA. The higher level of *per* mRNA at lower temperature might be the factor responsible for the high level of rhythmicity seen in the majority of the transgenic flies (Majercak et al. 1999).

However this remains to be investigated, as the 3' ends of *pseudoobscura-per* may not be spliced in the same way. The splicing efficiencies of *per* in *D. yakuba* and *D. santomea* does not exhibit thermal calibration which is consistent with the afro-equatorial habitat of this species where in day length and temperature exhibits little fluctuation throughout the year (Low et al. 2008).

The effect of temperature on the length of the average period of the locomotor activity in this study was also prominent and an overall trend toward shortening of period under 18^{o}C was seen in the positive controls (both natural and transgenic) and *pseudoobscura-per* transformant lines. The same trend was reported in previous studies using these fly lines (Piccin et al 2000; Hennessy 1999; Peixoto et al. 1988). However the difference of the average period for the *pseudoobscura-per* flies was large, 4.16h. Compared to the *D. pseudoobscura per* transgenics, the temperature compensation was much enhanced in double transgenics. The single *pseudoobscura-tim* transgenics were also temperature compensated. These results suggest that temperature compensation is disturbed more by heterospecific PER molecules than TIM molecules. This would fit with the general view that PER is important for thermal adaptation of the clock (Majercak et al. 2004; Sawyer 1997) than TIM, which is more important for light sensitivity of the clock (Zeng et al. 1996) plus photoperiodic effects mediated by the clock such as diapaus (Tauber et al. 2007).

The temperature dependent 3' per splicing event is mediated by phospholipase C (NORPA) and considered to play a very important role in the phase adjustment of the morning and evening activity (Collins et al. 2004, Majercak et al. 2004). Daan and Pittendrigh (1976) proposed a two oscillator system for controlling the morning and evening peak of activity which help animals to adapt to seasonal changes. They

proposed that on hot and long summer days, diurnal animals phase advance their morning activity and shift it to the late night, while simultaneously delaying their evening peak by shifting it to the early night. During the winter, these two peaks come close together, enabling the animal to take advantage of the day time heat. The E peak of activity in this study of *D. pseudoobscura* was in accordance to what has previously reported about these fly lines (Hennessey 1999). However surprisingly the *D. melanogaster* flies in my study showed no significant difference in the E peak under the two temperatures.

The phase analysis of the locomotor activity in the transgenic flies in LD12:12 also showed some very interesting results. Bywalez et al. (2012) found that M and E does not occur at a fixed time and respond differently to day length and temperature. They suggest that the two oscillators have different sensitivities and the phase of evening activity is more sensitive to high temperature, resulting in a delay. The phase of morning peak of activity was compared for the transgenic lines and it was revealed that *pseudoobscura-tim* transgenics (*w/Y; tim^{01}, tim^{ps} / tim^{01} (tim19 and 21)*) are active earlier under 25^{o}C than 18^{o}C which is the normal heat avoiding response by *D. melanogaster* flies controlled at least partially through the reduced per 3' splicing of the *dper* transcript (Majercak et al. 1999; Low et al. 2012). This splicing also delays the EP in hot days, generating the siesta. However these single *tim* flies cannot adjust their evening peak according to the temperature conditions and show an earlier EP on hot than cold days. When I crossed these flies onto the *pseudoobscura-per* background, I obtained the normal heat avoiding behaviour and the double transgenics produced a later evening peak at 25^{o}C than at 18^{o}C. Thus the normal hot day response requires both heterospecific PER and TIM partners revealing another possible example of

coevolution. This also points to the fact that the activity profiles of the flies under different temperature need an intact functional clock. So although the *tim* transgenes had an endogenous *melanogaster-per* but the flies were not very rhythmic specially in term of period. However when they get *pseudoobscura-per* they can interact better with the other clock component and bring out the temperature dependent specific response in the locomotor activity profiles.

In this study we not only see rescue of the circadian period in the double transgenics but also the role of *per* and *tim* in the circadian control of rhythms in LD under different temperatures and in species specific aspects of behaviour.

Chapter 5:

The Residual clock

5.1 Introduction

Wild-type *D. melanogaster* flies show a prominent bimodal locomotor activity profile under light dark conditions in the laboratory (Helfrich-Förster 2000) with pronounced morning and evening peaks (Hamblen-Coyle et al. 1992). However the study of locomotor activity profiles of the flies outside the laboratory under natural condition revealed that wild type as well as mutant per^{01} and tim^{01} flies display similar morning and evening activity bouts and also a third activity bout in the afternoon (called the "A" peak) under warmer condition which were absent in all previous studies from the laboratory (Vanin et al. 2012). per^{01} mutants have been reported to show some residual rhythmicity which generates weak activity rhythms under different light dark conditions in laboratory (Helfrich and Engelmann 1987; Helfrich-Forster 2001; Collins et al. 2005; Kempinger et al. 2009) .

An intact clock will suppress the effect of temperature as long as it is tolerable for the flies. Mennagazzi et al. (2012) reported that per^{01} showed more rapid locomotor responses to slow increase in temperature than wild flies. Bywalez et al. (2012) observed clock mutants under natural-like temperature cycles in the laboratory and reported the behaviour of the single mutants per^{01}, tim^{01} and the double mutant

per^{01}*; tim*^{01} flies to be different from the other clock mutants *Clk*^{jrk} and *cyc*^{01}. In most of the individual *per*^{01}, *tim*^{01} and *per*^{01}*; tim*^{01} flies, clear M and E peaks could be distinguished in their activity profiles under 31^{o}C/21^{o}C temperature cycles. The phase of their activity peak changed with the day length and seemed not to be varying with the temperature changes. The rhythmicity even continued after being released into constant conditions (DD) for one day on average and in a few individual flies for up to 5 days. The *Clk*^{jrk} mutants which could just follow the temperature cycle but not the light cycle did not show this rhythmicity after being placed under constant conditions. This suggests that natural-like temperature cycles allow the residual clock of *per*^{01} flies to run under constant condition for several days and are thus very potent zeitgebers.

The molecular mechanism of this residual clock in *per*^{01} is still unknown. Other clock genes such as *vrille (vri)* , *Par domain protein1 (Pdp1)* and *clockwork orange (cwo)* that are involved in additional feedback loops with CLK /CYC might be responsible for running this residual clock (Allada and Chung 2010). Specifically, natural or natural-like temperature cycles can help these transcripts to cycle in the absence of *per* and *tim*, resulting in the weak behavioural rhythms observed by Bywalez et al. (2012). My observation with the different heterospecific transgenic lines of chapter 4 which are mutant simultaneously for *per* and *tim* showed that flies that are totally arrhythmic in DD, nevertheless display residual rhythmicity in LD. This study aims to provide insight into the molecular mechanism controlling the residual *per*^{01} clock. I wanted to further study it in my transgenic flies and investigate the effect of temperature (if any). All the available HU (Houtenized) clock mutants were used for the analysis of residual rhythmicity under laboratory conditions in LD 12:12 at 25^{o}C.

Western blots were also performed to study whether any clock proteins are cycling in these mutants under LD.

5.2 Materials and Methods

5.2.1 Fly stocks

The transgenic flies created for the chapter 4 were also used here. These were *per^{01}/Y; tim^{+}; per ^{ps} / + (I20), per^{01}/Y; tim^{+}; per ^{ps} /+(I26), w/Y; tim^{01},tim ^{ps}* (lines 19 and *21*), *per^{01}/Y; tim^{01}, tim^{ps} /tim^{01}, tim^{ps}; per^{ps} / per^{ps}* (lines 19 and 35) and *per^{+}/Y; tim^{01}, tim^{ps} / tim^{01}, per^{ps}* (lines 19 and 21).The *w/Y; tim^{01}, tim^{mel} / tim^{01}* (line t28s) and *per^{01}/Y , w; per^{mel} /+*(line 2A) were the positive controls. The clock mutants on the HU background, *per^{01}; tim^{+}* , *per^{+}; tim^{01}* , *per^{+}/Y; tim^{+}; Clk^{jrk}* , *per^{+}/Y; tim^{+}; cyc^{01}* , and *per^{01}; tim^{01}* were used in this study. Another mutant line *per^{+}/Y; tim^{+}; Pdp1ε^{3135}/TM3-Sb* was also used (Zheng et al. 2009).

5.2.2 Data analysis

The data was analysed by using the "Befly" software developed by Dr. Ed Green (Allenbrandt et al. 2013) according to the procedure mention in chapter 2. "Prism" was used for the statistical analysis of the data.

5.2.3 Western blots

The cycling of CLK and PDP1ε protein was analysed through western blots (protocol in Chapter 2). About 50 fly head were collected at each time interval in LD 12:12 at 25^{o}C.

5.3 Results

5.3.1 Residual rhythmicity in transgenics

The LD12:12 activity profiles of the *melanogaster* transgenics *w/Y; tim^{01}, tim^{mel} / tim^{01}* (line t28s) and *per^{01}/Y , w; per^{mel} /+*(line 2A) showed bimodal activity. The flies show anticipation of lights on (red arrows in the Figure 5.1 A &B) and attain a peak in their activity after lights on. After this the flies do not show any activity during the day till in the late afternoon where they begin to show again an anticipation of lights off and attain the peak around that signal (Blue arrows in Figure 5.1A & B). The anticipation of lights on and off shows the presence of an endogenous clock and that flies are not merely responding to the light signals.

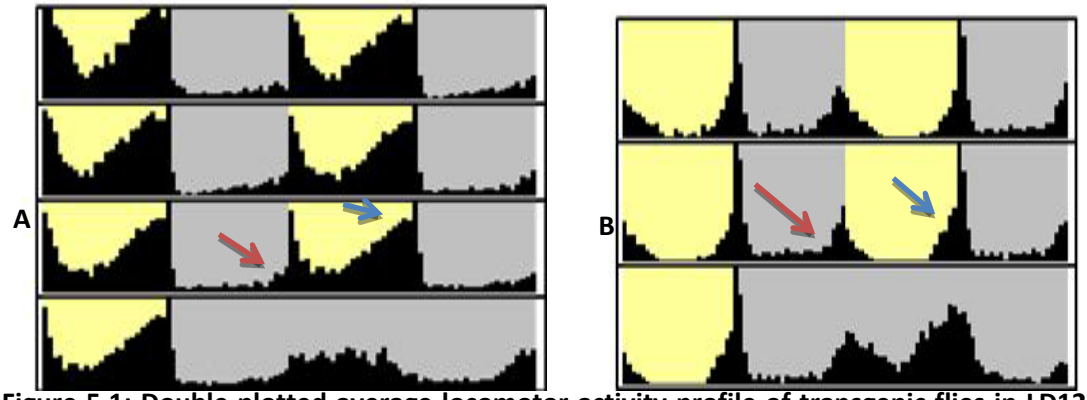


Figure 5.1: Double plotted average locomotor activity profile of transgenic flies in LD12:12. Red arrows shows morning anticipation and blue arrows shows evening peak of activity. A= *w/Y; tim^{01} tim ^{mel} /tim^{01}*(t28s) N=32, B= *w/Y, per^{01}; per^{mel}* /+(2A) N=32.

D. pseudoobscura-per transgenic flies (*per^{01}/Y; tim^{+}, per ^{ps} /+ I20* and *per^{01}/Y; tim^{+}; per ^{ps}/+ 126)* displayed an early peak of evening activity compared to the *melanogaster* transgenics (blue arrows Figure 5.2 A&B). The *pseudoobscura-tim*

transgenics *w/Y; tim^{01}, tim^{ps} / tim^{01}, tim^{ps}* (lines 19 & 21*)* also displayed a bimodal activity profile under LD 12:12 but the evening activity peak was also very early i.e. around ZT7 and ZT8 (blue arrows Figure 5.2 C&D). The double transgenic *per^{01}/Y; tim^{01}, tim^{ps} / tim^{01}, tim^{ps}; per^{ps} / per^{ps}* (lines 35 and 21) flies were completely arrhythmic in DD (see Chapter 4). However when their behaviour was analysed in LD 12:12 they showed rhythmic behaviour with an early peak of evening activity as in the single transgenic flies. However the anticipatory pre-lights on locomotor activity was absent and the flies only revealed behavioural masking due to the lights on signal. (Figure 5.2 E&F). The amount of activity is very low in DD and the individual fly analysis also shows no rhythmicity in the behaviour once the lights are off. Almost all of the locomotor activity is limited to the light period with a peak of activity around ZT 7-8.

This early evening component (blue arrows Figure 5.4) was also observed in the *per^{+}/Y; tim^{01}, tim^{ps} / tim^{01}, per^{ps}* (lines 19 and 21).

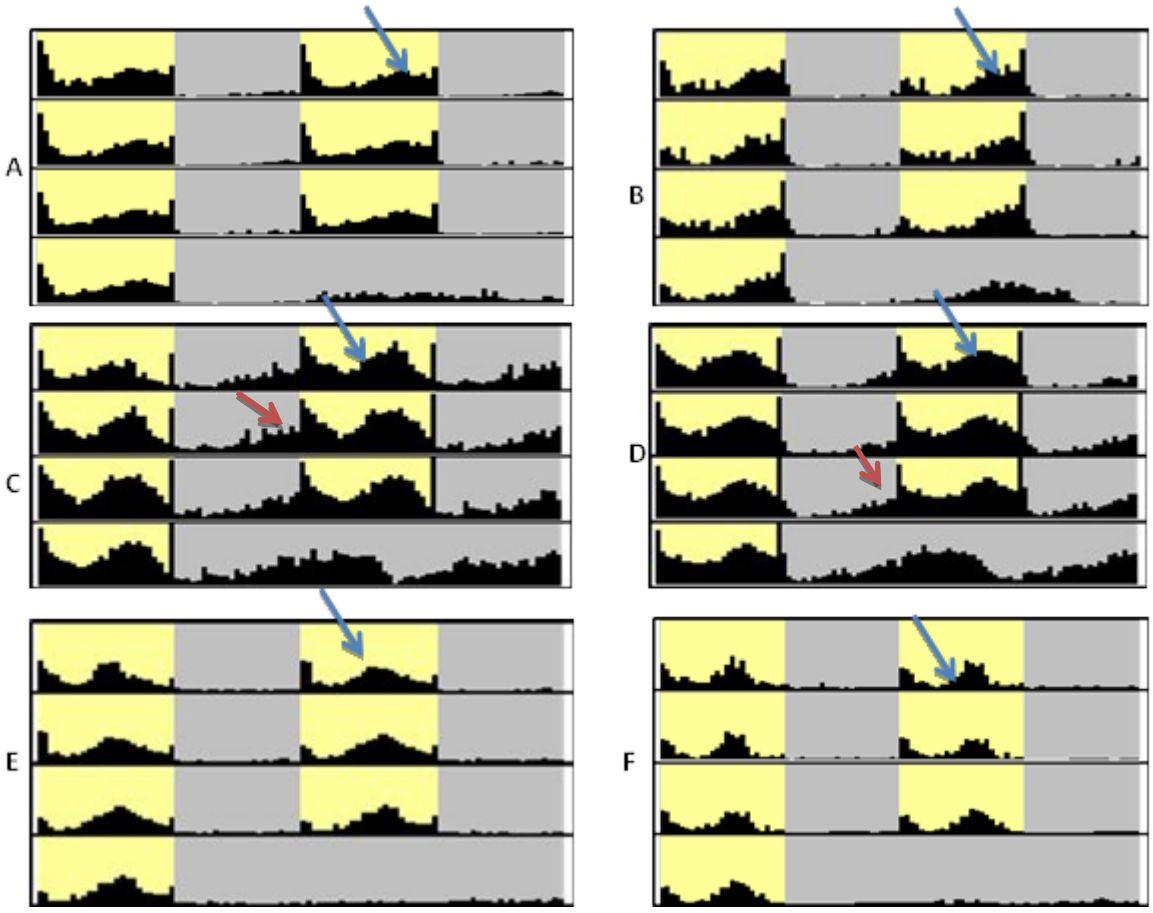


Figure 5.2: Double plotted average locomotor activity profile of transgenic flies in LD12:12. Red arrows shows morning anticipation and blue arrows shows evening peak of activity. A= *per^{01}/Y; tim^{+}; per ^{ps} / + (I20),* B= *per^{01}/Y; tim^{+}; per ^{ps} /+(I26),* C= *w/Y; tim^{01}, tim ^{ps} /tim^{01}(19),* D= *w/Y; tim^{01}, tim ^{ps} /tim^{01}(21)* , E= *per^{01}/Y; tim^{01}, tim ^{ps}/ tim ^{ps} , tim^{01}; per ^{ps} / per ^{ps}* (35), F= *per^{01}/Y; tim^{01}, tim ^{ps}/ tim ^{ps} , tim^{01}; per ^{ps} / per ^{ps}* (19) in LD 12:12 under 18^{o}C.

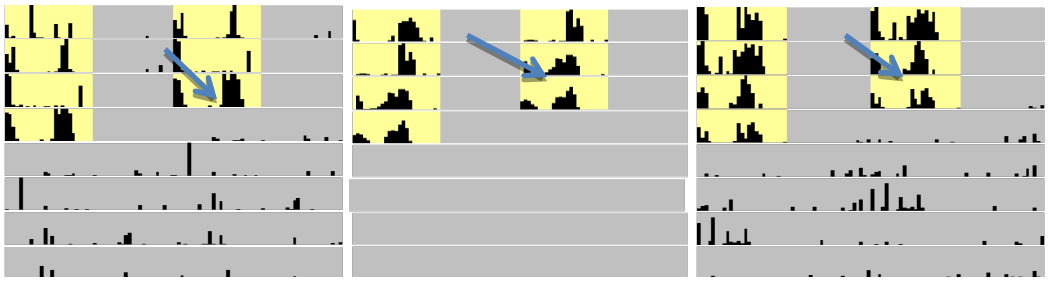


Figure 5.3: Locomotor activity profiles of individual flies of *per^{01}/Y; tim^{01}, tim ^{ps}/ tim ^{ps} , tim^{01}; per ^{ps} / per ^{ps}* (19) line under LD12:12 at 18^{o}C. Morning anticipation is absent but the evening peak is indicated by blue arrows.

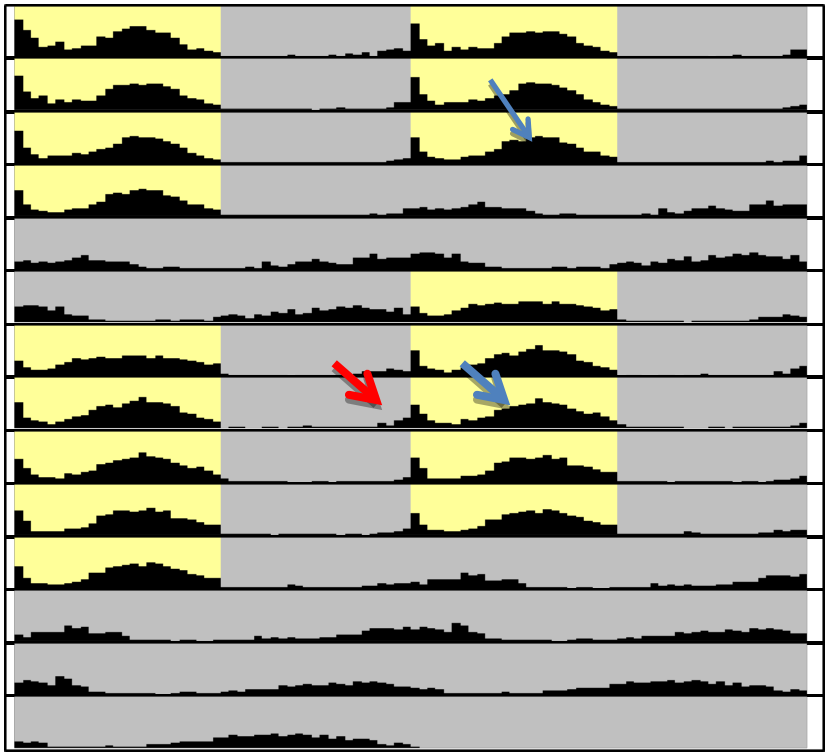


Figure 5.4: Locomotor activity profile of rhythmic *per^{+}/Y; tim^{01}, tim ^{ps} / tim^{01}, per^{ps}* (line 19) flies in LD 12:12 under 18^{o}C (N=30). Red and blue arrows point toward Morning and evening peaks respectively.

5.3.2 Residual rhythmicity in the HU lines

Residual rhythmicity was also seen in the *per^{01}; tim^{+} (HU)* mutant entrained under LD 12:12 at 25^{o}C for 10-12 days (Figure 5.5). After 10 days of entrainment the

flies showed rise in locomotor activity starting around ZT7 (blue arrow, Figure 5.5). They also showed a morning anticipation of activity before the lights on (red arrows on Figure 5.5). This experiment was repeated under 18^{o}C and it was observed that the day time activity still persists and the morning anticipation was also present (Figure 5.5). The evening peak is more scattered than it was on 25^{o}C but the major amount of locomotor activity is still limited to the day time only.

Although both of these lines are arrhythmic in DD, still the rhythmicity attained in LD is maintained in these flies for at least one day in DD on average. The individual flies in Figure 5.6 were placed in DD for one day after being entrained for 4 days in LD 12:12 and still they show morning anticipation as well as evening peak of activity (red arrows for morning anticipation and blue arrows for evening peak of activity in Figure 5.6).

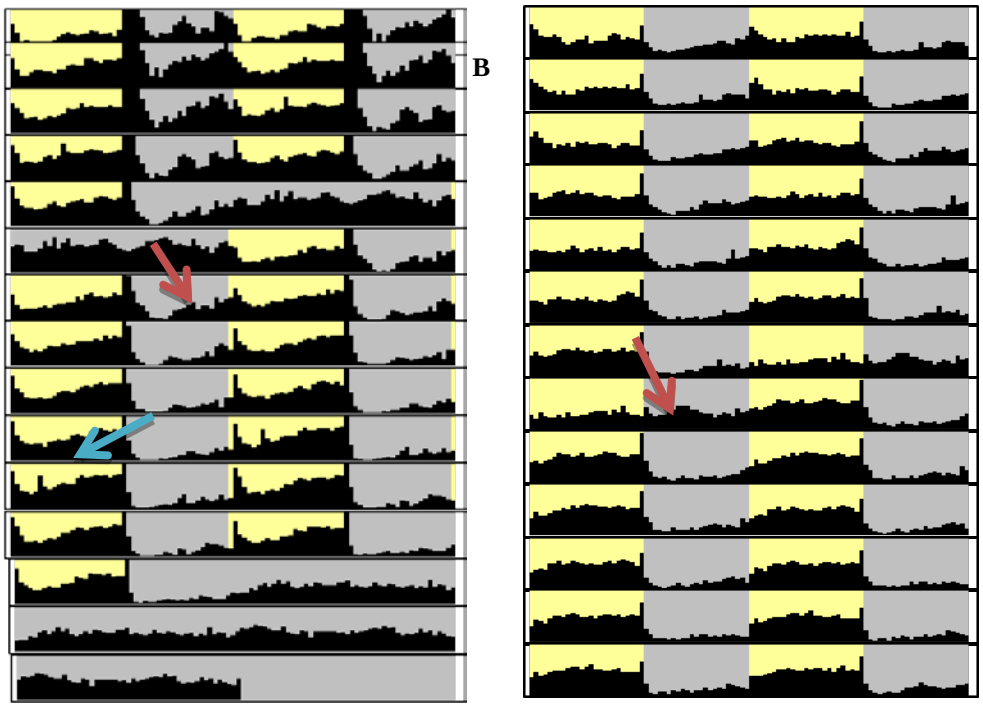


Figure 5.5: Locomotor activity profile of *per^{01}; tim $^{+}$(HU)* flies in LD 12:12 at 25 (A) and 18^{o}C (B) (red arrow indicate the morning anticipation and the blue arrow indicate the evening peak).(N=25)

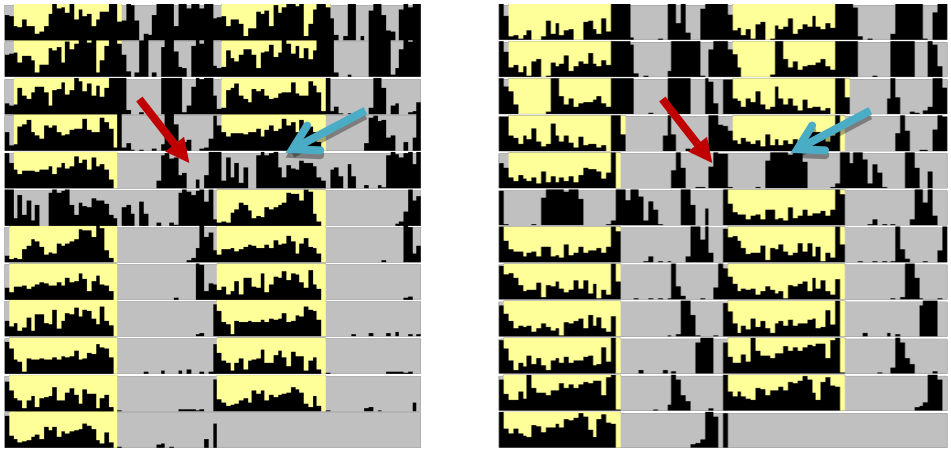


Figure 5.6: Locomotor activity profile of *per*^{01}*; tim*$^{+}$ *(HU)* individual flies in LD 12:12 at 25°C. (Arrow indicate the rhythmicity in the DD)

The same pattern was observed in the *per*$^{+}$*; tim*^{01} (HU) mutants (both red and white eyed flies) when entrained in LD 12:12 for 10-12 days (Figure 5.6 A). There is morning anticipation of activity before lights on. The activity then increases at about ZT7 and continues till lights off (Figure 5.6A). To further investigate this behaviour the double mutant *per*^{01}*; tim*^{01} (HU) was created and entrained in LD12:12 under 25°C for 10-12 days. This pattern of evening locomotor activity peak was also observed in the double mutant (Fig 5.6B). These flies also showed a morning anticipation of activity before lights are turned on (Red arrows Figure 5.6B)

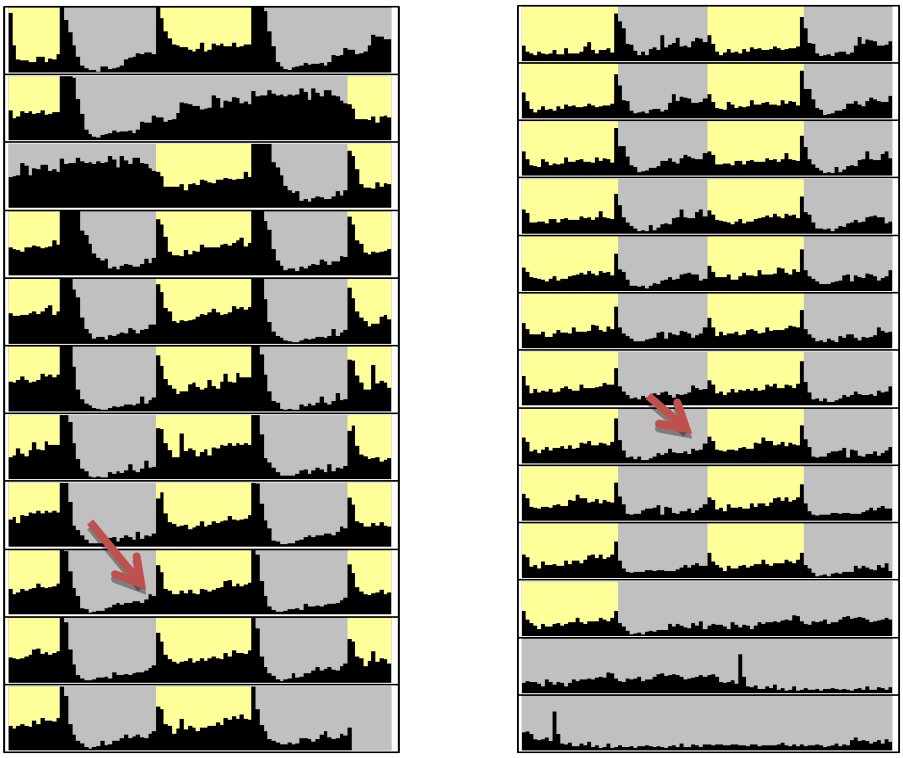


Figure 5.6 Locomotor activity profile of *per^{+}/Y; tim^{01}* (HU) (A) and ***per^{01}/Y; tim^{01}*** **(HU) (B) flies in LD 12:12 under 25^{o}C (N=32).**

Two more clock mutants *per^{+}/Y; tim^{+}; Clk^{jrk}* and *per^{+}/Y; tim^{+}; cycle^{01}* from the HU background (Figure 5.7 A&B respectively) and also a *Pdp1ε* mutant line (Figure 5.8) were also entrained for up to 12 days in LD at 25^{o}C. The *Pdp1ε* mutants was not in the HU background. These were *per^{+}/Y; tim^{+}; Pdp1 ^{3135}* mutants having a 4 bp deletion in an exon common to the *Pdp1* RD and RJ isoforms. The cytological location is on chromosome III (Zheng et al. 2009). The homozygous mutant flies were used for the experiment. However the results showed that all these lines are arrhythmic in LD even after entrainment for more than 10 day.

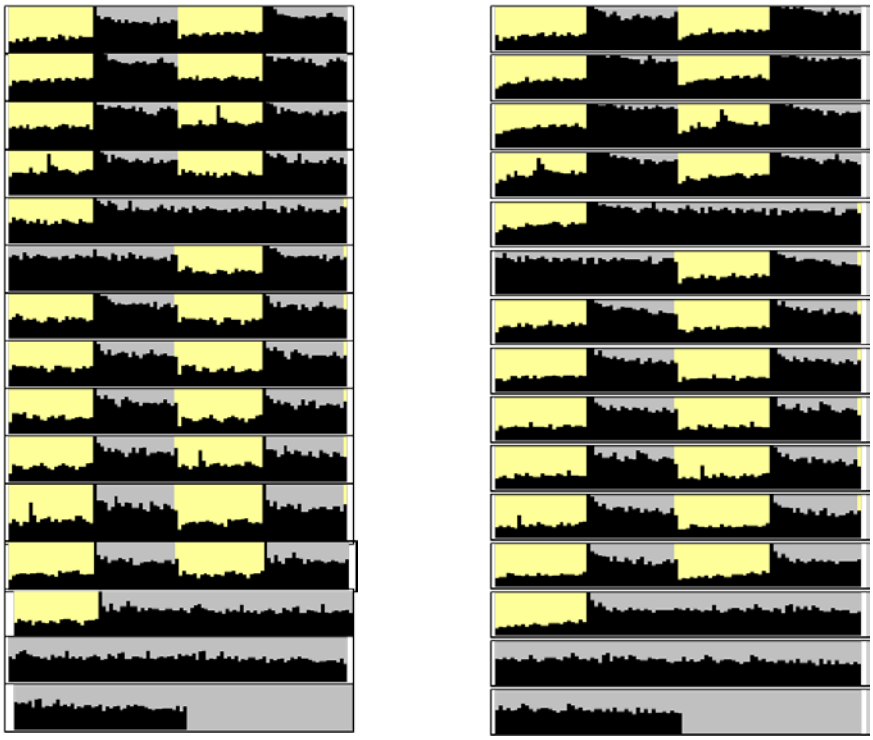


Figure 5.7: Locomotor activity profile of *per^{+}/Y; tim^{+}; Clk^{jrk}* (Hu) (A) and *per^{+}/Y; tim^{+}; cycle^{01}* (Hu) (B) flies in LD 12:12 under 25^{o}C (N=32).

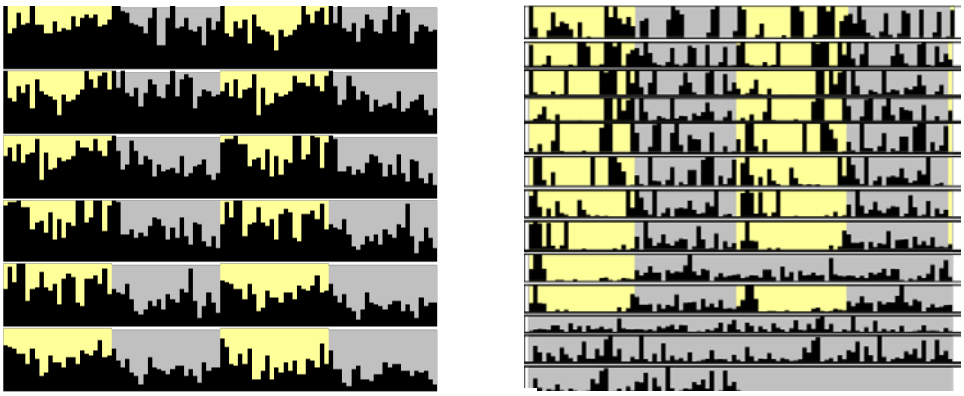


Figure 5.8: Activity profile of *Pdp1 ε* mutants (*per^{+}/Y; tim^{+}; Pdp1ε^{3135}*) (A= average of all flies, B= individual fly) under LD 12:12 at 25^{o}C (N =8).

The analysis for LD profile of the Hu flies showed that single *per* and *tim* mutant and double mutants are rhythmic in LD after up to 10 days of entrainment but *per^{+}/Y; tim^{+}; Clk^{jrk}* and *w/Y; tim^{+}; cycle^{01}* and *per^{+}/Y; +; Pdp1 ^{3135}* are still arrhythmic. So

it could be the translational product of any of these which is cycling in LD and resulting in these weak rhythms in the absence of PER and TIM.

5.3.3 Western blots

Western blots were performed to study which clock proteins (if any) might be cycling in the *per^{01}/Y; tim^{+} (HU)* mutants under LD and resulting in the rhythmic behaviour of these flies. As the *per^{+}; tim^{+}; Clk^{jrk}* mutants were arrhythmic in LD, the western blotting was done using CLK antibodies. The results showed that CLK is not cycling in the *per^{01}; tim^{+} (HU*) mutants (Figure 5.9).

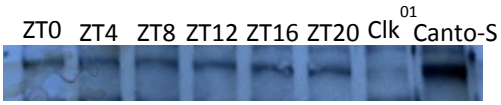


Figure 5.9: CLK protein level at different time points in the *per^{01}; tim^{+}* mutants under LD12:12 at 25^{o}C.

The next western blot was performed using the PDP1 (PAR domain protein) antibodies (Figure 5.10). This protein was observed to be cycling in the *per^{01}; tim^{+} (HU*) mutants. The collections of flies were performed for 6 time points in LD 12:12 at 25^{o}C. There was a significant difference ($F_{(5,12)}$=11.6, p=0.0003) in the level of proteins at the 6 different time points when normalised against HSP70 (Figure 5.11). The lowest amount of protein was detected on ZT4 which than start increasing at ZT8, reaching its maximum on ZT12 and stays almost at that level till ZT20.

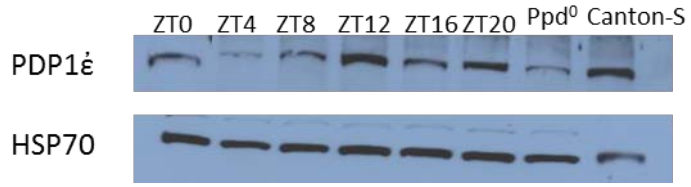


Figure 5.10: western blot showing protein level of PDP1 at 6 time points (ZT0-ZT20), *per^{+}/Y; +; Pdp1^{3135}* mutants and *D. melanogaster* control

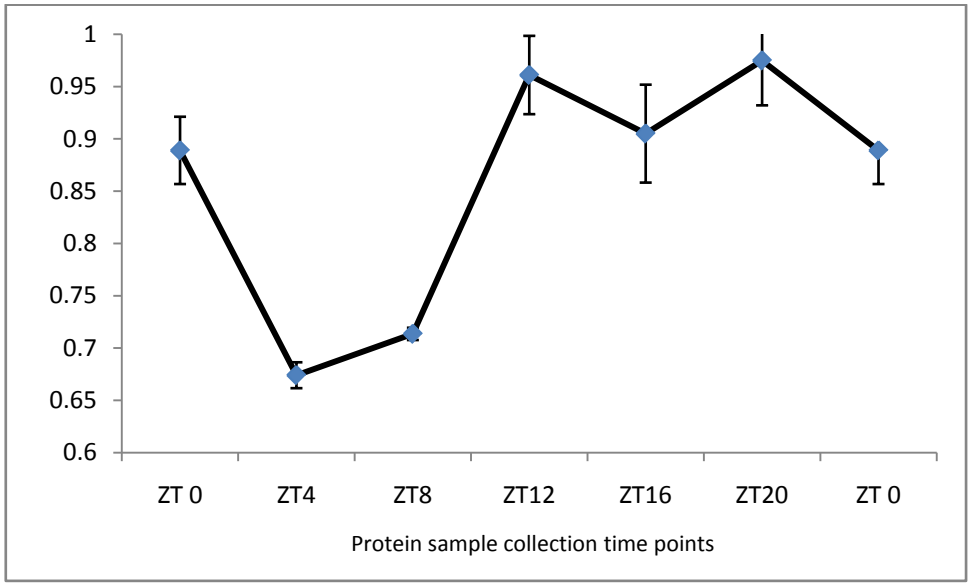


Figure 5.11: PDP 1 protein cycling in *per^{01}; tim^{+} (Hu)* flies under LD12:12 on 25^{o}C (N=3, $F_{(5,12)}$=11.6, p=0.0003).

5.4 Discussion

The *tim^{01}* flies carrying the *tim* transgenes from *pseudoobscura* and the homozygous double transgenics (*per^{01} /Y ; tim^{01}, tim ^{ps} /tim^{01}, tim ^{ps}; per^{ps}/per ^{ps}*) showed rhythmic behaviour in LD 12:12 as defined by the presence of a distinct evening peak of activity under both 25 and 18^{o}C. The homozygous double transgenics are totally arrhythmic when analysed under free run (DD) (Chapter4). The double

transgenic in the *per*$^{+}$ background (*per*$^{+}$ */Y; tim*^{01}*, tim* ^{ps}*/ tim*^{01} , *per* ^{ps}) also show this early peak of activity as compared to *melanogaster* transgenics. Another interesting finding was that the *per* and *tim* single and double mutants *per*^{01}*; tim*^{01} show this phenotype only after entrainment in LD 12:12 for about 10 days (Helfrich and Engelmann 1987) and they damp under lower temperatures. However my transgenic flies display them from the first day of their activity in LD 12:12 under both temperature conditions.

The HU single clock mutants *per*^{01} */Y; tim*$^{+}$, *per*$^{+}$*/Y; tim*^{01} and the double mutant *per*^{01}*/Y; tim*^{01} after entrainment under LD 12:12 for several days show not only the evening peak of activity but also display a morning anticipation of lights on. So the possibility remains that these mutants have a short-period residual clock which gives rhythmicity to these flies (Helfrich and Engelmann 1987). *Clk*^{jrk} severely disrupts the mechanism of the circadian clock and these flies completely lack the typical bimodal organization and only respond to the temperature changes (Allada et al. 1998, Bywalez et al. 2012). *per*$^{+}$*/Y; tim*$^{+}$*; Clk*^{jrk}, mutants do not show the LD rhythmic phenotype even after 12 days of entrainment. The western blots for CLK showed no cycling for this protein in *per*^{01} mutants however *Pdp1*ε was found to be cycling in these flies so that means that *per*$^{+}$*/Y; tim*$^{+}$*; Pdp1*ε^{3135} mutants should not behave rhythmically In LD. Indeed, this arrhythmic phenotype in these mutants has been shown by Zheng et al. (2009) and also observed in the present study.

Pdp1ε encodes seven isoforms however only one of them, is expressed rhythmically and was proposed to be an essential activator of *Clk* (Cyran et al. 2003). Zheng et al. (2006) demonstrated that CLK protein levels remains constant in the cell

but it is the phosphorylation state of the protein which determines it's binding to DNA and its transcriptional activator function. This might be the reason I could not see any cycling of CLK proteins in the per^{01} flies in my study.

Zheng et al. (2009) produced $Pdp1\varepsilon^{3135}$ mutants (used in my study) and showed that the effect on CLK abundance was small but its phosphorylation state was greatly reduced in these mutants. PDP1ε might affect phosphorylation of CLK in two ways (Zheng et al. 2009). One of it might be related to the PER dependent CLK phosphorylation. After entering the nucleus, PER/DBT complex dissociate and PER delivers the kinase DBT to CLK (Kim and Edery 2006; Yu et al. 2006) which phosphorylates CLK. In PDP1ε mutants there was considerable effect on PER levels (Zheng et al. 2009). So late at night when PER is trying to enter into the nucleus, a reduction in its level in these mutants will affect the phosphorylation of CLK (Kim and Edery 2006, Yu et al. 2006). The other mechanism for the reduced CLK phosphorylation might be through the control of a kinase which phosphorylates CLK (Zheng et al. 2009). However rescue of CLK in the $Pdp1\varepsilon$ mutants restores core clock function but not the behavioural rhythms which strongly suggest that this protein is not only important for the regulation of CLK but also has an independent regulatory role for other circadian outputs (Allada and Chung 2010). PDP1 ε has also a direct effect on PDF which is also an example of its direct role in the clock outputs (Zheng et al. 2009).

Based upon this all these functional roles of this protein we can conclude that $Pdp1\varepsilon$ probably be involved in running the residual clock in the absence of *per* and *tim.*

Chapter 6: Final Discussion

My study of the *cry* L232H polymorphism neither revealed any difference in the circadian photosensitivity nor in the distribution of these two alleles throughout Europe. However the phase variations in the males and females of the different genotypes in the Rende lines revealed an insight into a possible mechanism, although speculative, which might be responsible for the balanced polymorphism we observe in nature. Under some conditions, locomotor activity levels correlate with enhanced competitive mating for males (Kyriacou 1981), so the temporal difference in the locomotor phase might lead to disassortative mating which could contribute to the production of more heterozygotes resulting in intermediate frequencies of the two alleles in nature. This could be tested in the future with direct mating observations under conditions where the sexes and alleles are mixed and the different genotypes are labelled in some way (e. g Tauber et al. 2003). The observation that the locomotor phase difference we observed for this polymorphism is sex-related also needs further study and the use of different day lengths may help in separating further the activity phases of the flies and understanding this mechanism.

I could also successfully mimic the almost equal frequency in the wild of the two alleles in the laboratory under LD12:12 in constant 25^{o}C conditions in the Rende population. These results unusually show that a simple laboratory population experiment can sometime simulate the more complex environment of the wild, and suggest that the reason underlying this evident balancing selection may be

experimentally tractable (e.g Kyriacou 1985). However the detail sequence analysis of all these four alleles and also of the *per* and *tim* and few other clock genes will help in understanding this phenotype better. It will also provide evidence that is it only this SNP which is responsible for this phenotype or other genes are also contributing.

The level of rescue in the clock function of *D. melanogaster* by interspecific clock gene transformation was used to study the putative intermolecular coevolution between interacting clock proteins. PER and TIM are the two important clock proteins running the negative limb of the circadian clock feedback loops. TIM binds to the PER PAS domain of PER (Hardin 2011) and prevents its degradation (Kloss et al. 1998). Previous studies of interspecific PER rescue of rhythmicity are based upon the introduction of only one of the two proteins i.e. either PER or TIM in to *D. melanogaster* host (Piccin et al. 2000; Ousley et al. 1998 Nishinokubi et al. 2003). I used a co-insertion of the *D. pseudoobscura per* and *tim* transgenes into the corresponding double mutants to see if these two interacting protein together formed a coevolved module that could interact more efficiently with all the other clock proteins, CLK, CYC, DBT etc.

The study of these two transgenes separately revealed more than 50% rhythmicity in most lines but the period of activity was either very long (*pseudoobscura-per* transgenics) or very short (*pseudoobscura-tim* transgenics) as compared to controls. By introducing both transgenes simultaneously I observed that the period of the activity rhythm, if not the strength of rhythmicity, was rescued by the pair of hemizygous transgenes. These flies also showed a higher degree of temperature compensation for the period under 25^{o}C and 18^{o}C. The double

transgenics crossed into the *per*$^{+}$ background also showed higher levels of rhythmicity (as expected) and average periods at lower temperature as in wild *pseudoobscura* flies. These observation are consistent with the view that TIM and PER form a heterospecific coevolved module at least for the circadian period of activity suggesting they interact as a dimer more robustly with the other endogenous clock proteins, compared to a situation where only one of these negative regulators is heterospecific.

However a number of processes are involved in the circadian control of a 24 h period e.g. transcriptional activation, post transcriptional processes involving transcript stability (Stanewsky et al. 1997) and its transport to the cytosol, and even after the synthesis of proteins, there a number of post-translational processes that govern timekeeping by the core loop. The stability, nuclear localization and transcriptional repression of PER is controlled by its binding to TIM and also a number of kinases and phosphatases which control the phosphorylation state of PER and TIM (Hardin 2011). Strangely, flies with two copies of each *pseudoobscura-per* and *tim* transgene in the double mutant background showed arrhythmic behaviour in DD. However the LD analysis showed that these flies are not completely clock-less. Under LD 12:12 these flies have a *D. pseudoobscura* like activity profile with the absence of morning anticipation as well as a very prominent earlier evening peak of activity. This rhythmic behaviour was observed both under lower and higher temperatures. *per* and *tim* mutants have been previously studied and weak rhythms under LD after entrainment for 10-12 days have been observed (Helfrich and Engelmann 1987). My transgenic flies are in a *per*^{01}*;tim*^{01} background, but in contrast to the simple double mutant they show LD rhythms not only from the first day in LD but they are also very prominent and remain so at lower temperature. This suggests that it is not only the

residual *per*^{01}*; tim*^{01} clock which is running the short period in these flies but the homozygous *pseudoobscura per* and *tim* transgenes are also contributing in LD conditions. This dose-dependent phenotype suggests that hetero-specific PER-TIM proteins can only be tolerated up to a certain level of abundance after which there is interference with post-translational mechanisms.

These observation suggest that PER and TIM together form a coevolved model with the other clock proteins like CLK and CYC and they are able to interact with transcriptional-translational feedback loops in DD (as hemizygotes) and LD (also as homozygotes). The interaction of PER and TIM with the other clock proteins can be further directly studied using the yeast two hybrid system or S2 cells. The sequence comparison between *D. melanogaster* and *D. pseudoobscura* showed highest similarity for CRY and lowest for CLK. Transformation of these double transgenic flies also with *Clk,* or *cry* transgenes from *D. pseudoobscura* (technically very demanding in terms of the crosses) to see its effect on rhythmicity and period would also be very interesting. By comparing all three double transgenic lines we same differences in the level of expression which reflects in the behaviour of the flies. This could be the position effect of the different transgenes as these were old lines with p-element insertions. If these experiments are repeated again one can use PhiC31 integrase system (Sangiorgi et al 2008) to create the transgenic lines. This way all the lines will have the same location for the transgenes, so no genomic effect and the data will be comparable.

The bimodal activity profile of *D. melanogaster* is found to be under the control of several sets of neurons (Helfrich-Förster 2005). Under LD conditions in the laboratory, the PDF-positive sLNvs have been implicated in control of the morning locomotor activity forming a neuronal basis for the morning (M) oscillator, while the

fifth PDF-negative sLNv and LNds seem to be responsible for the evening activity and hence termed the evening (E) oscillator (Grima et al. 2004; Nitabach and Taghert 2008; Stoleru et al. 2004). The activity profile of *D. pseudoobscura* did not show morning anticipation of activity in LD. This seems to be an adaptive response of this species where the morning activity lost its selective advantage due to the long summer days of more northern climatic zones (Hermann et al. 2013). This type of activity profile has also been reported from high latitude species *D. montana* (Kauranen et al. 2012) and *D. virilis* (Dubruille and Emery 2008). Both of these species do not express PDF in the sLNv 's (or express at very low levels), which are the morning oscillator (Kauranen et al. 2012). Hermann et al (2012) reported some apparent differences in the PER expression in the clock neurons between Sophophora (to which *D. pseudoobscura* belongs) and Drosophila subgenuses Indeed, they reported similar observations in *D. pseudoobscura* that also showed reduced PDF immunostaining in the sLNvs.

However in my present study the *D. melanogaster* mutant carrying the *pseudoobscura-per* transgene alone as well as in combination with *pseudoobscura-tim* (the double transgenics) also show a *D. pseudoobscura*-like activity profile in LD with a lack of morning anticipation and an early evening peak. It would therefore be very interesting to study the M oscillators in the transgenic *D. melanogaster* which lack morning anticipation specially by looking at the expression levels of PER and PDF in these neurons. The *D. melanogaster* Pdf^{01} flies lack morning activity (Renn et al 1999) and also the ablation of PDF-positive sLNvs in *D. melanogaster* resulted in the loss of the morning anticipation in laboratory conditions (Shafer and Taghert, 2009). This provides a strong basis for the study of these neurons in my transgenics to see if the function of sLNvs has been suppressed in these flies.

Different subsets of clock neurons operate at high and low temperatures to mediate clock synchronization to temperature cycles. Gentile et al. (2013) reported that dorsally located clock neurons mainly mediate synchronization to higher (21-29^{o}C) and ventral clock neurons to lower (16-25^{o}C) temperature cycles. However by examining the expression of PER they observed that PER in the LNvs and LNds is required for entrainment to low temperature cycles. I also see a low temperature preference (in terms of rhythmicity) for *D. pseudoobscura* and single *per* or *tim* transformants. The level of *per* mRNA increases at low temperature (Majercak et al. 1999) which might lead to the high expression of PER in the LNds and LNvs resulting in better rhythmicity in these flies. The further study concentrating on the role of different set of neurons under different temperature in *D. pseudoobscura* flies and transgenics will help to understand better the evolutionary plasticity of circadian behaviour.

The rhythmic activity profiles in LD of the otherwise arrhythmic double transgenics flies led to the study of the residual rhythmicity in the clock mutants (per^{01} and tim^{01}). The cycling of the PDP1ε in the absence of PER, TIM and CLK cycling may reflect the independent regulatory role of this protein in controlling other circadian outputs (Allada and Chung 2010). My study revealed an insight into the molecular mechanism underlying residual rhythmicity in the otherwise arrhythmic homozygous double transgenic flies as well as in the clock mutants per^{01}. The next step is to investigate the level of VRI in these flies which is reported to compete with PDP1ε for the same *Clk* promoter (Cyran et al. 2003). The level of other clock proteins such as CYC and CRY should also be examined in these flies. This will help in identifying the

role of other clock protein as well as understanding the molecular mechanism of this enigmatic residual clock.

Chapter 7: Appendices

Location	Country of collection	Latitude	Longitude	Altitude (m)	Collection Date	N flies	CTT(L) frequency	CTA(H) frequency
Haifa	Israel	32.48N	34.59E	180	2002	42	0.5952	0.4048
Knossos	Greece	35.17N	25.9E	253	2002	44	0.6364	0.3636
Salice	Italy	40.23N	17.58E	34	2004	44	0.6591	0.3409
Rutigliano	Italy	40.56N	16.54E	100	2004	39	0.6026	0.3974
Bitetto	Italy	41.2N	16.45E	92	2004	43	0.5814	0.4186
Cavarzere	Italy	45.8N	12.4E	4	2004	38	0.4868	0.5132
Villorba	Italy	45.44N	12.14E	26	2004	9	0.5000	0.5000
Bolzano	Italy	46.30N	11.21E	262	2004	36	0.5139	0.4861
Corbières	Suisse	46.39N	7.6E	728	2004	17	0.5588	0.4412
Burgundy	France	47.17N	5.02E	245	2000	17	0.5882	0.4118
Wien	Austria	48.12N	16.22E	151	2004	12	0.4583	0.5417
Houten	Netherlands	52.1N	5.10E	2	2002	85	0.6588	0.3412
Leiden	Netherlands	52.9N	4.29E	-1	2000	16	0.6563	0.3438
Stockholm	Sweden	59.19N	18.4E	44	2008	17	0.5882	0.4118

	Korpilahti	Finland	62.1N	25.33E	120	2008	67	0.4925	0.5075
	Højbjerg	Denmark	56.7N	10.12E	2	2009	76	0.6513	0.3487
	Market Harborough	UK	52.28N	0.55W	93	2008	121	0.4793	0.5207
	Kibworth	UK	52.32N	0.59W	110	2008	161	0.5776	0.4224
	Gothenburg	Sweden	57.42N	11.58E	4	2008	35	0.4429	0.5571
	Nijar	Spain	36.58N	2.12W	356	2008	47	0.4787	0.5213
	Jumilla	Spain	38.28N	1.19W	510	2008	37	0.5676	0.4324
	Requena	Spain	39.29N	1.6W	692	2008	84	0.6012	0.3988
	San Sadurni D'anoia	Spain	41.25N	1.47E	162	2008	65	0.5385	0.4615
	La Rapita	Spain	40.37N	0.35E	10	2008	71	0.6479	0.3521
	Abaran	Spain	38.12N	1.24W	400	2008	26	0.5769	0.4231
	Fontanares	Spain	37.46N	3.43W	634	2008	58	0.5517	0.4483
	Algarrobo	Spain	36.46N	4.2W	86	2008	28	0.7321	0.2679

TABLE 7.1: Genotyping results for the L23H polymorphism in the 27 populations collected from different latitudes across the Europe.

Months	Allele Frequency		Genotype frequency			
A1	cry^{H}	cry^{L}	cry^{HH}	cry^{HL}	cry^{LL}	N
October start	90%	10%	90%	0%	10%	
October end	94.44%	5.56%	89%	11%	0%	36
November	96.50%	3.48%	95.34%	2.32%	2.32%	43
February	98.90%	1.14%	97.70%	3.30%	0%	44
March	99%	1%	98%	2%	0%	47
A2						
November start	90%	10%	90%	0%	10%	
November end	85.00%	15.00%	69.80%	30.20%	0%	43
February	96.80%	3.19%	93.60%	6.40%	0%	47
March	97%	3%	93.60%	6.40%	0%	47
B1						
October start	10%	90%	10%	0%	90%	
October end	14.47%	85.53%	10.53%	7.89%	81.58%	38
November	46.50%	53.48%	20.90%	51.16%	27.90%	43
February	77.11%	22.80%	54.30%	45.70%	0	46
March	87%	13%	74%	26%	0%	46
B2						
November start	10%	90%	10%	0%	90%	
November end	31.25%	68.75%	8.30%	45.83%	45.83%	24
February	82.55%	17.44%	69.80%	25.60%	4.70%	43
March	88%	12%	74.50%	26.50%	0%	47

Table7.2: Results of population cage experiment using Canton-S lines kept under LD 12:12 at 25^{o}C.

Months	Allele Frequency (%)		Genotype frequency (%)			Total flies
A1 Rende	cry^{H}	cry^{L}	cry^{HH}	cry^{HL}	cry^{LL}	
July	90%	10%	90%	0%	10%	
September	75.50%	24.50%	51%	49%	0%	47
November	65%	35%	46.50%	37.25%	16.20%	43
February	61%	39%	33.35%	55.50%	11.11%	45
May	53%	47%	28.30%	50%	21.70%	46
August	68%	32%	44.40%	47.20%	8.30%	36
September	58.50%	41.50%	29.80%	57.40%	12.80%	47
November	54%	46%	24.00%	61.00%	15.20%	46
April	49%	51%	32.00%	34.00%	34.00%	44
A2 Rende						
July	90%	10%	90%	0%	10%	
September	79.30%	20.70%	59%	41%	0%	46
February	63%	37%	34%	57.40%	8.50%	47
May	52%	48%	30.45%	44%	26%	46
August	48%	52%	18.20%	59%	22.70%	44
September	44%	56%	18.20%	52.30%	29.50%	44
November	36%	64%	12.80%	47%	40.40%	47
April	26.60%	73.40%	0.00%	53%	46.80%	47
B1 Rende						

August	10%	90%	10%	0%	90%	
November	33%	67%	8.70%	47.80%	43.50%	47
February	40.50%	59.50%	4.30%	65.20%	30.40%	46
April	41%	59%	11.10%	60.00%	28.90%	45
June	61.60%	38.40%	33.30%	57%	10.00%	30
August	50.00%	50.00%	16.70%	66.70%	16.70%	30
September	45.30%	54.70%	16.30%	58.10%	25.60%	43
November	56.40%	43.60%	36.20%	40.40%	23.40%	47
April	38.00%	62.00%	24.00%	28.30%	47.80%	46
B2 Rende						
August	10%	90%	10%	0%	90%	
September	6%	94%	0%	11%	89%	46
November	18%	82%	2.10%	31.90%	66%	47
February	45.70%	54.30%	20%	51.40%	28.50%	35
April	46.50%	53.50%	16%	60.50%	23.30%	43
June	38%	62%	12%	52%	36%	25
August	41%	59%	17.10%	60%	22.90%	35
September	25.60%	74.40%	11.60%	27.90%	58.13%	43
November	27.20%	72.80%	11%	32.60%	56.50%	46
April	52.00%	48.00%	25%	55.60%	20.00%	45

Table 7.3: Population cage result of Rend lines for cage A1, A2 (90%H : 10%L) and B1,B2 (90%L: 10%H).

		observed frequencies								Expected frequencies			
Cages	Month	cry^{HH} (AA)	cry^{HL} (Aa)	cry^{LL} (aa)	Total	2*AA+Aa	2*(AA+Aa+aa)	p=(2*AA+Aa/2*(AA+Aa)	q=(1-p)	p^2n	2pqn	q^2n	χ^2
A1	September	24	23	0	47	71	94	0.76	0.24	26.81	17.37	2.81	4.93
	November	20	16	7	43	56	86	0.65	0.35	18.23	19.53	5.23	1.41
	February	15	25	5	45	55	90	0.61	0.39	16.81	21.39	6.81	1.28
	May	13	23	10	46	49	92	0.53	0.47	13.05	22.90	10.05	0.00
	August	16	17	3	36	49	72	0.68	0.32	16.67	15.65	3.67	0.27
	September	14	27	6	47	55	94	0.59	0.41	16.09	22.82	8.09	1.58
	November	11	28	7	46	50	92	0.54	0.46	13.59	22.83	9.59	2.36
	April	14	15	15	44	43	88	0.49	0.51	10.51	21.99	11.51	4.44
A2	September	27	19	0	46	73	92	0.79	0.21	28.96	15.08	1.96	3.12
	February	16	27	4	47	59	94	0.63	0.37	18.52	21.97	6.52	2.47
	May	14	20	12	46	48	92	0.52	0.48	12.52	22.96	10.52	0.76
	August	8	26	10	44	42	88	0.48	0.52	10.02	21.95	12.02	1.49
	September	8	23	13	44	39	88	0.44	0.56	8.64	21.72	13.64	0.15
	November	6	22	19	47	34	94	0.36	0.64	6.15	21.70	19.15	0.01
B1	November	4	22	20	46	30	92	0.33	0.67	4.89	20.22	20.89	0.36
	February	2	30	14	46	34	92	0.37	0.63	6.28	21.43	18.28	7.35
	April	5	27	13	45	37	90	0.41	0.59	7.61	21.79	15.61	2.57
	June	10	17	3	30	37	60	0.62	0.38	11.41	14.18	4.41	1.18
	August	5	20	5	30	30	60	0.50	0.50	7.50	15.00	7.50	3.33
	September	7	25	11	43	39	86	0.45	0.55	8.84	21.31	12.84	1.29

	November	**17**	**19**	**11**	**47**	**53**	**94**	**0.56**	**0.44**	**14.94**	**23.12**	**8.94**	1.49
B2	**September**	**0**	**5**	**40**	**45**	**5**	**90**	**0.06**	**0.94**	**0.14**	**4.72**	**40.14**	0.16
	November	**1**	**15**	**31**	**47**	**17**	**94**	**0.18**	**0.82**	**1.54**	**13.93**	**31.54**	0.28
	February	**7**	**18**	**10**	**35**	**32**	**70**	**0.46**	**0.54**	**7.31**	**17.37**	**10.31**	0.05
	April	**7**	**26**	**10**	**43**	**40**	**86**	**0.47**	**0.53**	**9.30**	**21.40**	**12.30**	1.99
	June	**3**	**13**	**9**	**25**	**19**	**50**	**0.38**	**0.62**	**3.61**	**11.78**	**9.61**	0.27
	August	**6**	**21**	**8**	**35**	**33**	**70**	**0.47**	**0.53**	**7.78**	**17.44**	**9.78**	1.46
	September	**5**	**12**	**26**	**43**	**22**	**86**	**0.26**	**0.74**	**2.81**	**16.37**	**23.81**	3.07
	November	**5**	**15**	**26**	**46**	**25**	**92**	**0.27**	**0.73**	**3.40**	**18.21**	**24.40**	1.43

Table 7.4: Results of the Chi-square test for the genotype frequencies of *cry*^{HH}, *cry*^{H} and *cry*^{LL} in the Rende population cages.

Fly lines	25°C	18°C	Difference in min	F	p-value	Earliest
D. melanogaster Canton-S	11.69	11.77	0.07	$0.25_{(1,50)}$	0.618	18°C
D. pseudoobscura Flagstaff	11.45	10.67	-0.79	$17.98_{(1,59)}$	7.9e-05	18°C
w/Y; tim^{01} tim ^{mel} / tim^{01} (t28s	11.21	10.9	-0.32	$0.98_{(1,42)}$	0.328	18°C
per0, permel [2A]	11.72	12.04	0.32	$4.01_{(1,41)}$	0.052	25°C
per^{01}/Y; tim^{+}; per ^{ps} /+ (I26)	10.2	9.53	-0.68	$3.62_{(1,56)}$	0.062	18°C
per^{01}/Y; tim^{+}; per ^{ps} /+ (I20)	10.37	9.87	-0.51	$1.73_{(1,56)}$	0.193	18°C
w/Y; tim^{01}, tim ^{ps} / tim^{01} (tim19) MP	19.4	20.33	0.93	$6.3_{(1,73)}$	0.014	25°C
w/Y; tim^{01}, tim ^{ps} / tim^{01} (tim19)	6.41	8.61	2.2	$45.29_{(1,77)}$	2.6e-09	25°C
per^{01}/Y;tim^{01},tim^{ps}/tim^{01}; per^{ps}/+ (19)	10.57	7.79	-2.77	$51.22_{(1,60)}$	1.3e-09	18°C
w/Y; tim^{01}, tim ^{ps} / tim^{01} (tim21) MP	19.58	20.85	1.27	$11.02_{(1,54)}$	0.002	25°C
w/Y; tim^{01}, tim ^{ps} / tim^{01} (tim19)	6.53	8.24	1.71	$17.95_{(1,51)}$	9.4e-05	25°C
w/Y; tim^{01}, tim ^{ps} / tim^{01} (tim21)	8.86	7.81	-1.05	$4.86_{(1,50)}$	0.032	18°C
per^{+}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ (19) MP	21.86	22.38	0.52	$1.45_{(1,40)}$	0.235	25°C
per^{+}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ [19]	6.7	7.68	0.98	$11.2_{(1,47)}$	0.002	25°C
per^{+}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ [21] MP	23.01	23.41	0.4	$1.50_{(1,31)}$	0.229	25°C
per^{+}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ [21]	7.79	8.36	0.57	$1.83_{(1,39)}$	0.183	25°C

Table 7.5 : Phase of morning onset (MP) and evening peak of activity of the fly lines tested under 25°C and 18°C. Table shows the phase, the difference between the phase under two temperature conditions in minutes and also the results for the statistical analysis.

S.No	Fly lines under 25°C	Mean phase		Difference in hours	F	p-value
1	*D. pseudoobscura - D. melanogaster*	11.45	11.69	0.24	$4.14_{(1,59)}$	0.046
2	*w/Y; tim^{01} tim^{mel} / tim^{01}* (t28s)- *D. melanogaster*	11.21	11.69	0.48	$8.06_{(1,60)}$	0.006
3	*per^{0}/Y, per^{mel}/+ [2A] - D. melanogaster*	11.72	11.69	-0.03	$0.03_{(1,59)}$	0.859
4	*w/Y; tim^{01} tim^{mel} / tim^{01}* (t28s) - *per^{0}/Y, per^{mel}/+ [2A]* (31 & 30)	11.21	11.72	0.51	$7.83_{(1,59)}$	0.007
5	*w/Y; tim^{01}, tim^{ps} / tim^{01}* (tim19) - *per^{+}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ [19]*	6.41	10.57	4.16	$119.50_{(1,61)}$	< 1E-12
6	*tim 21 [w ; tim^{0},tim^{ps}] - per0; tim ps, tim0; per ps [21]*	6.53	8.86	2.33	$22.29_{(1,43)}$	0.000025
	18°C					
1	*D.pseudoobscura -D. melanogaster* 18C (31 & 21)	10.67	11.77	1.1	$25.00_{(1,50)}$	7.43E-06
2	*w/Y; tim^{01} tim^{mel} / tim^{01}* (t28s) - *D. melanogaster*	10.9	11.77	0.87	$7.62_{(1,32)}$	0.009
3	*per^{0}/Y, per^{mel}/+ [2A] - D. melanogaster*	12.04	11.77	-0.27	$2.73(_{1,32})$	0.108
4	*w/Y; tim^{01} tim^{mel} / tim^{01}* (t28s) - *per^{0}/Y, per^{mel}/+ [2A]* (31 & 30)	10.9	12.04	1.14	$10.31_{(1,24)}$	0.004
5	*w/Y; tim^{01}, tim^{ps} / tim^{01}* (tim19) - *per^{01}/Y;tim^{01},tim^{ps}/tim^{01}; per^{ps}/+ [19]*	8.61	7.79	-0.82	$6.01_{(1,76)}$	0.016
6	*w/Y; tim^{01}, tim^{ps} / tim^{01}* (tim21)- *per^{01}/Y;tim^{01},tim^{ps}/tim^{01}; per^{ps}/+ [21]*	8.24	7.81	-0.43	$1.18_{(1,58)}$	0.282

Table 7.6: Comparison of phase of activity among the different fly lines under 25°C and 18°C. Table shows the phase, the difference between the phase under two temperature conditions in minutes and also the results for the statistical analysis.

Table Analyzed	Data 1		
One-way analysis of variance			
P value	0.009		
P value summary	**		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	6		
F	3.503		
R square	0.2715		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	243.2		
P value	< 0.0001		
P value summary	***		
Do the variances differ signif. (P < 0.05)	Yes		
ANOVA Table	SS	MS	
Treatment (between columns)	3.431	0.6863	
Residual (within columns)	9.207	0.1959	
Total	12.64		
Newman-Keuls Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	Summary
w; tim^{01}, tim^{ps} / tim^{01} (tim21)) *vs* ***w/Y, tim^{01}***	-0.693	Yes	*
w; tim^{01}, tim^{ps} / tim^{01} (tim21) *vs* ***per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ (19);***	-0.068	No	ns
w; tim^{01}, tim^{ps} / tim^{01} (tim21) *vs* ***D. melanogaster***	-0.0121	No	ns
w; tim^{01}, tim^{ps} / tim^{01} (tim21) *vs* ***w; tim^{01}, tim^{ps} / tim^{01} (tim19)***	-0.0058	No	ns
w; tim^{01}, tim^{ps} / tim^{01} (tim21) *vs* ***per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ (19)***	-0.0030	No	ns
per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ (21) *vs* ***w/Y, tim^{01}***	-0.6899	Yes	*
per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ (19) *vs* ***per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ (21)***	-0.065	No	ns
w; tim^{01}, tim^{ps} / tim^{01} (tim19) *vs* ***D. melanogaster***	-0.0091	No	ns
per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ (21) *vs* ***w; tim^{01}, tim^{ps} / tim^{01} (tim19)***	-0.0027	No	ns
w; tim^{01}, tim^{ps} / tim^{01} (tim19) *vs* ***w/Y, tim^{01}***	-0.6872	Yes	*
w; tim^{01}, tim^{ps} / tim^{01} (tim19) *vs* ***per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ (19)***	-0.062	No	ns
w; tim^{01}, tim^{ps} / tim^{01} (tim19) *vs* ***D. melanogaster***	-0.0063	No	ns
D. melanogaster *vs* ***w/Y, tim^{01}***	-0.6808	Yes	**
D.melanogaster vs per^{01}/Y; tim^{01}, tim^{ps}/tim^{01}; per^{ps}/+ (21)	-0.056	No	ns

Table 7.7: Results of data analysis for *tim* qPCR (analysis performed using "Prism")

Summary of all Effects; design: (pdp.sta)						
1-TIME						
	df Effect	MS Effect	df Error	MS Error	F	p-level
1	5	0.038736	12	0.003323	11.65696	0.000287
Newman-Keuls test; PROTIEN (pdp.sta)						
Probabilities for Post Hoc Tests						
MAIN EFFECT: TIME						
	{1}	{2}	{3}	{4}	{5}	{6}
	.8889810	.6791800	.7082761	.9346986	.8590502	.9388671
ZT 0 {1}		0.003895	0.006301	0.350716	0.536932	0.555496
ZT4 {2}	0.003895		0.548172	0.001298	0.006498	0.001536
ZT8 {3}	0.006301	0.548172		0.002223	0.007748	0.00285
ZT12 {4}	0.350716	0.001298	0.002223		0.280313	0.931
ZT16 {5}	0.536932	0.006498	0.007748	0.280313		0.367326
ZT20 {6}	0.555496	0.001536	0.00285	0.931	0.367326	

Table 7.8: Statistical analysis of PDP protein level in the *per*^{01},*tim*$^{+}$ flies using "Statistca 5".

Chapter 8: References

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