NATURAL ENTRAINMENT OF THE *DROSOPHILA MELANOGASTER* CIRCADIAN CLOCK

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Abstract

In the laboratory, the circadian clock of *Drosophila* has been studied by analysing locomotor activity rhythms in either rectangular light-dark cycles in constant temperature or temperature cycles in constant light. In this way, the molecular components of the circadian clock have been characterised and their entrainment to the environment has been dissected. In nature, however, light-dark cycles and temperature cycles occur together and represent a dynamic environment. The aim of this work is to study the entrainment of *D. melanogaster* to natural conditions.

The locomotor activity profile of wild-type flies in natural conditions has been described in detail. In particular, the position of the morning and evening components have been analysed with relation to the environment. It is found that temperature comprises the principal environmental cue that determines the timing of both morning and evening behaviour. Morning behaviour appears to be more responsive to the dynamic environment of the morning, apparently responding to light intensities as low as 0.0006 lux. Apart from wild-type flies, mutants for the canonical clock genes (per^{01}, tim^{01}, $Clock^{jerk}$, $per^{01}tim^{01}$) as well as the output and input genes Pdf^{01} and cry^{0}also show temporally regulated behaviour in natural conditions, suggesting the presence in 'clockless' flies of a residual timing mechanism. This work has revealed that clockless mutants can affect different parts of the circadian behavioural profile, and that GAL4 mediated disruption of the network of circadian neurons in the brain, can have unanticipated phenotypes.

Preliminary molecular work has also revealed that the effect of light is dominant over that of temperature on the cycling of TIM. A de-coupling of this cycling among different neuronal groups in the brain is observed in natural conditions. Finally, an antibody has been generated against CRY, the dedicated circadian photoreceptor.

Acknowledgements

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Chapter 1: Introduction

Most organisms, from simple prokaryotes to complex eukaryotes, exhibit daily rhythms in behaviour, physiology and biochemistry. These rhythms have a period of approximately 24 hours and are termed circadian rhythms (*circa* = about, *diem* = day). Circadian rhythms are endogenous in nature but are kept in synchrony with the environment through various external cues like light and temperature. This property of synchronisation of the clock is termed 'entrainment' and is an integral part of all circadian rhythms. However, these rhythms also persist in the absence of these external cues (constant conditions) (Pittendrigh, 1967) and this is also characteristic of the circadian clock in all organisms. Another property of the circadian clock is to maintain this 24 hour rhythm in a variety of physiologically viable temperatures, a phenomenon called temperature compensation.

It has been known for a while that the presence of a functional circadian clock which is in synchrony with its environment is of advantage to the overall fitness of an organism. This was first exhibited in *Drosophila* (Sawyer *et. al.*, 1997) and cyanobacteria (Ouyang *et al.*, 1998) and most recently in the pitcher-plant mosquito *Wyemyia smithii* (Emerson *et al.*, 2008). The clock has also been shown to effect immunity in *Drosophila melanogaster*, as measured by its susceptibility to bacterial infection (Shirasu-Hiza *et al.*, 2007) as well as in humans in which sleep rhythms modulate immunity (Majde and Krueger, 2005).

As shown in figure 1.1, the endogenous circadian clock is composed of an input pathway, a central core and an output pathway. The input pathway transmits information about the external environment to the core oscillator. This is comprised of a network of genes, their mRNA and protein products, some of which oscillate with a 24-

hour period. The network of proteins acts upon downstream genes that give rise to rhythmic outputs.

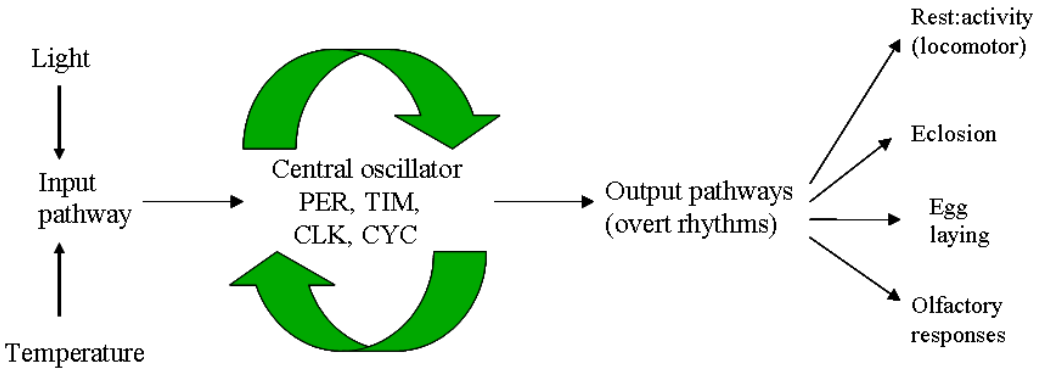


Figure 1.1: Basic mechanism of the circadian clock. It involves an input pathway (light and temperature), a central driving oscillator comprising of core clock genes and output pathways (behavioural rhythms).

1.1 Evolution of the clock

It has been speculated that the circadian clock evolved in order to enable the organism to avoid the damaging effects of the ultra-violet component of light on DNA. This can be observed in the form of daily vertical migration between the surface of water and the bottom of the ocean by zooplankton (Reviewed by Gehring and Rosbash, 2003; Tauber *et al.*, 2004). The fact that circadian rhythms have been observed in a variety of organisms ranging from fungi to humans reinforces the genetic and universal nature of the circadian clock. Clock mutants in *Chlamydomonas reinhardi* were identified by observing defects in phototactic growth assays (Bruce, 1972). In the cyanobacteria *Synochochoccus* circadian rhythms were identified in luciferase-imaging assays of mutants (Kondo *et al.*, 1994). The fungus *Neurospora crassa* exhibits circadian patterns of conidiation which are under the control of clock genes (Feldman

and Hoyle, 1973). In fact, *frq*, a core clock gene in *Neurospora* was one of the first clock genes to be identified and cloned (McClung *et al.,* 1989). In *Arabidopsis thaliana*, the classic plant model, there is an observable circadian rhythm in leaf movements (Millar *et al.*, 1995). In animals, the fruit-fly *Drosophila melanogaster* exhibits a circadian pattern of eclosion (emergence of adult from pupa) at the population level and locomotor activity of each individual fly (Konopka and Benzer, 1971). Mice too show a behavioural (amongst others) circadian phenotype of wheel-running activity (Vitaterna *et al.*, 1994). Since their discovery, the basic mechanism of the input pathways, core oscillators and output pathways have been elucidated in these organisms and found to retain some similar properties across these diverse species.

1.2 Clock Outputs

The circadian clock in *Drosophila melanogaster* manifests itself as a number of rhythmic phenotypes such as eclosion (emergence of adult flies from pupa) (Konopka and Benzer, 1971), locomotor activity, olfactory (Krishnan *et al.*, 1999) and egg-laying rhythms (Allemand and David, 1984). Of these, the most commonly used phenotypes are eclosion of populations and locomotor activity of individual flies. In fact, the first genetic link to behaviour was established using the eclosion rhythm of *D. melanogaster* as the phenotype (Konopka and Benzer, 1971).

In *Drosophila*, numerous independent microarray studies of fly heads have shown that a minority of gene transcripts cycle with a period of approximately 24 hours (Keegan *et al.*, 2007; McDonald and Rosbash, 2001; Ueda *et al.*, 2002; Wijnen *et al.*, 2006). These genes are believed to be directly, or indirectly, under the control of clock genes. Genetic screens have already revealed the function of a number of genes that lie downstream of the central oscillator. One such gene, *lark*, is important for gating the

eclosion of flies (Newby and Jackson, 1993). LARK is known to be an RNA-binding protein and it also plays a vital role in development because homozygous mutants are lethal. However, this gene does not seem to play any role in locomotor activity rhythms (McNeil *et al.*, 1998). Recently, a gene, *E74*, downstream of *lark* has been identified which regulates circadian eclosion rhythms (and not locomotor activity rhythms) in a manner similar to *lark* mutants (Huang *et al.*, 2007). This reinforces the fact that there are possibly different pathways stemming from the central oscillator that are responsible for different rhythmic outputs.

Another downstream clock gene, *ebony*, seems to have no role in eclosion but effects locomotor activity rhythms (Newby and Jackson, 1991). It does this by functioning as a β-alanyl-biogenic amine synthase in glial cells. Glia, in turn, are known to effect neuron excitability thereby effecting motor neurons responsible for locomotor activity (Suh and Jackson, 2007). Another gene, *takeout*, affects the circadian control of feeding behaviour along with locomotor activity (Sarov-Blat *et al.*, 2000). The most extensively studied output gene in *Drosophila* is *Pdf* (Renn *et al.*, 1999) (described in section 1.5). Flies mutant for this gene show altered locomotor activity rhythms and a complex cycling of the core clock gene *period* in the clock neurons in the brain (Yoshii *et al.*, 2009).

1.3 Molecular clock of *D. melanogaster*

The first clock gene to be identified in *Drosophila* was termed *period* (*per*) (Konopka and Benzer, 1971). It was discovered in a mutagenesis screen of rhythmic eclosion behaviour. Mutants for this gene have either a 19 h rhythm (per^S) or a 28 h rhythm (per^L) or they are completely arrhythmic (per^{01}) in constant darkness (DD). All these mutations were mapped to the same locus on the X-chromosome. Further

molecular analysis revealed that per^S involves a single nucleotide substitution in the fifth *per* exon that changes a serine residue into an asparginine. The allele per^L is also a single nucleotide substitution in the third exon of *per* that results in a valine to aspartic acid amino acid substitution (Yu *et al.*, 1987; Baylies *et al.*, 1987; Colot *et al.*, 1988). The per^{01} phenotype is caused by a substitution *(C464T)* that changes a glutamine into a stop codon (Yu *et al.*, 1987).

The next clock gene to be identified (Sehgal *et al.*, 1994) and cloned (Gekakis *et al.*, 1995 ;Myers *et al.*, 1995) was termed *timeless* (*tim*). Soon after its identification, it was discovered that TIM protein is degraded in the presence of light (Hunter-Ensor *et al.*, 1996; Lee *et al.*, 1996; Young *et al.*, 1996; Zeng *et al.*, 1996). It was also found that the mRNA transcript as well as the protein products of *per* and *tim* cycle with a robust rhythm of approximately 24 hours driven by a negative feedback loop (Hardin *et al.*, 1990; Hardin, 1994; Zerr *et al.*, 1990).

The transcription of *per* and *tim* is promoted by two transcription factors, CLOCK and CYCLE (Allada *et al.*, 1998; Rutila *et al.*, 1998). Both these proteins have a PAS domain (P=PERIOD, A=ARNT, S=SINGLE-MINDED) known to be involved in protein-protein interactions. They also have a basic Helix-Loop-Helix (bHLH) domain responsible for DNA binding. These transcription factors bind to E-box regions *(CACGTG)* found in most clock genes (reviewed by Kyriacou and Rosato, 2000) including *per* and *tim*. The *per* and *tim* transcripts are then translated into their respective proteins. These proteins heterodimerise (Gekakis *et al.*, 1995) and translocate back into the nucleus (Curtin *et al.*, 1995).

In the nucleus, the PER-TIM complex inhibits the transcription of their own genes by acting upon the CLK-CYC complex (Darlington *et al.*, 1998). In particular, the DNA-binding property of the CLK-CYC complex is compromised (Lee *et al.*,

1999). It is believed that PER alone can inhibit the functioning of the CLK-CYC complex (Rothenfluh *et al.*, 2000). The translocation of the PER-TIM heterodimer has recently been questioned by fluorescence resonance energy transfer (FRET) studies which indicate that the proteins dissociate before their movement to the nucleus (Meyer *et. al.*, 2006).

Thus PER and TIM regulate their own transcription through this negative feedback loop (Glossop *et al.*, 1999). CLK and CYC promote the transcription of two other genes, *vrille* (Blau and Young, 1999) and *Pdp1ε* (Lin *et al.*, 1997). In turn, *vrille* is known to inhibit and *Pdp1ε* to promote clock transcription (Cyran *et al.*, 2003). Due to this, the levels of the *Clk* transcript also cycle (Glossop *et al.*, 1999) even though the CLOCK protein is constitutively expressed in circadian neurons (Houl *et al.*, 2006). This constitutes another negative feedback loop interlocked with the PER-TIM loop described above. Recently, however, there has been some uncertainty about the role of *Pdp1ε* in the clock because a reduction in PDP1ε by RNA-interference or its over-expression does not disrupt oscillator function but only behavioural rhythmicity (Benito *et al.*, 2007). On the other hand, more recent work has re-enforced the isoform specific role of *Pdp1ε* in the core oscillator (Zheng *et al.*, 2009). Another recently identified gene whose expression appears to be mediated by CLK is *clockwork orange* (*cwo*). It has been identified by both microarray studies (Lim *et. al.*, 2007) and an RNA interference screen (Matsumoto *et. al.*, 2007). *cwo* is rhythmically expressed and is found to be localised in the master clock of the brain (Kadener *et. al.*, 2008). It has been shown to function as both a transcriptional activator and repressor and mediates the amplitude of rhythms in flies (Matsumoto *et. al.*, 2007; Richier *et al.*, 2008).

Along with the transcripts, the protein levels of PER and TIM also oscillate, albeit with a significant delay compared to the transcripts (Curtin *et al.*, 1995). This

delay is partly due to the presence of a cytoplasmic localisation domain (CLD) (Saez and Young, 1996). The other mechanism responsible for this delay in accumulation is phosphorylation and degradation of PER and TIM. PER is known to be phosphorylated by the protein DBT encoded by the gene *double-time* (*dbt*) which is the homologue of mammalian casein kinase Iε (Price *et al.*, 1998). Casein Kinase 2α (CK2α) is also a kinase which is involved in phosphorylation of PER and TIM but its mechanism of action is still largely unknown (Akten *et al.*, 2003; Lin *et al.*, 2002; Lin *et al.*, 2005; Meissner et al., 2008). Hyper-phosphorylated PER is then degraded by an F-box protein called SLIMB (Grima *et al.*, 2002; Ko *et al.*, 2002). On the other hand, phosphorylated PER is stabilised by protein phosphatase 2A (PP2A). The other clock protein, TIM is phosphorylated and targeted for degradation by SGG, encoded by *shaggy*, the *Drosophila* homologue of the vertebrate *Glycogen synthetase kinase* (*Gsk*) (Martinek *et al.*, 2001). Hyperphosphorylated TIM is in turn degraded by another F-box protein called JETLAG in a light-dependent manner (Koh *et al.*, 2006). TIM is de-phosphorylated and stabilised by protein phosphatase 1 (PP1) (Fang *et al.*, 2007). TIM degradation in the presence of light mentioned above also contributes to a delay in protein accumulation. These cycling mRNAs and proteins along with the negative feedback loops described above constitute the transcription-translation oscillator (TTO) which lies at the core of the circadian oscillator in *Drosophila* (Reviewed by Zheng and Sehgal, 2008).

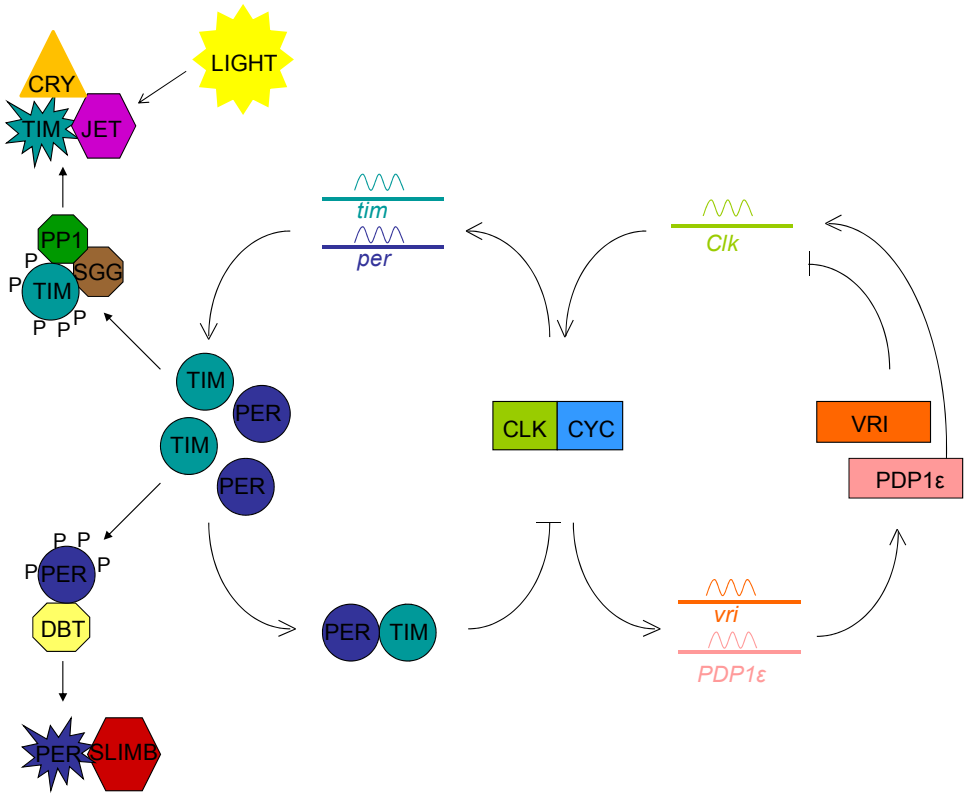


Figure 1.2: Diagrammatic representation of the central oscillator of the circadian clock of *D. melanogaster*. It is composed of two interlocked feedback loops with CLK and CYC at its core which in turn control the expression of clock genes *per*, *tim*, *vrille* and *Pdp1ε* (details in text).

1.4 Molecular clock in other organisms

The transcription-translation oscillator (TTO) model (along with various post-translational modifications) described above for the core of the *Drosophila* circadian clock is believed to be conserved across species. However, in micro-organisms, numerous circadian systems have been shown to function even without rhythmic transcription. One such example of this phenomenon is seen in the form of 24-h phosphorylation rhythms of core clock proteins (KaiA, KaiB and KaiC) in cyanobacteria which are retained *in vitro*. They do not require any rhythmic transcription of these clock genes and thus challenge the widely accepted TTO model of the circadian clock (Nakajima *et al.*, 2005). *Acetabularia* exhibit circadian rhythms in

photosynthesis and these rhythms are retained even when the cells are enucleated thus eliminating the possibility of them being generated by a transcriptional-translational oscillator (Mergenhagen and Schweiger, 1975). Along with the examples stated above, some work in the fungus *Neurospora* has reported conidiation rhythms that persist in mutants for genes that constitute the *frq*-TTO described for this organism (reviewed by Lakin-Thomas, 2006). These rhythms are believed to be generated by FRQ-less oscillators (FLOs) (reviewed by Iwasaki and Dunlap, 2000).

The mammalian core oscillator, of the other hand, is strikingly similar to that described for *Drosophila*, albeit more complex (reviewed by Hastings *et al.*, 2008). For instance, there are four *period* homologues in mammals – *mPer1*, *mPer2* and *mPer3* and *mPer4* (Zylka *et al.*, 1998; Clayton *et al.*, 2001), although *mPer4* is a pseudo-gene (Gotter *et al.*, 2001). There is only one *mTim* but it does not seem to encode the binding partner of any of the mPERs (Gotter *et al.*, 2000). Instead, this role is played by the product of mammalian *cryptochrome*, encoded by the paralogues *mCry1* and *mCry2*. In *Drosophila*, *cry* is the dedicated circadian photoreceptor (Stanewsky *et al.*, 1998) (described in section 1.6.1). The positive elements of the clock seem to be similar between flies and mammals. *mClock* binds to *Bmal1/MOP3* (homologue of *dcyc*) to promote the transcription of the *mPer* and *mCry* paralogues. *Rev-Erbα* is believed to be the mammalian analogue of *vrille* as it inhibits *mClock*. These genes in mammals constitute the transcription-translation oscillator that lies at the core of the mammalian circadian clock.

1.5 Where is the clock located in *Drosophila*?

In *Drosophila* and mammals, independent circadian clocks have been identified in most parts of the body (Glossop and Hardin, 2002). However, they all have a master

clock which is believed to synchronise these numerous peripheral clocks. In mammals, including humans, this master clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus region of the brain (Ralph *et al.*, 1990).

In *Drosophila*, the developmental larval stage displays the presence of an endogenous circadian clock, like adults, as measured by *per* mRNA cycling (Saez and Young, 1988). An independent clock has also been identified in the prothoracic gland of the pupa of flies. In adult flies, clocks have been isolated in numerous tissues such as the antennae (Tanoue *et al.*, 2004), malphigian tubules (Giebultowicz *et al.*, 2000; Hege *et al.*, 1997) and photoreceptors (Cheng and Hardin, 1998). A peripheral clock has recently been identified which is responsible for circadian cuticle deposition in the furca in the epidermis of adults (Ito *et al.*, 2008). Apart from these peripheral clocks, a 'master' clock that controls locomotor behaviour is located in the fly brain.

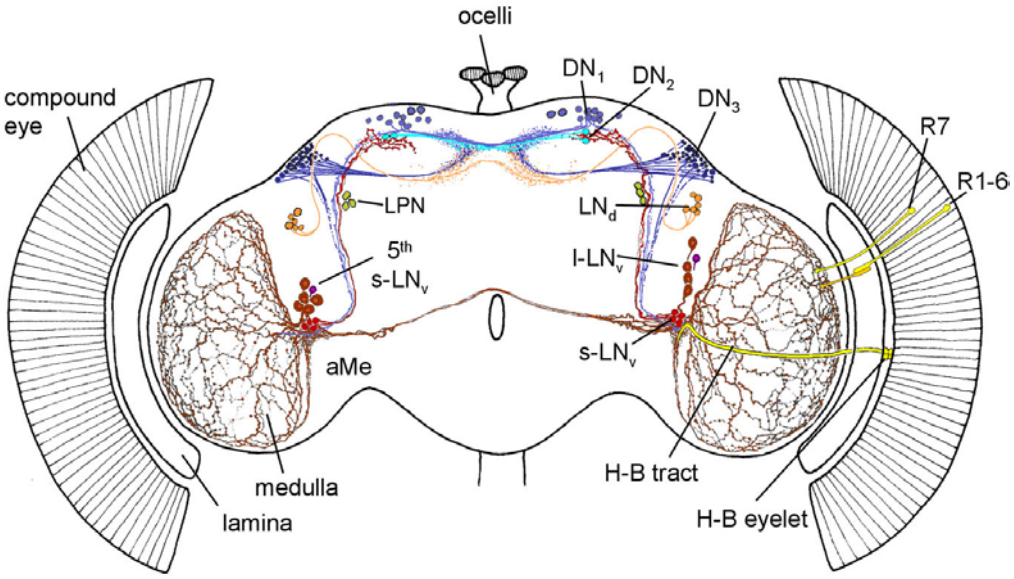


Figure 1.3: Diagrammatic representation of the fly brain showing the location of the different classes of clock neurons. The compound eyes and ocelli are also indicated in the figure (figure taken from Helfrich-Forster *et al.*, 2007).

In particular, approximately 150 neurons in the fly brain express the core clock genes *per* and *tim* (Helfrich-Forster, 2005). These are largely classified into two groups, dorsal and lateral neurons, based on their location in the brain (reviewed by Dubruille and Emery, 2008). The lateral neurons are further divided into three sub-groups, the lateral posterior neurons (LPNs), the ventral lateral neurons (LNvs) and the dorsal lateral neurons (LNds). The ventral lateral neurons are further classified, based on their relative size, into small and large ventral lateral neurons (sLNvs and lLNvs respectively). The dorsal neurons, on the other hand, are divided into three groups based on their relative positions and are called the DN1s (approximately 17 in number), the 2 DN2s and the large group (more than 40) of DN3s. Amongst the DN1s, two are anterior in location (DN1a) and the remaining are posterior (DN1p). The two anterior DN1s are the adult versions of the larval circadian neurons. Since the classification of clock neurons is currently based on their location, all the neurons within each group do not necessarily have the same functions (described later). Rather, these neurons seem to be organised into a complex network in which different groups of cells communicate with each other to manifest circadian rhythms of locomotor activity (Nitabach, 2005; Peng *et al.*, 2003).

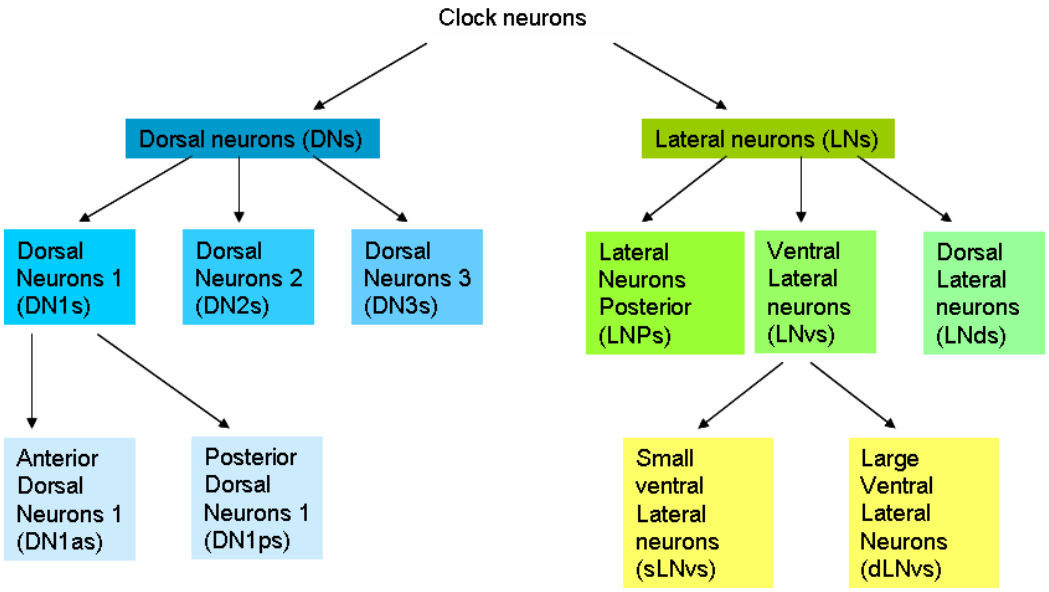


Figure 1.4: Diagrammatic representation of the classification of known clock neurons in the fly brain.

The means by which different neurons communicate is not entirely understood. However, the functions of two of neuropeptides have been studied. The neuropeptide Pigment Dispersing Factor (PDF) is the homologue of crustacean Pigment Dispersing Hormone (PDH) (Helfrich-Forster, 2005). It is believed to be essential for normal functioning of the circadian clock as uncovered by mutants and cell ablation studies (Renn *et al.*, 1999). PDF is expressed in the large ventral lateral neurons and four of the five small ventral lateral neurons. These sLNVs send projections to the dorsal region of the brain (Lin *et al.*, 2004; Mertens *et al.*, 2005) which express the G-Protein Coupled Receptor (GPCR) *Han*, a PDF receptor (Hyun *et al.*, 2005; Lear *et al.*, 2005). The arborisations of these projections change their morphology in a circadian manner (Fernandez *et al.*, 2008). This structural plasticity is a possible mechanism involved in generating circadian locomotor behaviour. PDF appears to cycle in the small cell termini of ventral lateral neurons and this cycling is lost in circadian mutants (Park *et*

al., 2000). However, a lack of PDF cycling does not result in any aberrations of fly locomotor activity (Kula *et al.*, 2006).

A neuropeptide called IPNamide has also recently been identified in circadian neurons (Shafer *et al.*, 2006). It is expressed in the anterior DN1s and represents another putative signalling factor still to be characterised. In fact, it is already known that circadian locomotor activity of *Drosophila* is controlled by multiple neuropeptides (Taghert *et al.*, 2001). For example, *Drosophila* neuropeptide F (NPF) is expressed in some LNds and all 4 PDF-expressing sLNvs (Lee et *al.*, 2006; Johard et *al.*, 2009). Another neuropeptide called ion transport peptide (ITP) has been found to be expressed in one LNd and the 5th non-PDF-expressing sLNv (Johard et *al.*, 2009). Other neurons in the brain also affect the overt behaviour of locomotion, either directly or through the clock network. These neurons also produce dopamine and it has been demonstrated that mutations in the *Drosophila* dopamine-2 like receptor genes (D2R) significantly reduce locomotor levels, which can be rescued pharmacologically (Draper *et al.*, 2007). The mushroom bodies (MBs), which are known to be responsible for behaviours involving learning and memory (Zars, 2000), are also believed to affect locomotor activity. In particular, it has recently been shown that MBs suppress locomotor activity. This was established by using mutants, chemical ablation of precursor cells and the targeted expression of tetanus toxin (TNT) in the MBs themselves (Martin *et al.*, 1998). The MBs may act on locomotion through Kenyon cells (Martin *et al.*, 1998). Thus, many other clock-dependent or independent pathways affecting locomotor activity in flies remain to be characterised.

Another aspect of communication between neurons has been used to study the neuronal control of circadian locomotor activity. This involves the hyper-excitation of neuronal activity by expressing sodium channels (NaChBac) or its silencing by

expressing potassium channels (dORK) in specific clock neurons (Nitabach *et al.*, 2002; Ren *et al.*, 2001). Specifically, it has been established that electrical silencing of the sLNvs causes disruption of circadian rhythms in DD (Nitabach *et al.*, 2002). This observation (along with others described in section 1.6.1.3) enforces the pacemaker nature of these neurons. On the other hand, hyper-excitation of these neurons results in the desynchronisation of the circadian network which is manifested as complex multiple rhythms (Nitabach *et al.*, 2006). Thus, these studies also support the view that the clock neurons in the fly brain operate together as a complex network and influence each other directly or indirectly. This line of work, which involves measuring the activity of individual neurons (Krishnan *et al.*, 2008; Park and Griffith, 2006), can help unravel the mystery of how the molecular clock in the brain manifests as behavioural rhythms.

1.6 Entrainment of the circadian clock

The circadian clock described above is endogenous in nature. However, it is kept in synchrony with the external environment by cues such as light and temperature. They constitute the input pathways that feed environmental information to the core oscillator of the clock.

1.6.1 Entrainment by light: Cryptochrome and the visual system

Light constitutes one of the most important environmental cues that synchronises the clock. It transmits information to the core of the circadian clock through the visual system of the fly and also through a dedicated blue-light photoreceptor Cryptochrome (CRY) (Stanewsky *et al.*, 1998; Ishikawa *et al.*, 1999). CRY belongs to the DNA photolyase family of proteins that are responsible for

repairing DNA damage caused by ultra-violet light (Todo, 1999). It has a photolyase domain and FAD-binding domain with flavin as the primary and methyltetrahydrofolate (MTHF) as the secondary chromophore (Thompson and Sancar, 2002). In *Drosophila*, however, CRY does not repair DNA. Instead, it acts as a blue-light photoreceptor with an absorbance peak at a wavelength of 420nm (reviewed by Lin and Shalitin, 2003; Ashmore and Sehgal, 2003).

CRY is a 596 amino acid protein (Ishikawa *et al.*, 1999) and is expressed in the pacemaker neurons of the brain as well as in peripheral tissues (Emery *et al.*, 2000a). Recently, immunocytochemistry has shown that in the brain CRY is expressed in all sLNVs (including the non-PDF expressing cell), the lLNvs, about half of the LNds, the two anterior DN1s and some of the posterior DN1s. Thus, the different classes of neurons are heterogeneous with respect to CRY expression (Yoshii *et al.*, 2008).

The photoreceptor function of CRY is evident from behavioural analysis of a missense mutant, cry^b that appears insensitive to constant bright light, because unlike the wild-type, these flies remain behaviourally rhythmic (Emery *et al.*, 2000b). In fact, in constant light the locomotor activity of cry^b flies splits into two components - one with a short period (<24 hours) and another with a long period (>24 hours) (Yoshii *et al.*, 2004). However, cry^b flies do entrain to light-dark cycles indicating a possible residual activity of the CRY^B protein. Recently, a null mutant for the *cry* gene (cry^0) has been engineered which retains these properties of cry^b defective flies, and so residual circadian light sensitivity in these mutants may be due to the opsin based canonical visual system (Dolezelova *et al.*, 2007).

CRY is degraded by light (Lin *et al.*, 2001). Recently, genetic screens have identified a number of components that are able to degrade CRY in a light-dependent manner. These genes are involved in proteosome complexes, regulating redox states

and signal transduction (Sathyanarayanan *et al.*, 2008). It is believed that CRY transmits light information to the clock through light-dependent interaction with the core clock proteins TIM (Ceriani *et al.*, 1999) and possibly PER (Rosato *et al.*, 2001). The CRY-TIM interaction involves another protein called JETLAG (Koh *et al.*, 2006) which is also involved in the light-dependent degradation of CRY (Peschel *et al.*, 2009). It has been demonstrated that this interaction is mediated by the carboxy-terminus of the CRY protein. This has been demonstrated using CRYΔ which lacks the C-terminal end (approximately last 20 amino acids). CRYΔ is constitutively active and binds PER and TIM in light and dark (Dissel *et al.*, 2004). This C-terminal region of CRY is extremely diverse between species while the N-terminal region is more conserved.

CRY1 and CRY2 (Hsu *et al.*, 1996) are known to be a part of the core oscillator of the mammalian circadian clock (reviewed by Sancar, 2004). As described above, in the master clock of the fly brain, CRY acts as a circadian photoreceptor. However, cry^b mutants disrupt the clock of the antennae as measured by bioluminescence in LD and DD suggesting that they may contribute to core clock function in the periphery (Krishnan *et al.*, 2001). This phenomenon was also observed in various other peripheral tissues such as legs, heads, bodies and wings (Levine *et al.*, 2002). In addition, it has been demonstrated that CRY functions as a transcriptional repressor of *Clk* in these peripheral tissues (Collins *et al.*, 2006). Thus fly CRY appears to incorporate some characteristics of its mammalian relatives.

The obvious candidate for entrainment of the *Drosophila* circadian clock to light is its visual system through retinal and extraretinal photoreceptors (indicated in figure 1.3). In support of this it has been shown that PER cycles in the visual system of the fly (Zerr *et al.*, 1990). *D. melanogaster* has 7 eyes – 2 compound eyes, 3 ocelli and 2 Hofbauer-Buchner (H-B) eyelets present as a cluster of photoreceptors under the retina.

These components of the visual system are believed to be linked to the circadian clock by neuronal connections through the small and large ventral lateral neurons which project into the optic lobes (Helfrich-Forster *et al.*, 2002). Rhodopsins are the photoreceptors of the visual system in *Drosophila*. In the phototransduction pathway, rhodopsins, when activated by light, are converted into metarhodopsins which in turn bind to G-proteins. This complex then activates Phospholipase C (PLC) encoded by *norpA* and opens two calcium permeable channels called the Transient Receptor Potential (TRP) and TRP-Like channel (TRPL) (reviewed by Hardie and Raghu, 2001; Hardie, 2001; Pak and Leung, 2003). It is believed that this cascade of events forms an essential part of the transduction of light information by the eyes to the clock. Nevertheless cry^{b} flies, lacking functional CRY, are still able to entrain to light-dark conditions, via the canonical opsin-based pathway (Stanewsky *et al.*, 1998), implying that CRY is not the only light input pathway into the circadian clock. Indeed, flies mutant for all known components of the visual pathway ($glass^{60j}$) and cryptochrome (cry^{b}) have been reported to be visually and circadian blind (Helfrich-Forster *et al.*, 2001) supporting the additional role of the visual system in circadian entrainment.

The compound eyes have a broad range of light absorbance extending into the red end of the spectrum and it has recently been shown that the rhodopsins present in them are responsible for circadian entrainment of the clock when subjected exclusively to red light (Hanai *et al.*, 2008). The compound eyes are also believed to be responsible for entrainment to extreme photoperiods (Rieger *et al.*, 2003). The light absorbance of the H-B eyelets peaks at a wavelength of approximately 480nm while cryptochrome absorbance peaks at a value of 420nm. This indicates some degree of redundancy in the molecules responsible for photoreception of the circadian clock (reviewed by Ashmore and Sehgal, 2003). It is thus believed that the different components of the visual system

cooperate with the dedicated blue-light photoreceptor cryptochrome in ensuring the entrainment of the *Drosophila* circadian clock to light.

1.6.2 Neuronal control of entrainment by light

In constant temperature of 25°C and 12 h light, 12 h dark (LD12:12) cycles male flies exhibit a bimodal pattern of activity with a morning peak at the lights-on transition and an evening peak at the lights-off transition. These peaks are preceded by anticipatory bouts of activity a few hours before the light transition that is present in wild-type flies but absent in per^{01} mutants (Grima *et al.*, 2004) (also see figure 1.5).

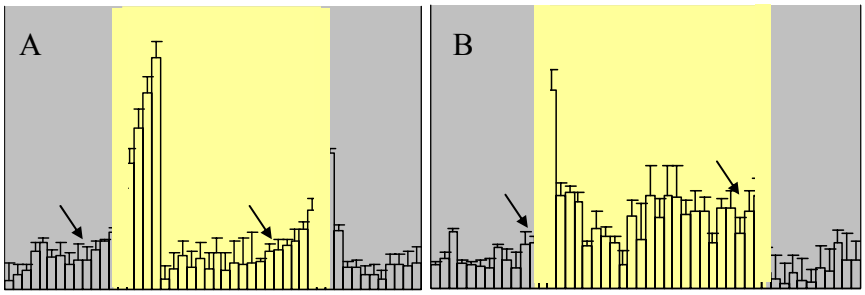


Figure 1.5: Average locomotor activity profiles in LD 12:12 and constant temperature of 25°C. (A) wild-type (WTALA) and (B) per^{01} flies. The area in yellow is the light phase and in grey is the dark phase of the LD cycle. The arrows indicate anticipatory activity in WTALA which starts a few hours before the light transition and is lost in per^{01} mutants.

By rescuing *per* function in various sets of neurons in a per^{01} background, it has been demonstrated that different neurons are responsible for the anticipatory morning and evening locomotor components (Grima *et al.*, 2004). In particular, the PDF-expressing small ventral lateral neurons (sLNVs) control the morning anticipation of lights on and are called the morning cells. On the other hand, the dorsal lateral neurons (LNds) control the evening component and are referred to as the evening cells. Another group came to similar conclusions using similar methods (Stoleru *et al.*, 2004).

However, this study included a group of dorsal neurons (DN1s) and the 5th sLNv along with the LNds in its definition of the evening cells. Despite these minor differences, both these studies confirmed the long-standing hypothesis that the *Drosophila* circadian clock is composed of a dual oscillator (Pittendrigh and Daan, 1974).

Grima *et al.* (2004) also showed that the PDF-expressing sLNvs are necessary and sufficient to drive locomotor activity rhythms in DD. This has also been established by using *disco* mutants that lack lateral neurons and are arrhythmic in constant darkness (Helfrich-Forster *et al.*, 2007). Veleri *et al.* (2003) also confirmed this finding by using a *per-luciferase* (*luciferase* reporter gene which lies under the control of the *per* promoter) construct as a real-time measure of circadian transcription and finding that the oscillations in sLNvs are robust and self-sustained. Also, recently, it has been established that the clock in evening cells is dominant in constant light conditions (LL) (Murad *et al.*, 2007; Stoleru *et al.*, 2007). Soon afterwards, it was shown that light activates behavioural output from evening cells and inhibits it from morning cells, reinforcing their functions in light and dark respectively (Picot *et al.*, 2007). Thus, it has been postulated that the morning cells are dominant in short photoperiods representing winter conditions while the evening cells play a dominant role in the longer photoperiods of summer (Stoleru *et al.*, 2007), proving appropriate seasonal circadian responses. It is evident from the studies described above that the network of neurons involved in circadian behaviour is complex.

1.6.3 Entrainment by temperature

Apart from the input of light information to the clock as described above, there are other environmental cues that are able to entrain the circadian clock. One of these *Zeitgebers* (time-givers) is temperature (Wheeler *et al.*, 1993). It is known that LL

induces arrhythmicity in wild-type flies (Konopka *et al.*, 1989). However, when LL is accompanied by temperature cycles as small as 2°C in amplitude, a circadian locomotor activity rhythm is obtained in wild-type flies (Matsumoto *et al.*, 1998). At the neuronal level, it has been established that the DN1s (a component of the evening cells), along with the LPNs, are responsible for entrainment to temperature cycles in constant light (Miyasako *et al.*, 2007). In DD, however, apart from these neurons, the PDF-expressing morning cells are necessary and sufficient for synchronisation of circadian rhythms in temperature cycles (Busza *et al.*, 2007).

Rhythmic activity in temperature cycles is accompanied by circadian rhythms in molecular oscillations of the core clock proteins PER and TIM (Yoshii *et al.*, 2005). The proteins peak at the middle to late cryophase whereas the peak of locomotor activity occurs at late thermophase under these conditions. Activity rhythms are also present in the clock mutants - per^{01}, tim^{01} and $Clock^{jerk}$ under these conditions of LL and temperature cycles. However, the mutants do not show any 'anticipatory' activity (see figure 1.6) indicating that these rhythms in wild-type flies are clock-driven.

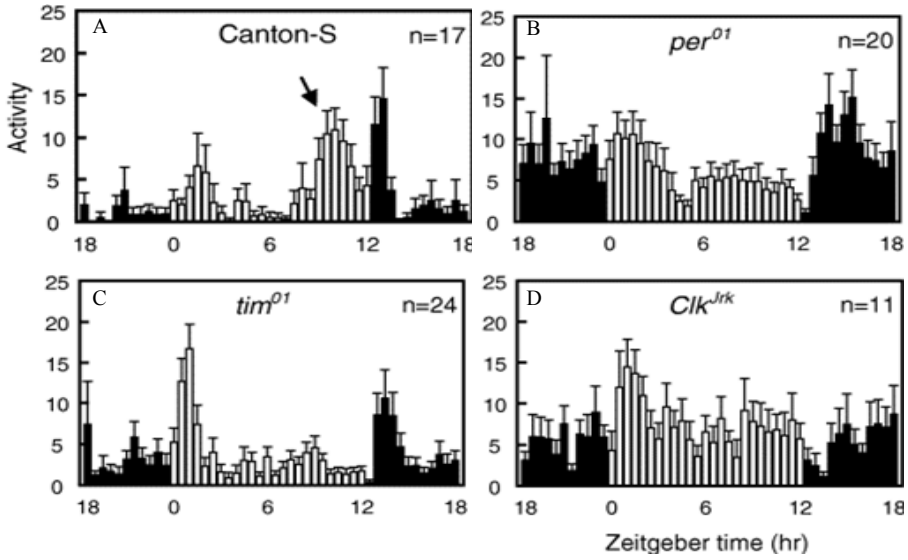


Figure 1.6: Activity profiles of wild-type (A) Canton-S and mutants (B) *per*^{01}, (C) *tim*^{01} and (D) *Clk* ^{jerk} in constant light and temperature cycles of 25°C (black activity bars):30°C (white activity bars). As indicated by the arrow, Canton-S anticipates the temperature transition while the mutants do not (figure taken from Yoshii *et al.*, 2005).

The clock-like nature of rhythms in temperature cycles is enforced by the observation that thermosensors in the antennae are not necessary for these rhythms, as determined by *luciferase* imaging (Glaser and Stanewsky, 2005). By screening with *per-luc* in temperature cycles a mutant, *no-circadian temperature entrainment* (*nocte*), was identified which was behaviourally arrhythmic and also lacked rhythms in *per-luciferase* transcription (Glaser and Stanewsky, 2005).

A few links between temperature cycles and core clock gene products have already been established. For example, it is known that heat pulses induce rapid degradation of both PER and TIM (Sidote *et al.*, 1998). Heat pulses administered in the early part of the night result in a phase delay of activity behaviour. This phenomenon is lost in *cry*^{b}, indicating that the effect is mediated by CRY, which gives this dedicated photoreceptor an additional role in temperature entrainment (Kaushik *et al.*, 2007).

At the transcriptional level, it is known that a light pulse stimulates *tim* mRNA expression at cold temperatures (18°C) and not at hot temperatures (Chen *et al.*, 2006). Another transcriptional effect of temperature on *tim* is through the alternative splicing of the last intron of the gene. Unspliced *tim* encodes a protein approximately 3.5 kD smaller than spliced *tim*. The spliced *tim* transcript is referred to as tim^{cold} but its function has yet to be demonstrated (Boothroyd *et al.*, 2007). Another effect of temperature, that on splicing of *per*, is better understood. At low temperature and short photoperiods mimicking winter conditions, the splicing of the *per* 3' untranslated region (UTR) is enhanced, leading to an early accumulation of PER protein and a subsequent advance in the evening peak of activity (Majercak *et al.*, 1999). This is presumably ecologically advantageous because it would ensure that the bulk of the fly's activity would occur during the middle of the day in winter, when the temperatures are comparatively warm. At high temperatures and long photoperiods, representative of summer, *per* splicing is inhibited and the peaks of activity are shifted towards dawn and dusk, leading to a 'siesta', presumably during the hotter parts of the day and thereby ensuring that the majority of the activity occurs during the cooler parts of the day.

norpA (no-receptor-potential-A) is a gene which encodes Phospholipase C (PLC) (Bloomquist *et al.*, 1988). Mutants for *norpA* show an earlier upswing of PER at both low and high temperatures, via enhanced *per* splicing. Thus *norpA* provides an important signalling component for temperature information into the circadian clock (Collins *et al.*, 2004; Majercak *et al.*, 2004) alongside its well established function in the phototransduction pathway described in section 1.6.1.

1.7 Natural alleles of core clock genes

It is believed that *D. melanogaster* originated in sub-saharan Africa and then spread to the Old World about 10-15Kya (David and Capy, 1988). The environmental inputs into the clock may mean that clock genes have evolved local adaptations to cope with the dramatically different demands of, for example, an African, versus a Swedish environment. One such example is provided by the natural alleles of *per* (reviewed by Costa and Kyriacou, 1998) in which the repetitive coding minisatellite, *ACNGGN,* that encodes a series of perfect Thr-Gly repeats is highly polymorphic in both length and sequence. The hexapeptide (Thr-Gly)3 forms a β-turn (Castiglione-Morelli *et al.*, 1995). The number of Thr-Gly repeats varies from 14 to 24 repeats. In Europe and Australia, the main prevalent alleles are those with 14, 17, 20 or 23 repeats (Costa *et al.*, 1992; Sawyer *et al.*, 2006). The distribution of the two most frequent alleles (17 and 20 repeats) follows a latitudinal cline with *Thr-Gly*$_{17}$ predominant in southern and *Thr-Gly*$_{20}$ in northern Europe (Costa *et al.*, 1992). Behavioural studies of flies hemizygous for these alleles revealed that *Thr-Gly*$_{20}$ maintains a slightly shorter period of about 23.7 h at a wide range of temperatures, thus exhibiting almost perfect temperature compensation, whereas *Thr-Gly*$_{17}$, shows a 24 h period at hot temperatures, but this shortens significantly as the temperature is reduced (Sawyer *et al.*, 1997). In this way, the two natural alleles are aptly suited to their northern and southern European thermal environments, with *Thr-Gly*$_{17}$ predominant in lower latitudes with comparatively stable warmer annual temperatures, and *Thr-Gly*$_{20}$ in the higher latitudes with their colder and more variable thermal environment. This seems to provide an example of balancing selection acting on the two natural alleles of *period* (Sawyer *et al.*, 1997). Indeed, neutrality-type tests on the Thr-Gly regions suggest the operation of balancing selection (Rosato *et al.*, 1997).

The repetitive region has also coevolved interspecifically with its immediate flanking regions (Peixoto *et al.*, 1993; Peixoto *et al.*, 1998; Rosato *et al.*, 1997), and transformants carrying *per* constructs with flanking regions from one species ligated to the repeat of another can completely disrupt rhythmicity, or generate extreme temperature-sensitive, yet robust periods (Peixoto *et al.*, 1998). The Thr-Gly polymorphism has also been characterised in *D. simulans* (Wheeler *et al.*, 1993) and found to be in linkage disequilibrium with its neighbouring flanking regions in natural populations suggesting balancing selection (Rosato *et al.*, 1994). Remarkably, African *per* alleles contain chimeric repetitive and flanking regions, again revealing some extreme temperature-sensitive circadian behaviour (Rogers *et al.*, 2004a; 2004b). Natural selection therefore appears to have shaped this repetitive region, both intra and interspecifically, with respect to temperature compensation of the clock.

Another example of a natural polymorphism in a core clock gene is the length polymorphism in *tim* whose 5' sequence contains two start methionine codons *(ATG)* (Rosato *et al.*, 1997). These can generate a long and short isoform of the TIM protein which differ by 23 amino acids within the *ls-tim* variant. The presence of a *G* nucleotide deletion between the two *ATG*s in the *s-tim* variant results in a stop codon 19 residues after the first methionine, and prevents the formation of the longer TIM isoform. The distribution of the two alleles also follows a latitudinal cline with *ls-tim* prevalent in southern Italy while *s-tim* predominates in northern Europe. The *ls-tim* variant is not present in sub-saharan Africa, which is where *D. melanogaster* originated (David and Capy, 1988). In fact *ls-tim* is a new allele that originated in southern Italy about 7-8000 years ago and is spreading by directional selection throughout Europe (Tauber *et al.*, 2007). The *ls-tim* and *s-tim* variants also shows a difference in the female's photoperiodic diapause at low temperatures. This protective physiological response to

oncoming winters is enhanced in *ls-tim* females, so even under long days, they move into reproductive arrest, suggesting insensitivity to photoperiods (Tauber *et al.*, 2007). Indeed *ls-tim* flies are also less responsive to a light pulse than *s-tim* flies when administered at both ZT15 and ZT21, over a range of temperatures, and among several natural populations as well as corresponding transformants (Sandrelli *et al.*, 2007). Thus a generalised photoinsensitivity in *ls-tim* may account for both circadian and diapasue phenotypes. This can probably be explained by the fact that in *ls-tim* flies, which produce both the L-TIM and S-TIM isoforms, TIM is more stable under light-dark cycles in fly heads (Sandrelli *et al.*, 2007). This may be because S-TIM interacts more strongly with CRY than L-TIM, as observed using the yeast two-hybrid system (Sandrelli *et al.*, 2007). As enhanced diapauses and reduced circadian photosensitivity would appear to be beneficial to fitness in a seasonal environment such as Europe that have exotic LD regimes compared to Africa, this may explain why the *ls-tim* allele appears to be spreading from southern Italy (Pittendrigh *et al.*, 1989; Tauber *et al.*, 2007).

The two examples illustrated above highlight how natural allelic variations in clock genes are adaptive to their local environment. They also emphasise the importance of placing the study of *Drosophila* circadian behaviour in a more 'natural' context.

1.8 Aims

Until very recently, laboratory studies of *D. melanogaster* circadian behaviour have used single males or females placed in rectangular or block cycling conditions, with lights turning on and off suddenly, usually at constant temperature. In nature, however, the light intensity increases and decreases gradually during dusk and dawn.

Also, at night there is the presence of moonlight which varies in intensity depending on the phase of the moon (from brightest at full-moon to least intensity at new-moon) and so lights may never be completely 'off' at night, depending on atmospheric conditions. Also, flies perceive a completely different spectrum of light in nature, which ranges from ultra-violet to infra-red and this kind of solar spectrum is conventionally not used in laboratory behavioural studies. The intensity of solar light is extremely high (10's or 100's of thousands of lux) whereas the range of light intensity used in most laboratory experiments is from 500 to 2000 lux. Apart from the quality and quantity of solar and moonlight perceived by flies in natural conditions, a number of other environmental stresses that flies experience and cope with in nature are also ignored in the laboratory. For example, most laboratory work is done in constant temperature (18°C, 25°C and 29°C), whereas in nature, the temperature varies gradually over the course of a day with the lowest daily temperature occurring just after sunrise and the temperature peak in the afternoon. Ambient temperature is also usually different on two consecutive days. As mentioned above, locomotor activity of *Drosophila* is studied using single males or females placed in glass tubes, and thus they are socially isolated during the course of the behavioural assay. In nature, however, we have observed that flies are rarely found alone but instead live in large numbers together.

The aims of this project are to place laboratory studies of circadian rhythms of *Drosophila* into a more ecological context. This has been done by studying the entrainment of flies to natural conditions by placing the standard equipment used to measure locomotor activity outside. This work has been done at two different latitudes - Leicester, UK (52°N38) and Padova, Italy (45°N25) because the environmental conditions vary at different latitudes. In particular, the activity profiles of wild-type flies (Chapter 3) has been analysed in detail. The effect, if any, of the presence of moonlight

in nature and in the mimicked laboratory conditions (applied as dim light during the 'dark' phase) (see also Bachleitner *et al.*, 2007; Kempinger *et al.*, 2009) on the activity profile of wild-type flies has also been explored (section 3.2.6). The environmental conditions have been closely monitored during all experiments at these two latitudes and Standard Operating Protocols (SOPs) have been established to mimic natural conditions in the laboratory. Some work has also been done to test the effect of any social factors, for example, the presence of an additional female or male, on the locomotor activity of an individual fly (section 3.2.7) (see also Fujii *et al.*, 2007). In order to examine the activity profile of wild-type and mutant flies in greater detail, an attempt has been made to distinguish the morning and evening components of locomotor activity in the wild, and examine these components in clockless mutant flies (chapters 4 and 5). At the molecular level, the cycling in the abundance of clock proteins in heads and brains has been investigated by performing western blots and immunocytochemistry (ICC) on clock proteins from flies collected after being entrained to natural conditions (chapter 6). In addition, a polyclonal antibody has been generated against cryptochrome – the dedicated circadian blue-light photoreceptor (chapter 7).

The data reported in chapters 3, 4 and 5 have been generated as part of a collaboration between the Department of Biology of the University of Padova and the Department of Genetics of the University of Leicester, granted by the European Commission (6th Framework Programme; Project EUCLOCK N° 018741). The recording of locomotor activity was performed mostly by Stefano Montelli in Padova (a PhD student at the Department of Biology, University of Padova) and then analysed jointly by him, me and Stefano Vanin. Also, as part of the collaboration, the ICC data reported in Chapter 6 has been generated together with Pamela Menegazzi (a PhD student at the Department of Biology, University of Padova).

Chapter 2: Materials and Methods

2.1 Fly stocks, media and growth conditions

2.1.1 Fly stocks

The various wild-type and mutant strains of *Drosophila melanogaster* used for locomotor activity experiments are listed below.

Wild-type strains

- *Canton-S* is a wild-type strain of *Drosophila* which has been maintained in the laboratory for a number of decades. It is used as a control in most published circadian work.
- WTALA is a collection of isofemale lines collected in Northern Italy at the approximate latitude of 46°N in September-October 2004 and was obtained from Prof. Costa (University of Padova, Italy).
- HU is collection of isofemale lines collected in Houten, Holland in August-November 2000 at the approximate latitude of 52°N (Tauber *et. al.*, 2007).

Mutant strains

Most of the mutants were created by the mutagenesis of *CantonS*. However, in the past few decades, they have been bred in the laboratory and also been through many generations of balancers. Thus, their genetic background is largely unknown.

- w^{1118} is a deletion of the *white* gene (Zachar and Bingham, 1982).

- The *per*^{01} loss of function allele is the result of a nucleotide substitution in the coding region of *per* (codon 464 in the fourth exon) resulting in a premature stop codon (Yu *et al.*, 1987).
- *per*^{S} is a missense mutation resulting in an amino acid replacement at position 589 (Yu *et al.*, 1987). *per*^{S} flies have an endogenous period of approximately 19 hours in constant darkness (Konopka & Benzer, 1971) and show an early onset of the evening peak under entrained LD conditions (Hardin *et al.*, 1992).
- *per*^{L} is a missense mutation in the PAS domain giving an endogenous period of approximately 29 hours (Konopka & Benzer, 1971).
- The *tim*^{01} loss of function mutation results from a frame-shift caused by the deletion of 64 base pairs resulting in the formation of a truncated TIM protein (Myers *et al.*, 1995).
- The *per*^{01}*;tim*^{01} strain was generated in the laboratory (Collins *et. al.*, 2005).
- *Clock*^{jerk} encodes a premature stop codon and is arrhythmic in constant darkness (Allada *et al.*, 1998).
- *Pdf* ^{01}is a nonsense mutation in the coding region of the *Pigment-dispersing factor* gene producing a premature stop codon (Renn *et al.*, 1999*)*.
- In *cry*^{0} the entire coding region for the *cryptochrome* gene was replaced with the mini-*white*$^{+}$ gene through homologous recombination (Dolezelova *et al.*, 2007).
- *cry*^{b} has a nucleotide substitution in the coding region resulting in an amino acid substitution in the flavin binding domain of the protein. This allele of *cry* is considered to be a hypomorph and has little, if any, functional CRY protein (Stanewsky *et al.*, 1998).

- The loss of function *norpA*^{p41} mutation is a 351 base pair deletion in *no-receptor potential (norpA)*, two-thirds of which is in the coding sequence. (R. Stanewsky, personal communication).
- The *w;uascycΔ103;+* responder line has a P-element insertion (on the second chromosome). CYCΔ lacks the DNA binding domain of *cycle* and thereby binds to endogenous *Clock* but does not act as a transcriptional activator thus behaving as a dominant negative and stops the functioning of the circadian clock in the cells where it is expressed (Tanoue *et al.*, 2004).
- The *ywuashiduasreaper;+;+* responder line has a P-element insertion on the X-chromosome which contains the sequence encoding *hid* and *reaper* genes. *head involution defective* (*hid*) and *reaper* are pro-apoptotic genes in *Drosophila* which induce cell death where they are expressed.
- *yw;pdfgal4;+* is a line which drives the expression of the gene of interest in PDF^{+} cells (mainly the small and large ventral lateral neurons) using the promoter sequence of *Pdf*.
- *yw;timgal4;+* is a line which drives the expression of the gene of interest in all known clock cells through the *tim* promoter.
- The *yw;timgal4;uashacry16.1* strain of flies has the promoter *tim* constitutively driving the expression of HA-*cry* (obtained from Dr. Ezio Rosato).

2.1.2 Media and growth conditions

The flies were grown in glass bottles or vials on sugar food (231.5g sugar, 50g agar, 41g dried yeast, 5l water, 50ml 20% Nipagen in ethanol). They were maintained either at 25ºC (their life cycle is approximately 10 days at this temperature) during experiments or at 18ºC (20 day life cycle) for long term storage of stocks. At both

temperatures, they were subjected to a light regime of 12 h of light and 12 h of darkness (LD12:12) wherein the lights are switched on from 8 AM till 8 PM.

2.2 Locomotor activity experiments

2.2.1 Experimental set-up

Single male flies (unless otherwise stated), 1-4 days old, were placed in small glass tubes which are 0.5 cm in diameter and approximately 8 cm long. They were filled with sugar food (see Section 2.1.2) up to about 2 cm and sealed with a small black cap (TriKinetics Inc) at one end and the other end was sealed with a cotton bung. These tubes were placed individually in channels of locomotor activity monitors (TriKinetics Inc, Waltham, MA, USA) which are used to record the activity data of single flies. There is an infra-red beam of light which runs through the monitor and a locomotor activity event is recorded every time the beam is interrupted by the fly moving along the tube. The data was recorded every 15 mins and transferred to a computer and then summed into half hour bins for further analysis.

The monitors were housed in light boxes equipped with 15 light emitting diodes (LEDs) programmed to turn on and off using timers (Müller) according to the experimental light regime. The light boxes were placed into incubators (Scientific Laboratories Supplies Ltd) that maintained the experimental temperature. The LEDs of each light box were electrically wired such that they could be turned on as 3 sets of 5 LEDs each if so required. This was especially important when moonlight was mimicked in the laboratory. For this, one row of LEDs was left on at minimum intensity during the ‘dark’ phase of the LD cycle. 4 of the 5 LEDs were covered using thick black

material and only one was left exposed. The exposed LED was then darkened using a black marker to minimise the amount of light emitted by it.

When experiments were being performed in natural conditions, the TriKinetics locomotor activity monitors were kept outside and connected to a computer. Even though this environment is not an exact representation of the habitat of fruit-flies, it enables us to monitor their activity in 'natural' light and temperature cycles. For this purpose, it was ensured that no artificial light reached the location where the flies were kept. Also, the monitors were protected from rain. Most importantly, the chosen location was not under direct sunlight and the monitors were placed in the shade of leaves and walls. This was found to be critical because, if direct sunlight was incident on the monitors, the infra-red beam was disrupted and false locomotor activity events were recorded. As a control, along with activity tubes containing flies, a few empty tubes were randomly distributed amongst the channels in every activity monitor. If any events were recorded in these empty tubes, the average number of these events over a 30-minute bin was subtracted from the activity of each fly before it was analysed further. In this way, the effect of any 'fake' activity was excluded from the analysis. However, if a large number of spurious counts occurred during an experiment it was excluded from the analysis of the evening component of locomotor activity.

2.2.2 Monitoring the Environment

An environment monitor (TriKinetics Inc) was used to record the ambient temperature in degrees C, light intensity in lux and humidity of the conditions during all the experiments conducted in the wild. The conversion of light intensity from Lux to the more appropriate μEinstein/m^2/sec depends on the wavelength of light and thus on

the light source itself. An approximate measure of conversion is 1000lux = 16-20μEinstein/m^2/sec.

2.2.3 Data collection and Analysis

The locomotor activity monitors were connected to a computer using a Power Supply Interface Unit (TriKinetics Inc) and the data from them was collected using the DAMSystem2.1.3 software (TriKinetics Inc). The data for the days of interest was then extracted using the DAMFileScan102 software (TriKinetics Inc). Only flies that survived till the end of the experiment were used for all subsequent analysis. Locomotor activity rhythms are routinely analysed for three parameters – period (τ), phase (φ) and amplitude (A).

The period of each individual fly was calculated by autocorrelation and also by a high resolution spectral analysis using the CLEAN algorithm (Rosato and Kyriacou, 2006). In the autocorrelation analysis, the data (collected in 30 minute bins) is shifted point by point and compared to itself to create correlation coefficients (thus called autocorrelation) which are then plotted together. As seen in figure 2.1A, for example, a wild-type fly shows an autocorrelation peak of 24 hours which appears above the 99% confidence limit level. Along with autocorrelation, the locomotor activity of each fly is also subjected to CLEAN spectral analysis. The raw data are then randomised 100 times to generate the confidence limits (Rosato & Kyriacou 2006). Flies with a single detectable peak above the 99% confidence limit (see figure 2.1B) were included in the computation of the average period of a genotype. Those with multiple peaks above the 99% confidence limit were regarded to have multiple rhythms. All others were considered to be arrhythmic. To be classified as rhythmic, a fly had to show both a significant autocorrelation and spectral analysis. The data obtained after CLEAN was

assembled together and further processed using BeFly, a collection of macros generated in the laboratory by Edward Green.

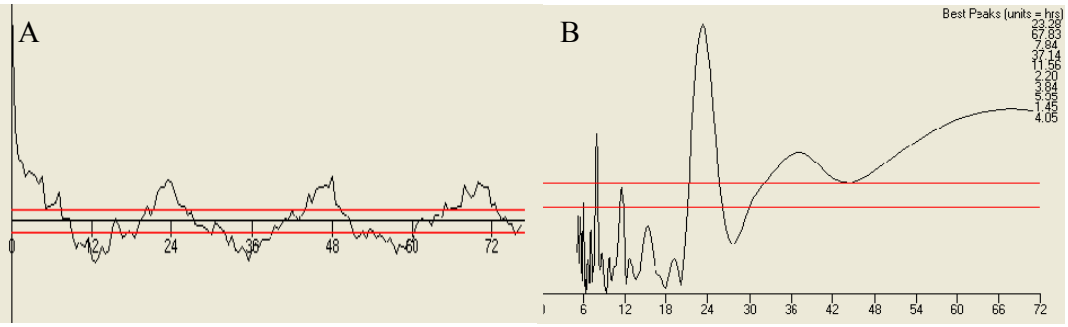


Figure 2.1: The analysis of the period of a single wild-type fly entrained to LD12:12 at 25°C and then released into constant darkness (DD). (A) The autocorrelation plot shows a single strong peak at 24 hours. (B) The result of the CLEAN algorithm also shows a single peak at 23.28 hours thereby confirming the autocorrelation result. In both figures the 95% confidence limits are shown in red. In B the upper red line represents the CLEAN 99% confidence limit.

In this work, for the experiments conducted in natural conditions, the locomotor activity of each fly was analysed using CLEAN and autocorrelation as described above in order to determine whether or not the flies entrained to these conditions. If the flies were found to be arrhythmic in this analysis, it was concluded that they were unable to entrain to the external environmental cues.

The average activity histogram (actogram) of entrained flies was plotted using Microsoft Excel® 2003. The actograms were then used to determine the parameters studied for all the natural activity data that had been collected (see figure 2.2). In particular, the onset, peak and offset of the activity burst in the morning and evening were determined using the actograms generated for each fly (details of analyses in chapters 4 and 5 respectively).

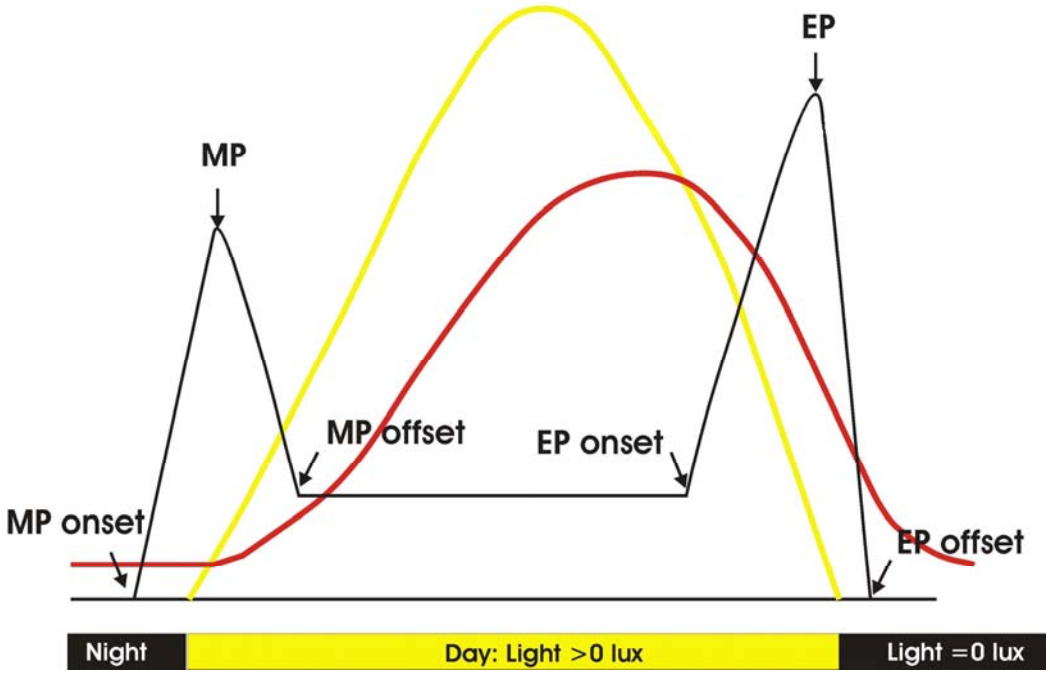


Figure 2.2: Diagrammatic representation of locomotor activity recorded in natural conditions. The activity is represented in black, temperature in red and light intensity in yellow. For the morning and evening bursts of activity, the onset, peak and offset were determined for each fly for every genotype used.

2.3 DNA extraction, amplification and visualisation

2.3.1 DNA Extraction

A single male fly was used for extraction of genomic DNA to be used in PCR reactions. Fly squashes were prepared using 50µl of freshly prepared Squishing Buffer which is composed of 10mM Tris-HCl pH 8.2, 1mM EDTA, 25mM NaCl, and 200 µg/ml Proteinase K (freshly added each time). Fly preparations were incubated at room temperature for 30 min and then at 95°C for 5 min to inactivate the Proteinase K. Stock was made of fly lines by freezing males and storing at –80°C.

2.3.2 DNA Amplification – Polymerase Chain Reaction (PCR)

All PCR reactions were done using the DYAD™ DNA Engine Peltier Thermal Cycler. The reagents used in every PCR are listed in table 2.2. The primers used, along with the components of the 5X PCR Buffer are listed in appendix 1. The standard cycling conditions for all PCR reactions are stated in table 2.3.

Table 2.2: Standard components of a PCR.

PCR Component	Concentration
DNA	Upto 1µg
11.1X Buffer	1X
Forward Primer	5pmoles
Reverse Primer	5pmoles
Taq DNA Polymerase	1Unit
Water	To make final volume of 20 or 50µl

Table 2.3: Standard cycling conditions for PCR.

PCR Step	Temperature	Time (min:sec)
Initial Denaturation	92°C	2:00
Denaturation	92°C	0:30
Annealing	Specific for primers used (ref appendix 1)	0:30
Extension	72°C	0:30 – 1:10 (dependent on size of PCR fragment)
Repeated steps 2, 3 and 4 for 35 cycles		
Final Extension	72°C	10:00

2.3.3 Restriction Digestion

The amplicons generated by PCR were subjected to restriction digestion by the appropriate enzyme obtained from New England Biolabs®. 1µl (20 units) of enzyme was used for every reaction and the appropriate buffer was used at a concentration of 1X. If necessary, 1X BSA was also added to increase the efficiency of the enzymatic

reaction. Distilled water was used to make up the final volume of the reaction (20-30µl). All digestions were incubated at 37°C overnight.

2.3.4 DNA Visualisation

DNA fragments were separated by Agarose Gel Electrophoresis. All gels were made by melting Seakem® LE Agarose or 3:1 Nuseive Agarose in 1X TBE Buffer (10X TBE contains Tris 0.89M, EDTA-Na2-Salt 0.02M and Boric Acid 0.89M). Ethidium Bromide (0.5µg/ml) was added to the melted gel and it was allowed to set. PCR product was loaded onto the gel using an appropriate amount of 5X Gel Loading Buffer (consisting of Ficoll and Bromophenol Blue in 5X TBE buffer) to make a final concentration of 1X. Gel electrophoresis was done using 1X TBE as running buffer and a voltage of 80-140V. The control used to determine the size of the DNA fragments was either Fullranger 100bp DNA ladder (Norgen) or λ DNA (digested with *Hind*III) and ϕ DNA (digested with *Hae*III). The agarose gel was then visualised using a ultra-violet trans-illuminator and photographed using Gene Genius from Bio Imaging System.

2.3.5 DNA Sequencing

Plasmid DNA, purified from bacteria by doing mini-preps (see section 2.4.2.2) was sequenced using the service provided by the Protein Nucleic Acid Chemical Laboratory (PNACL) situated at the University of Leicester itself. The chromatograms of DNA sequences retrieved were analysed using Chromas Version 1.45 (available freely at http://www.technelysium.com.au/chromas.html).

2.4 Bacterial Cloning Procedures

2.4.1 Bacterial Strains, Media and Growth Conditions

Three strains of the bacterium *E. coli* were used for cloning purposes - XL1Blue, BM25.8 and BL21. They were grown in Luria Broth (LB) with continuous vigorous shaking at 250 rpm at a temperature of 37ºC (unless otherwise stated). Appropriate antibiotics were added to the media to enable selection of the bacterial strain harbouring the plasmid in use. Table 2.4 lists the antibiotics used for selecting the different bacterial strains along with their working concentrations.

Table 2.4: Working concentrations of antibiotics used to select various *E. coli* strains used.

Strain	Antibiotic	Working Concentration
XL1Blue	Tetracycline (TET)	15µg/ml
BM25.8	Kanamycin (KAN) and Chloramphenicol (CAM)	50µg/ml and 34µg/ml respectively
BL21	-	-

2.4.2 Plasmids

2.4.2.1 pGEX6p-1

pGEX plasmid constructs containing full-length *cry* cDNA and another construct with only its N-terminal portion fused to the coding region of *glutathione S-transferase* (GST) were obtained as a gift from Ralf Stanewsky (Emery *et al.*, 1998).

2.4.2.2 Plasmid purification

Plasmid DNA was extracted from bacterial cells by performing a mini-prep using a QIAprep® Spin Miniprep kit (QIAGEN).

2.4.3 Transformation

Bacterial cultures were grown in LB till they reached an optical density of 0.5 and at that point they were peletted by spinning at 2500 rpm for 15 min. The peletted cells were resuspended in ice-cold Transformation and Storage Solution (TSS) pH 6.5 which is luria broth media enhanced with 30mM Polyethylene Glycol (PEG_{3350}), 50mM $MgCl_2$ and 5% DMSO. This buffer made the cells chemically competent and suitable for transformation and the cells were then stored at -80°C.

The chemically competent bacterial cells were transformed by giving them a heat-shock at 42ºC for 2 min and then immediately placing them on ice for 2 min. Transformed cells were allowed to recover for 2 h by incubating at 37ºC and 250rpm in LB. The cells were then plated onto appropriate selection medium plates (containing antibiotic for the bacterial stain as well as the plasmid) and incubated overnight at 37ºC without shaking.

2.5 Protein Extraction

2.5.1 Bacterial Cultures

GST-CRY fusion protein over-expressed using the pGEX6p-1 plasmid was extracted from the different bacterial strains using the GST Gene Fusion System handbook (GE Healthcare). The same manual was used to attempt to perform affinity chromatography for the purification from protein extracts.

2.5.2 Drosophila Heads

Flies were collected at different time points by freezing in liquid nitrogen and stored at -80ºC and their heads were used for western blots. Heads were collected in

eppendorf tubes kept on dry ice by vortexing the frozen flies and using a sieve to separate the heads from the bodies. A volume of extraction buffer (0.1% TritonX, 10mM EDTA, 1mM DTT, 0.5mM PMSF, 10µg/ml Apsotinin, 5µg/ml Leupeptin, 5µg/ml Pepstatin in HEMG; HEMG is 0.1M KCl, 20mM HEPES pH 7.5, 50% Glycerol) which was double that of the approximate volume of heads was added to the samples and then placed on wet ice. The heads were homogenised for approximately 1 min using a sterile plastic pestle while keeping the samples on wet ice the entire time. The debris was separated by spinning the samples at 13,000 rpm for 5 min at 4ºC. The supernatant containing the proteins was retained and quantified using Bradford Reagent (Sigma-Aldrich) (see section 2.6). They were then equalised (usually to a minimum OD of 0.3) using extraction buffer before loading onto an SDS-polyacrylamide gel.

2.6 Protein Quantification

Bradford Assay

Protein samples extracted from *Drosophila* heads were quantified by mixing 1µl of sample with 200µl of Bradford's Reagent (Sigma-Aldrich) and 800µl distilled water (1:5 dilution) and incubating at room temperature for 5 min and then measuring the optical density (OD) at 595 nm using a Spectrophotometer (Eppendorf).

2.7 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Appropriate amounts of 3X loading buffer (188mM Tris HCl pH 6.8, 6% v/v SDS, 30% v/v Glycerol, 15% v/v βMercaptoethanol, 0.03% w/v Bromophenol Blue) were added to the samples and they were boiled for 3 min at 95ºC. They were then loaded onto the gel along with a pre-stained broad-range molecular weight marker

(BioRad Laboratories or New England Biolabs®). An acrylamide gel is divided into two parts – the stacking gel and the resolving gel. Their components (6% gel used to visualise TIM, 10% gel used to visualise CRY) are listed in tables 2.5 and 2.6 respectively. The gels were run using running buffer (2.5mM Tris, 0.25M Glycine, 0.1% v/v SDS) at 100V for approximately 2 h for a small gel while the large gel was run over-night (approximately 17 h) at 40V.

Table 2.5: Components of the stacking gel portion of a polyacrylamide gel

Component (Stock)	Stock Concentration	Volume used in ~10ml
Tris HCl pH 6.8	1M	1ml
SDS	20%	50µl
APS	25%	20µl
TEMED	100%	10µl
Acrylamide	30%	1.5ml
Water		7.3ml

Table 2.6: Components of the resolving gel (6% and 10%) portion of a polyacrylamide gel. A final volume of 20ml was used to make a small gel while the volume was doubled to 40ml for a large gel.

Component (Stock)	Stock Concentration	6% Gel (Volume used in ~10ml)	10% Gel (Volume used in ~20ml)
Tris HCl pH 8.8	2M	2ml	3.7ml
SDS	20%	50µl	100µl
APS	25%	80µl	80µl
TEMED	100%	12µl	12µl
Acrylamide	30%	2ml	6.6ml
Water		5.8ml	8.6ml

2.8 Visualisation of proteins

2.8.1 Coomassie Blue Staining

Gels were stained by incubating with coomassie blue (45% Methanol, 10% Acetic Acid, Brilliant Blue) overnight and destained by incubating with Destaining

Solution (45% Methanol, 10% Acetic Acid) for a couple of hours changing the solution a couple of times.

2.8.2 Western Blotting

Proteins extracted from *Drosophila* heads (see section 2.5.2) were run on an SDS-Polyacrylamide gel (see section 2.7) as described above. The stacking gel was removed from the resolving gel and discarded. The proteins on the gel were then electrically blotted onto Nitrocellulose (Whatman® Protran®) in blotting/transfer buffer (composed of 40mM Tris, 40mM Glycine, 0.375% SDS, 20% Methanol) at 400mA for approximately 2-3 h. The nitrocellulose membrane was blocked by incubating in 5% milk buffer which is composed of non-fat milk powder and TBST (composed of 300mM NaCl, 0.05% Tween, 20mM Tris) for one hour at room temperature to prevent binding of primary antibody to any non-specific proteins. After blocking, the membrane was incubated in the primary antibody over-night at 4°C (for a list of antibodies, their source and working concentrations, see table 2.7). The membrane was then washed three times for 15 min each in TBST. It was then incubated in the appropriate secondary antibody (see table 2.7) for 2 h at room temperature. The membrane was then washed again three times for 15 min each in TBST to remove any unbound secondary antibody. A chemiluminescence reaction (details below) was performed by pouring a visualisation solution containing 0.1M Tris pH 8.5 and approximately 220mM luminol, 100mM cumaric acid and 0.01% Hydrogen Peroxide onto the membrane for 1 min. The membrane was then wrapped in cling film and placed in a light-proof cassette and processed further.

The secondary antibody used is conjugated to Horse Radish Peroxidase (HRP) enzyme. Upon exposure to hydrogen peroxide, HRP catalyses a chemiluminescence

reaction between luminol and cumaric acid which can then be captured on X-Ray photographic film (Fujiflim). Depending on the strength of the reaction, the film was exposed for a certain amount of time (for example, 10 sec, 30 sec, 1 min, 10 min) and then developed using an automated developer.

Table 2.7: List of primary and secondary antibodies used in Western Blotting along with their source and working concentrations.

Antibody	Source	Animal in which it was raised	Working Concentration
ά -TIM	Prof. Rouyer	Rat	1:1,000
ά -CRY	Self	Guinea Pig	1:1,000
ά –HA	Sigma-Aldrich	Mouse	1:10,000
ά –HSP	Sigma-Aldrich	Mouse	1:10,000
ά –Rat	Sigma-Aldrich	Goat	1:8,000
ά –Guinea Pig	ABCam Ltd	Goat	1:10,000
ά –Mouse	Sigma-Aldrich	Goat	1:6,000

2.9 Immunocytochemistry (ICC)

2.9.1 Brain dissection

Flies were collected at the appropriate time points (every three hours for a 24-hour time course collected in natural conditions) and fixed in 4% Paraformaldehyde (PFA) by incubating for approximately 3 h on a rotating wheel. They were then washed three times for 15 mins each in Phosphate Buffer Saline (PBS) which is composed of 140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4. The brains of these flies were dissected under a microscope with the help of two pairs of fine forceps. A small Petri dish containing a few drops of PBS on a layer of silicon was placed under the microscope. A fly was placed in the drop of PBS and the head of the fly was held with the forceps in the right hand and the body in the left hand. The body was removed and discarded and the head retained in the right hand. The proboscis of the fly was the first

to be removed and discarded. The right forceps were then used to hold one eye through the hole created by the removal of the proboscis and the left forceps were also inserted into that hole to hold the other eye. By gently applying pressure, the two eyes were pulled apart in opposite directions to reveal the brain underneath. Before continuing, it was ensured that all the cuticle and connective tissue was removed from the surface of the brain to minimise non-specific fluorescence.

2.9.2 Incubation with buffers and preparation of slides

The dissected brains were placed into sieves which were prepared by cutting the top half of eppendorf tubes and sealing one end using a Nylon Net with 80µm open area and 32% mesh opening (Product code: PA17XX) obtained from Gandenzi, SRL, Italy. These sieves were placed in NUNC 24-well tissue culture plates with approximately 700µl of each solution. This volume allowed the buffer to enter the sieve and thereby come in contact with the brains there. The brains could then be transferred between wash-buffers and antibody solutions by moving the sieve between wells and without any direct handling of the brains themselves. All incubations were done on a shaking plate to allow constant movement of the buffer solutions.

In this manner, the brains were washed five times for 6 mins each in 0.3% PBST (PBS + 0.3% TritonX). They were then permeabilised for 10 mins in 1% PBST. They were then blocked for 2 h with 1% Bovine Serum Albumin (BSA) to minimise non-specific binding of the primary antibody. After blocking, the brains were incubated in the primary antibody which was diluted in 0.3% PBST which contained 1% BSA and also had 0.1% Sodium azide. The presence of sodium azide prevents the contamination of the antibody solution with bacteria and thereby allows the re-use of the antibody. The brains were incubated in primary antibody for the required period of time (between 3

days and 1 week) at 4°C. They were then washed five times for 6 mins each in 0.3% PBST. After washing, they were blocked again for 1 h with 1% BSA. The brains were then incubated in the appropriate secondary antibody (raised against the animal in which the primary antibody was generated) at 4°C which was conjugated with a fluorophore. The brains were again washed 5 times for 20 mins each. Table 2.8 lists the antibodies used for the immunocytochemistry experiments conducted.

Table 2.8: List of primary and secondary antibodies used for immunocytochemistry

Antibody	Source	Animal in which it was raised	Fluorophore	Working Concentration
ά –TIM	Gift from Prof. Rouyer	Rat		1:10,000
ά –CRY	Self	Guinea Pig		1:500
ά –PDF	Developmental Studies Hybridoma Bank	Mouse		1:600
ά –Rat	Sigma-Aldrich	Goat	Alexa Flour594/568	1:500
ά –Guinea Pig	ABCam Ltd	Goat	Cy2	1:500
ά –Mouse	Sigma-Aldrich	Goat	AlexoFlour488/ Cy5	1:500

Once the protein of interest had been labelled with the appropriate antibody, the brain was mounted onto slides (VWR) with a drop of mounting medium which comprises of 80% glycerol and 3% propyl gallate and covered with a coverslip of 0.1mm thickness (VWR). The slides were stored in the dark at 4°C. They were visualised as described below.

2.9.3 Visualisation of brains

The slides containing brains stained for the different antibodies were either visualised on the laser scanning Olympus FV1000 Confocal microscope or Nikon 80i Microscope with QICAN Fast Camera. Wherever possible, two images were taken for

each brain – one for the right and one for the left hemisphere. Individual images were taken of planes at different depths in order to create a Z-series for each brain. The size of the sections forming a Z-series was either 0.44μM (if using 40X objective) or 1.4 μM (if using 20X objective). The optimal microscopic settings, in particular the laser gain, amplifier gain, amplifier offset and laser intensity were determined for each experiment. These settings are described in the relevant chapters.

2.9.4 Quantification of signal

When quantifying the signal of a clock gene across different time points, in order to generate its cycling profile, the microscopic settings mentioned above were kept constant not only for all the brains of a time point but also for all the time points. The images were viewed and quantified using ImageJ Version1.42g (available freely at http://rsb.info.nih.gov/ij/). The average pixel intensity for each neuron was measured along with the signal from its corresponding background area. The final amount of signal was calculated using the formula:

Intensity = (Signal – Background)/Background * 100

2.10 Statistical Analysis

All graphs were made using Microsoft® Excel 2003 or OriginPro 8.0. All statistical analyses in this report were done using Statistica 8 (StatSoft®).

Chapter 3: Results - Wild-type flies in natural conditions

3.1 Introduction

The locomotor activity rhythm of *Drosophila* has been extensively studied in the laboratory and provides a robust phenotype for the investigation of the molecular network of the circadian clock in flies. However, the environmental conditions of rectangular light-dark cycles and constant temperature that are imposed on flies in these laboratory experiments are very different from what they experience and cope with in nature. The environment in the wild is dynamic and multiple parameters such as light intensity and temperature are constantly changing.

In the work reported in this chapter, the environmental parameters constituting a typical summer's day at both experimental locations, Leicester and Padova, have been established and an attempt has been made to mimic these standard operating protocols (SOPs) in the laboratory. Locomotor activity of wild-type flies, WTALA, has been recorded and compared in these conditions. The activity of wild-type flies was also recorded in natural conditions at the two locations and analysed in detail (see section 2.2). The effect of light and temperature on this behaviour has been described for single males. The effect of moonlight on locomotor activity has also been investigated (see also Bachleitner *et al.*, 2007; Kempinger *et al.*, 2009) to check whether conclusions drawn from laboratory experiments hold true in natural conditions. Along with this, some work has been done to study the effect, if any, of various social combinations such as male couples, female couples and male-female couples on locomotor activity in nature and in the laboratory (see also Fujii *et al.*, 2007).

3.2 Results

3.2.1 Canton-S behaves similar to natural strains in mimicked 'natural' conditions

Canton-S is a strain of flies that has been maintained in the laboratory for many decades. In order to check if this long-term laboratory rearing has had any effect on behaviour, its locomotor activity profile has been compared to the two natural strains that have collected relatively recently from the wild – WTALA and HU (see section 2.1). When the typical 'natural' conditions of a mid-summer day in Leicester are mimicked in the laboratory (LD 19:5, temperature 22:14°C), the behaviour of Canton-S (figure 3.1A) appears to be similar to both natural stains (figure 3.1B and C). However, Canton-S does show a larger amount of nocturnal activity and a more evident startle-response to the lights-off transition. Also, its morning peak under such conditions is reduced when compared to the natural strains. For these reasons, all subsequent work in natural conditions was done using the natural strains WTALA or HU and their activity profile was considered to be representative of Canton-S.

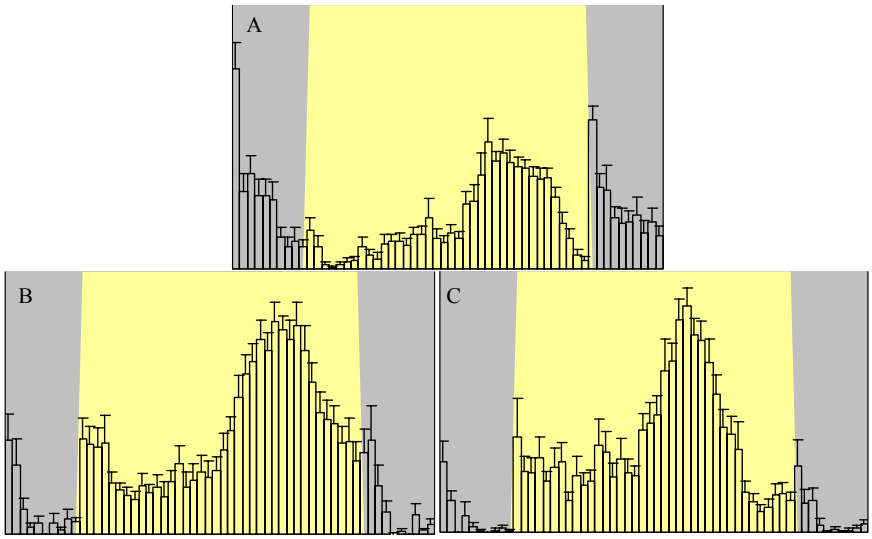


Figure 3.1: Activity profile in LD 19:5 and cycling temperature 22:14°C. (A) Canton-S (n=31), (B) WTALA (n=27), (C) HU (n=30). The light phase is in yellow and dark phase in grey. The scale on the Y-axis is the same in order to make them comparable. Canton-S appears to be different from WTALA and HU when 'natural' conditions are mimicked in the laboratory.

3.2.2 Mimicking SOPs in the laboratory

The environmental conditions constituting a typical summer's day in Leicester and Padova were established from field experiments (and termed Standard Operating Protocols – SOPs) and an attempt was made to mimic these photoperiods and temperature cycles in the laboratory. The longest photoperiod recorded for Leicester was 19:5 (referred to as 'long days') while that for Padova was 16:8 ('short days'). The ambient temperature in this season ranged from a maximum of 21°C to a minimum of 14°C (referred to as 'cold') in Leicester while in Padova the temperature range was 30:22°C ('warm'). In order to mimic the natural environmental as closely as possible, the light intensity was ramped, but due to equipment limitations, this ramp was from 0 to 600 lux with a 3-step increase which took 1.5 h. Also, the temperature in the incubator took approximately 1.5 h to increase from the night time to day time

temperature. Thus, there was also a gradual ramp in temperature cycles. These SOPs were applied to the laboratory in four combinations:

1. Long photoperiod (19:5) cold temperature (21:14°C) – Leicester (figure 3.2A)
2. Long photoperiod (19:5) and warm temperature (30:22°C) (figure 3.2B)
3. Short photoperiod (16:8) and cold temperature (21:14°C) (figure 3.2C)
4. Short photoperiod (16:8) and warm temperature (30:22°C) – Padova (figure 3.2D)

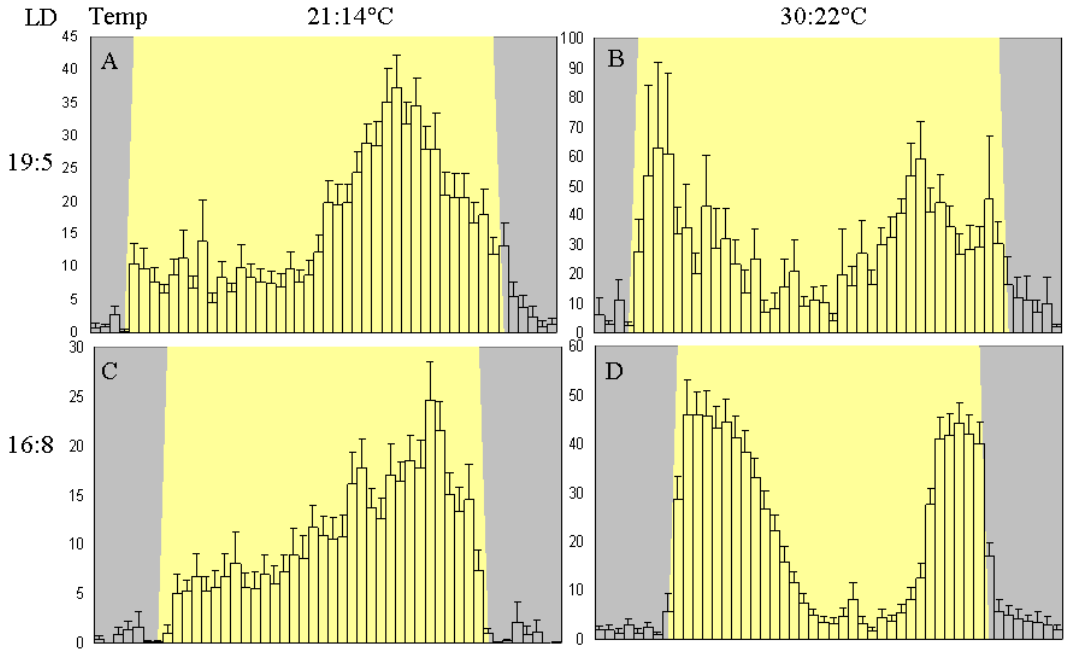


Figure 3.2: WTALA activity profile in natural SOPs mimicked in the laboratory. (A) LD 19:5, temperature 21:14°C (n=29), (B) LD 19:5, temperature 30:22°C (n=12), (C) LD 16:8, temperature 21:14°C (n=32), (D) LD 16:8, temperature 30:22°C (n=32).

At warm temperature cycles, in both long and short photoperiods (figure 3.2B and D), WTALA flies show a bimodal activity pattern with a slight shift in the phase in the evening peak as it occurs earlier in longer photoperiods than shorter ones. In cold

temperature cycles, the activity appears to be unimodal with a reduction in the morning component irrespective of the length of the day (figure 3.2A and C).

3.2.3 Flies are bimodal in hot and unimodal at cold temperatures in the wild

It has been shown in the laboratory that the activity profile of wild-type flies in LD conditions depends on the ambient temperature. In particular, the activity is bimodal at hot constant temperatures such as 25°C and 29°C with a morning peak around the time of lights-on and an evening peak which occurs around lights-off. The suppression of activity in-between these two peaks is called the 'siesta' and is believed to occur in order to enable the animals to avoid the hottest parts of the day. This is because of the observation that, at colder constant temperatures of 18°C, this siesta is reduced and the evening peak shifts towards the middle of the day (reviewed by Blau and Ruthenfluh, 1999). This phenomenon can also be observed in cycling temperatures as reported in section 3.1.

In figure 3.3, the solar light intensity has been represented in yellow, temperature in red and activity in bars. In the data obtained from Padova on 3rd July 2008 (figure 3.3A), when temperatures range from 22°C at night to 33°C in the day, the activity is bimodal with clear morning and evening peaks (indicated by arrows). However, note the suggestion of a third peak during the hottest part of the day (see below). In Leicester, in data obtained in the same season on 16th July 2008 (figure 3.3B), flies show unimodal activity with a broad evening peak (see arrow) which starts earlier in the day. This is probably because the temperature range in Leicester was from a minimum of 14°C to a peak of 19°C on this day. This observation of temperature effecting the distribution of activity in the day seems to be consistent between laboratory and natural conditions.

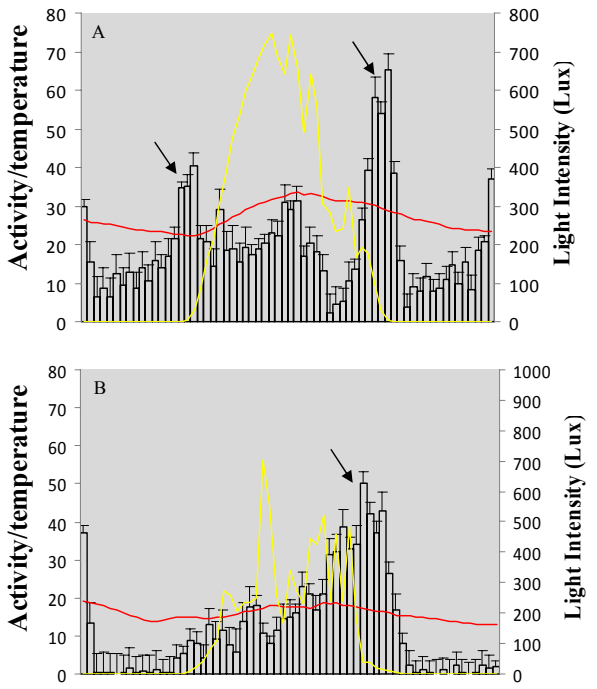


Figure 3.3: Activity profile of WTALA in natural conditions. (A) Padova - hot (n=26) and (B) Leicester – cold (n=20). The light intensity is represented in yellow, temperature in red and activity in bars. The activity appears to be bimodal (or even trimodal) in hot temperatures and unimodal in cold (see arrows for morning and evening peaks).

3.2.4 An 'Afternoon peak'

Closer inspection of the activity profile of WTALA flies in hot natural conditions showed that the middle-day activity differs from that reported in the laboratory. In the laboratory, at hot temperature, a male fly shows highly reduced activity levels (siesta) in the time between the morning and evening peaks (Majercak *et. al.*, 1999). However, in relatively hot natural conditions (data recorded on 4th July 2008, temperature ranging from 23-33°C), after the offset of morning activity, flies seem to steadily increase their activity and manifest an additional peak in the afternoon (see arrows in example fly in figure 3.4A and average in 3.4B and also figure 3.3A). The percentage of flies which have a peak in the afternoon is greater in hotter days than

relatively cooler ones such that on very hot days (average day temperature > 29°C), 100% of the flies exhibit this phenotype (see figure 3.5).

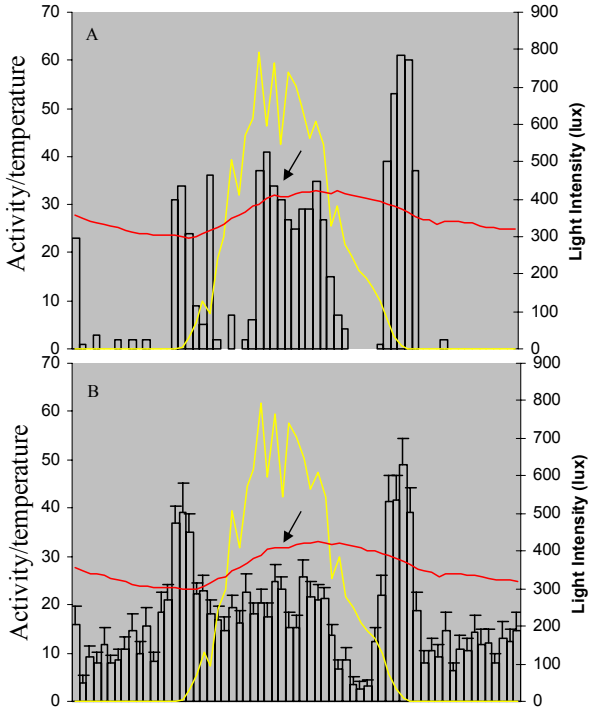


Figure 3.4: Activity profile of WTALA flies indicating the 'afternoon peak'. (A) Single male, (B) average of 26 flies. The 'afternoon peak' (see arrows) is distinct from the morning and evening peaks and occurs during the hottest part of the day.

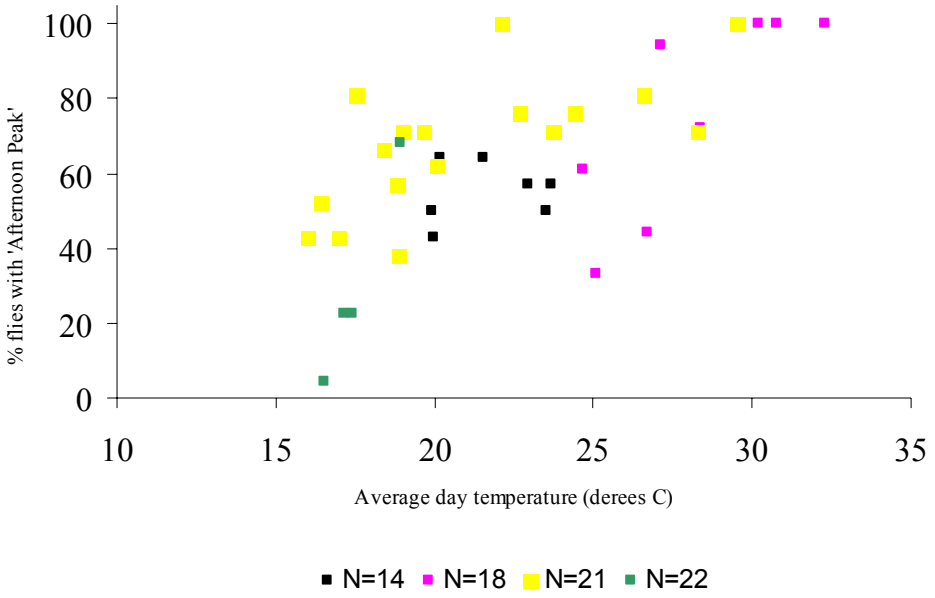


Figure 3.5: The percentage of flies with an 'afternoon peak' depends on day temperature and increases in hot days so that when the average day temperature is around 29°C, 100% flies have an 'afternoon peak'. Different colours have been used to represent the number of flies used on each day.

In order to determine the position of the siesta in natural conditions, the time in the middle of the day at which the activity of WTALA dropped to the minimum was determined for many days across different temperatures, and compared (anchored) to the time at which the temperature was at its peak. Thus, negative values would indicate that the siesta occurs earlier than the temperature peak for that day while positive values imply the presence of siesta in the later part of the day. Figure 3.6 shows that the position of the siesta in natural conditions depends on the average temperature of the day and shifts to the later part of the day during hot days ($R^2 = 0.68103$, $p < 0.0001$). This is consistent with laboratory studies which show that the position of the siesta is modulated by temperature and occurs later on hotter days primarily due to alternative splicing of *per* (Chen *et al.*, 2007; Collins *et al.*, 2004; Low *et al.*, 2008; Majercak *et al.*, 1999). However, in the laboratory, the siesta is not preceded by an 'afternoon peak' as it is in natural conditions.

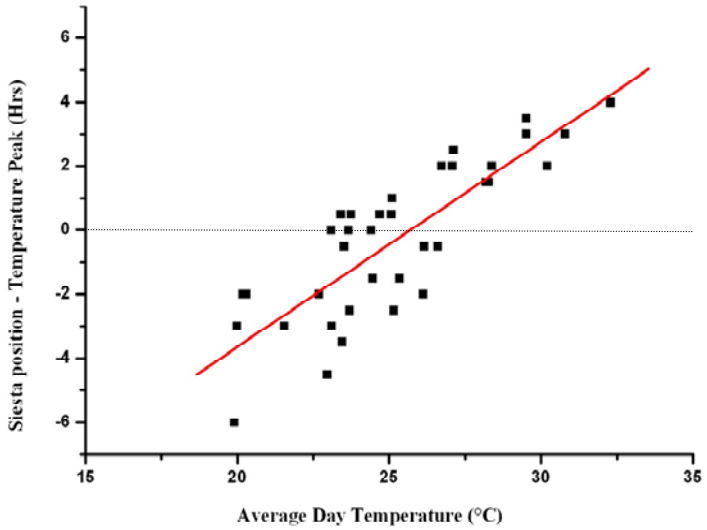


Figure 3.6: Position of siesta represented as the number of hours before or after the temperature peak that it occurs. It has been plotted against the average temperature of the day and a significant positive correlation indicates that the siesta occurs later in hotter days than colder ones ($R^2 = 0.68103$, $p < 0.0001$). For each point, the number of flies is greater than or equal to 10 ($N \geq 10$).

3.2.5 Nocturnal activity depends on temperature during the night in natural conditions

The nocturnal activity in wild-type flies has been quantified as the proportion of activity during the night compared to the total activity during the entire day (Σ night activity/ Σ night+day activity). It was found that this proportion of nocturnal activity depends partly on the ambient temperature during the night ($R^2 = 0.13167$, $p = 0.000132$) (figure 3.7). From figure 3.7, it appears that the relationship between the proportion of nocturnal activity and night temperature is more linear over the 10-20°C range of temperature. This could be because this temperature is the most physiologically viable for the fly's activity. However, a re-analysis over this range does not change this correlation between the proportion of nocturnal activity and temperature ($R^2 = 0.1498$, $p = 0.0004$).

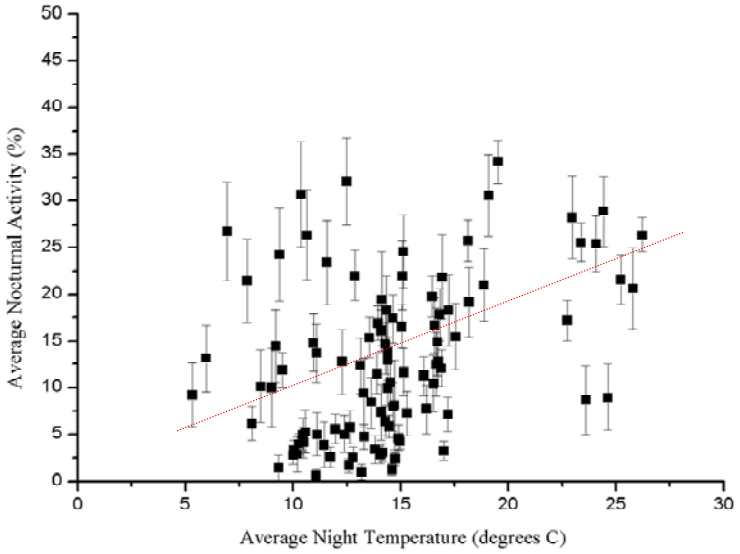


Figure 3.7: Proportion of nocturnal activity is related to the average temperature during the night (R^2 = 0.13167, p = 0.000132). For each point, the number of flies is greater than or equal to 10 (N≥10). A re-analysis of the data set between the 10-20°C range of temperature does not significantly change the correlation (R^2 = 0.1498, p = 0.0004).

3.2.6 Effect of moonlight on nocturnal activity in laboratory and natural conditions in WTALA

The activity of single male wild-type flies was recorded in the laboratory by mimicking the conditions of a typical Italian summer day (LD 16:8 and temperature cycles of 30:22°C) (figure 3.8). Half the flies were maintained in darkness during the 8 h dark phase (blue) but for the other half there was a very low level of illumination (<<1 lux; see section 2.2.1) to mimic the presence of moonlight (red). The proportion of nocturnal activity was calculated in the manner described above and compared using ANOVA. It was found that single males show a significant increase in the amount of nocturnal activity in the presence of 'moonlight' (p ~ 0). Apart from this, it was also observed that the presence of low levels of illumination in the night causes a shift in the phase of the evening peak of flies. However, this shift in peak is not consistent with the

observations of Bachleitner and colleagues (Bachleitner *et al.*, 2007) as it does not shift into the night to make flies nocturnal. Instead the evening peak occurs earlier than the flies that experienced a dark night (see arrows in figure 3.8). The direction of the shift also eliminates the possibility that the presence of moonlight is mimicking a long photoperiod as this would shift the peak to the right (Majercak *et al.*, 1999).

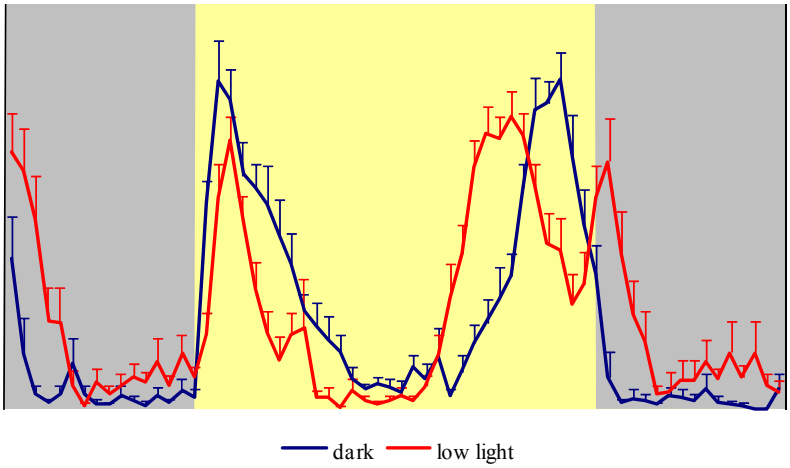


Figure 3.8: Activity profile of single males when a typical Italian summer day is mimicked in the laboratory (LD16:8, temperature 30:22°C). The activity was recorded with (n=17, red) and without (n=17, blue) a low level of illumination (<<1 lux) during the dark phase. Males show a significant increase in proportion of nocturnal activity in the presence of low levels of illumination at night ($p \sim 0$) and also shift their evening peak as it occurs earlier than in flies which experience a dark night phase.

In order to check whether the observation made in the laboratory was also true in natural conditions, the proportion of nocturnal activity of single males was computed for the nights when the illumination of the moon was either 98-100% (full moon) or 0-1% (no/new moon). The information about the phases of the moon was obtained from the United States Naval Observatory (USNO) Astronomy Application Department Online Database. In order to ensure that the day lengths were similar, the data chosen for the comparison between full-moon and no-moon nights was taken from contiguous recordings. Also, since it has been demonstrated above (section 3.2.5) that the nocturnal activity of flies in natural conditions depends on the average night temperature, this

parameter was also taken into consideration during the analysis. The regression lines of the proportion of nocturnal activity in full-moon and no-moon nights when plotted against average night temperature have been compared by using Students t-test on the slopes and intercepts. The data has also been compared by Analysis of Covariance (ANCOVA) using temperature as a covariate. This data is reported in figure 3.9 and shows that there is no significant difference between the nocturnal activity on dark nights with no moon or relatively bright full-moon nights ($p_{slopes} = 0.9772$, $p_{intercepts} = 0.8699$, ANCOVA, $F_{(1,22)}=0.04048$, $p=0.84239$).

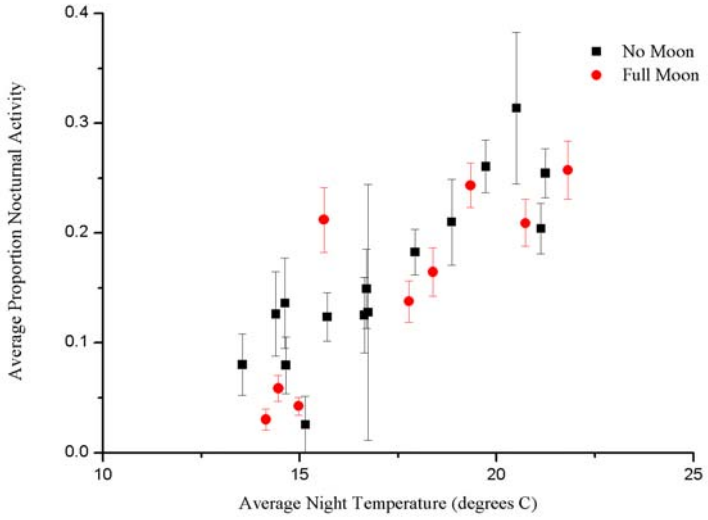


Figure 3.9: Proportion of nocturnal activity on full-moon (red) and no-moon (black) nights compared to the average night temperature. There is no significant difference between the two conditions when data is compared by using an ANCOVA or a Student t-test on the slopes and intercepts of the two regression lines ($p_{slopes} = 0.9772$, $p_{intercepts} = 0.8699$, ANCOVA, $F_{(1,22)}=0.04048$, $p=0.84239$)

3.2.7 Effect of social combinations on nocturnal activity in the laboratory and natural conditions

In order to examine whether the presence of another fly of the same or opposite sex has an effect on the locomotor activity profile of a single fly, two flies were placed

together in an activity tube. The locomotor activity of wild-type single males and females, and also male couples (MM), female couples (FF) and male-female couples (MF) was recorded as usual in the laboratory as well as in natural conditions in Leicester and Padova and the proportion of nocturnal activity was computed as described in section 3.2.5.

It has recently been demonstrated that in LD 12:12 at 25°C, when male flies are placed with a female in a 2D arena, they become nocturnal instead of diurnal when their 'close-proximity' is used as a measure of activity (Fujii *et al.*, 2007). This observation was tested by recording the locomotor activity of MF, MM and FF couples in the laboratory in LD 12:12 and temperature cycles of 25:20°C (figure 3.10). Moonlight was also mimicked as described in section 2.2.1. The profile of single male and single female flies is reported in figure 3.10A and B respectively but the nocturnal activity of MF couples was only compared to MM and FF couples to control for the presence of two animals in a tube (couples) instead of just one (single flies). MF couples show a significant increase in nocturnal activity as compared to both MM and FF couples (ANOVA, $F_{(2, 547)} = 73.207$, $p_{MF/MM} \sim 0$, $p_{MF/FF} \sim 0$). This observation is in accordance with that of Fujii and colleagues (Fujii *et al.*, 2007) and extends the study to show that the phenomenon of increased nocturnal activity also occurs in temperature cycles.

Further, in order to place this study in an ecologically sound context of longer photoperiods and more realistic 'natural' temperatures, the activity of these social combinations was recorded in LD 16:8 with a temperature cycle of 30:22°C and by providing a low level of moonlight during the 'dark' phase (Padova summer day). Under these conditions, there was no significant difference between the proportion of nocturnal activity of MF couples and MM or FF couples (ANOVA, $F_{(2, 319)} = 1.9256$, $p_{MF/MM} = 0.9595$, $p_{MF/FF} = 0.2232$) (see figure 3.11 A, B and C). However, it can be seen

from the figure that the startle effect after light-off is stronger for MM and FF couples than MF couples. Thus, the analysis was repeated by excluding the first 4 hours of the 'dark' phase. An ANOVA revealed that the nocturnal activity of MF couples in the second half of the night is significantly greater than MM ($p \sim 0$) and FF ($p \sim 0$) couples.

In order to investigate whether these social combinations show any difference in nocturnal activity in natural conditions, the activity of MF, MM and FF couples was recorded in various seasons in Leicester (figure 3.12 A, B and C) and Padova (figure 3.12 D, E and F). It has been demonstrated above (section 3.2.5) that in natural conditions, the amount of nocturnal activity of single males is dependent on the average temperature during the night. The proportion of nocturnal activity of the couples, too, was thus compared with an Analysis of Covariance (ANCOVA) using the average night temperature as a covariate. It was found that across a broad transient of temperatures, there was no significant difference between the nocturnal activity of MF couples and that of MM or FF couples (ANCOVA, $F_{(2,311)} = 4.288$, $p_{MF/MM} = 0.0519$, $p_{MF/FF} = 0.3475$). Thus, the observation that heterosexual social interaction makes flies more nocturnal appears to be true for a variety of laboratory conditions but is not reflected in the dataset that has been analysed in natural conditions in this work.

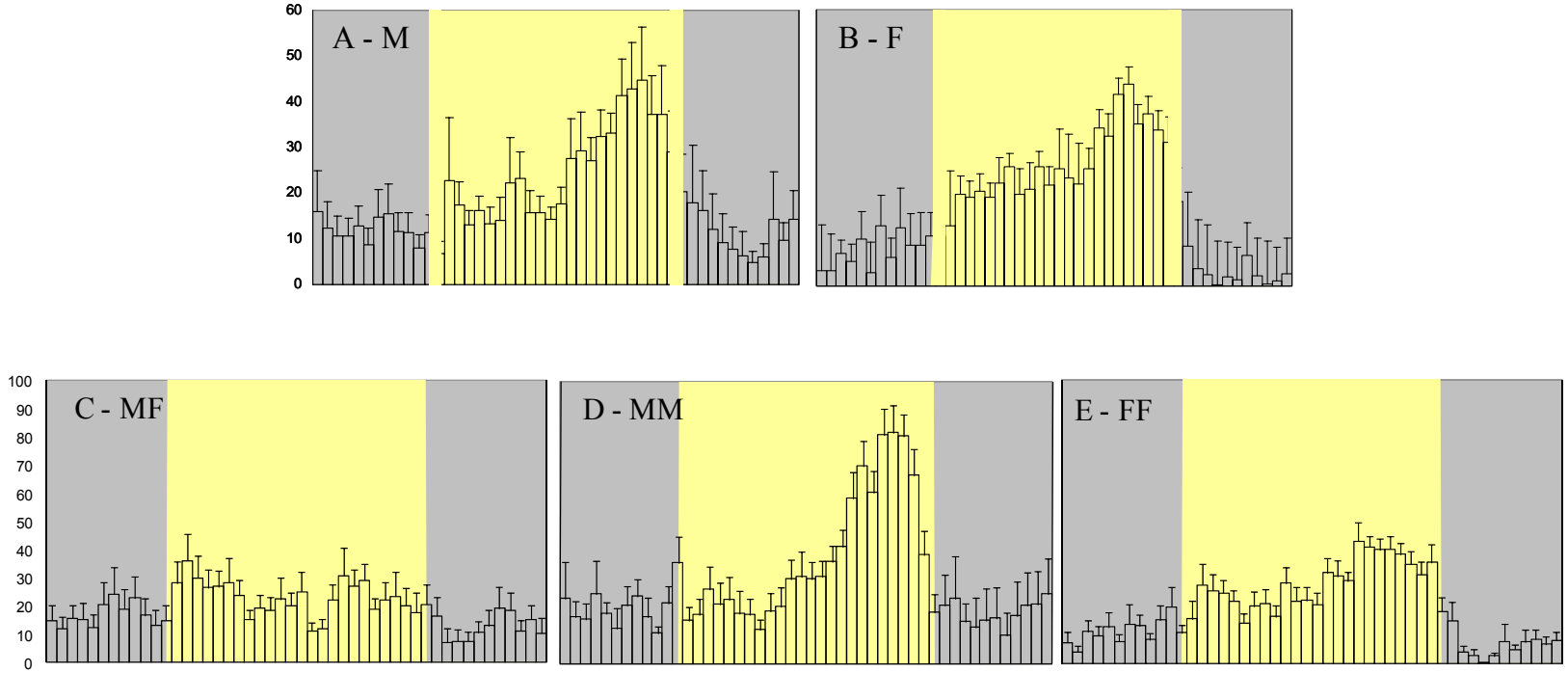


Figure 3.10: Locomotor activity profiles of various social combinations recorded in the laboratory in LD 12:12, 25:20°C temperature cycles and moonlight at night. (A) Single males (n=18), (B) Single females (n=18), (C) MF (n=19), (D) MM (n=17), (E) FF (n=19) couples. MF couples show a significant increase in proportion of nocturnal activity when compared to MM and FF couples in these conditions (ANOVA, $F_{(2, 547)} = 73.207$, $p_{MF/MM} \sim 0$, $p_{MF/FF} \sim 0$).

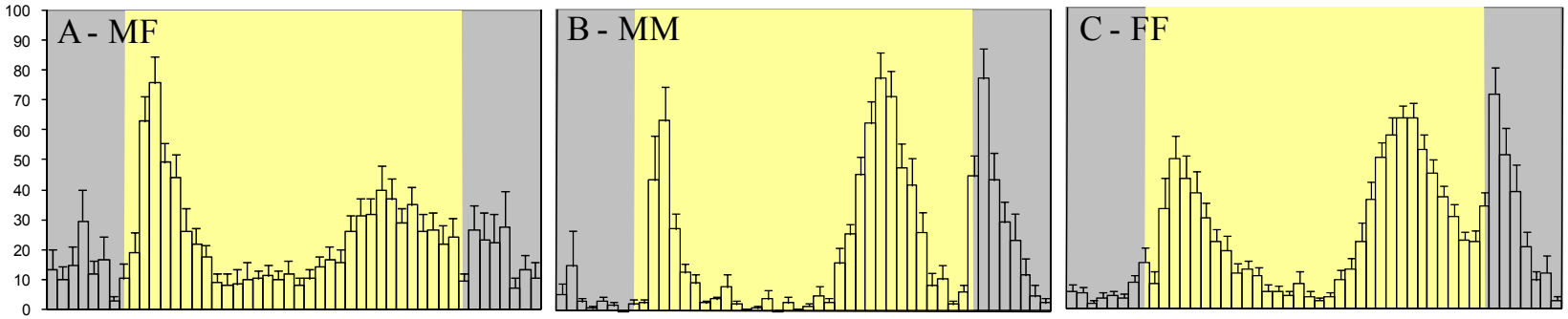


Figure 3.11: Locomotor activity profiles of various social combinations recorded in the laboratory under LD 16:8 and temperature cycles of 30:22°C (Padova summer day). (A) MF (n=13), (B) MM (n=15), (C) FF (n=18) couples. There is no significant difference in the nocturnal activity of the three social combinations (ANOVA, $F_{(2, 319)} = 1.9256$, $p_{MF/MM} = 0.9595$, $p_{MF/FF} = 0.2232$).

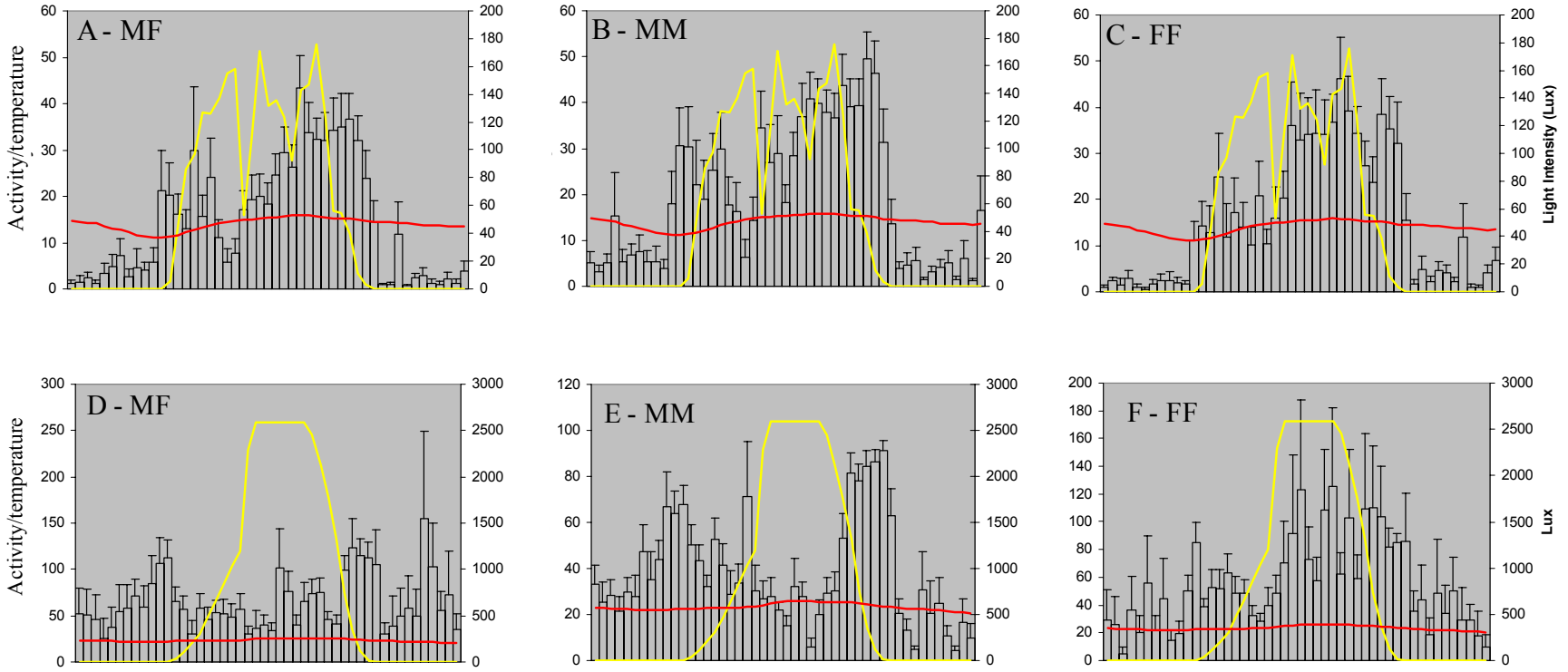


Figure 3.12: Activity profiles of various social combinations recorded in natural conditions. (A) MF - Leicester (n=25), (B) MM - Leicester (n=26), (C) FF - Leicester (n=24), (D) MF - Padova (n=6), (E) MM - Padova (n=9) and (F) FF - Padova (n=5). There is no significant difference between the nocturnal activity of the three social combinations across a broad transient of temperatures (ANCOVA, $F_{(2,311)} = 4.288$, $p_{MF/MM} = 0.0519$, $p_{MF/FF} = 0.3475$).

3.2.8 Effect of a combination of environmental cues on 'anticipation'

As described before, in laboratory conditions, wild-type flies anticipate changes in environmental conditions such as transitions of lights on to off and *vice versa.* In constant light and temperature cycles too, it has been shown that they anticipate the temperature transitions by increasing their activity a few hours before them (Yoshii *et al.*, 2005). In fact, a loss of anticipation of these transitions in both light and temperature cycles is characteristic of clock mutants in these conditions (see section 1.6).

As has been reported in section 3.2.2, SOPs representative of natural environmental conditions in Leicester and Padova were mimicked in the laboratory. It was expected that combining input cues of light and temperature in a synchronised manner (high temperature in the light phase and low temperature in the dark phase of an LD cycle) would enhance the 'anticipatory' activity of wild-type flies. On the contrary, such conditions seem to dramatically reduce, if not obliterate completely, any anticipatory activity. Figure 3.13 shows the activity profile of wild-type flies WTALA in LD 12:12 and constant temperature 25°C compared to that when temperature cycles of 25:14°C are imposed (A and B respectively). It was observed that, while wild-type flies clearly anticipate morning in LD 12:12 by increasing their activity some hours before the light transition, this anticipation was lost due to the additional cue of temperature cycles. Thus, combining cues by providing both cycling light and temperature unexpectedly causes a dramatic reduction in the morning anticipation of wild-type flies. This will be further explored under natural conditions in Chapter 4.

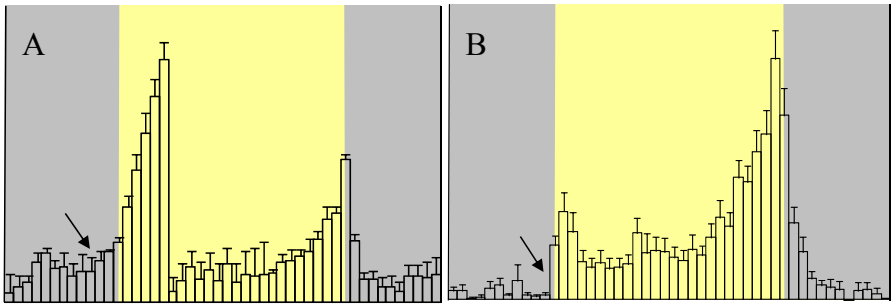


Figure 3.13: Activity profile of WTALA showing a reduction of morning anticipation upon combination of light and temperature cues. (A) LD12:12 constant temperature 25°C, (B) LD 12:12 temperature cycles of 25:14°C. The anticipation of morning which occurs in constant temperature is lost in temperature cycles (see arrows).

3.3 Discussion

In this chapter, the locomotor activity of wild-type flies has been analysed in detail in natural conditions or in those mimicked in the laboratory. For this work, natural strains WTALA and HU were used as they have been collected relatively recently from the wild and their activity looks rather similar to Canton-S, the standard laboratory control (see figure 3.1). Additionally, there appears to be less nocturnal activity in the natural strains and their morning and evening peaks are slightly better defined than Canton-S in mimicked natural conditions. Thus they have been used for all further analysis described in this work and their behavioural profile has been taken to be indicative of Canton-S too.

After monitoring the environmental conditions in Leicester and Padova at the two recording stations, Standard Operating Protocols have been established that are representative of a typical summer's day in each location. These have been mimicked in the laboratory by cycling both temperature and light in the incubator. It is known that flies are bimodal at high and unimodal at low temperatures and the molecular mechanism of this influence of temperature on the activity profile of flies is being investigated (Chen *et al.*, 2007, Low *et al.*, 2008). These studies are typically done by

cycling light and keeping temperature constant. However, from this study, it appears that the temperature-dependent modulation of the distribution of activity in the day is retained in temperature cycles both in the wild and also when natural conditions are mimicked in the laboratory (figure 3.2 and 3.3).

However, some differences were observed between activity in natural conditions and that in the laboratory. For instance, on hot days in the wild, flies show an additional third peak of activity which occurs in the middle of the day, between the morning and evening peaks (figure 3.4). In fact, on hotter days the number of flies with an 'afternoon peak' increases when compared to cold days (figure 3.5). This peak occurs during the hottest period of the day which is usually associated, from laboratory observations, with a 'siesta'. This 'afternoon peak' could be the result of the stress of extremely hot temperatures and an attempt of the flies to escape from this heat which causes an increase in activity. It could also be explained as high temperatures favouring an increased level of activity. Also, in the wild, flies do show a 'siesta', the position of which is modulated by temperature. On hot days, the position of the siesta shifts to the latter part of the day (figure 3.6). However, it appears that this shift in the position of the siesta occurs as a result of the manifestation of the additional 'afternoon' peak. Such an 'afternoon peak' has not yet been reported in any laboratory experiments and represents a novel aspect of fly locomotor behaviour uncovered in natural conditions. It also contradicts laboratory assumptions that flies show a siesta at high temperatures in an attempt to avoid movement in the hot parts of the day. It would be interesting to study this 'afternoon peak' in the laboratory by imposing very high temperatures (>30°C) during the middle of the light phase and checking for a subsequent increase in locomotor activity of flies. Once the conditions required for the presence of the 'afternoon peak' are established, its underlying mechanisms can be further investigated.

Upon investigating factors effecting nocturnal activity in flies, it was observed that elevated temperatures during the night increase nocturnal activity (figure 3.7). In the laboratory, it has been observed that there is an effect of low levels of illumination at night (moonlight) at the molecular and behavioural level. There is a shift of the morning and evening peaks into the night and this is attributed to the shift in phase of PER-cycling in the 5th small ventral lateral neurons (5th sLNv) (Bachleitner *et al.*, 2007). A quasi-mimicking of natural conditions in the laboratory supports this effect of moonlight on the proportion of nocturnal activity (figure 3.8) even though the conditions used in this work differ greatly from those used by Bachleitner and colleagues. Apart from the relatively long photoperiods used in this study, the amount of light illuminating the 'dark' phase was much higher and the light-dark cycles were accompanied by temperature cycles too. In natural conditions, however, comparing the nocturnal activity between contiguous full-moon and no-moon nights showed no significant differences (figure 3.9). In support of this, it has been observed by collaborating colleagues in the Department of Biology at the University of Padova that there is no change in the phase of PER-cycling of the 5th sLNv in the presence or absence of moonlight (Menegazzi, P. and Costa, R., personal communication). Thus, the molecular data obtained in these conditions is in accordance with the behavioural data reported here.

In the laboratory, it has recently been observed that the presence of social stimuli, in particular, heterosexual interactions, can make flies nocturnal as opposed to diurnal (Fujii *et al.*, 2007). However, this study did not use locomotor activity as the behavioural phenotype. For this reason, some work has been done to investigate whether these social factors affect locomotor activity behaviour as well. Experiments done in the laboratory demonstrate that this phenomenon appears to be true for standard

laboratory conditions (figure 3.10). A look at the profile of the locomotor activity of male-female couples shows that they have less day-time activity compared to male or female couples. This could be the reason why their proportion of nocturnal activity is greater. When 'natural' photoperiods and temperatures are mimicked in the laboratory (figure 3.11), there is again a difference between the nocturnal activity of male or female couples and heterosexual male-female ones. Interestingly, homosexual couples show a larger startle effect at the lights-off transition than heterosexual ones. This could be due to an increased aggression between the flies. In experiments conducted in natural conditions over various temperatures (figure 3.12) it was found that male-female couples and same-sex couples have the same proportion of nocturnal activity (which in turn is dependent on temperature). These observations indicate that heterosexual interactions do not result in a shift in the endogenous circadian clock in natural conditions.

The anticipation of the transitions of light or temperature in cycling conditions is an important property of the circadian clock wild-type flies. It was expected that, in the laboratory, a combination of external cues, such as cycling both light and temperature in phase, would result in enhanced morning and evening 'anticipation'. However, on the contrary, the morning anticipation that is seen in LD 12:12 and constant temperature is lost when temperature cycles are imposed. This suggests that morning anticipation, which in flies provides the cardinal definition of rhythmicity in LD cycles, and has major implications for the study of the neuronal network underlying rhythmicity (Grima *et. al.*, 2004; Stoleru *et. al.*, 2004), is a laboratory phenomenon. This observation led to the examination of morning and evening peaks, in particular their onset, in further detail in chapters 4 and 5 respectively.

3.4 Conclusions

- Flies are unimodal at low and bimodal at high temperatures in natural conditions and when these conditions are mimicked in the laboratory.
- On hot days in the wild, flies show a third peak of activity which occurs during the hottest part of the day and has been termed the 'afternoon peak'.
- The proportion of nocturnal activity of flies increases with an increase in the average temperature during the night.
- In natural conditions, the presence of moonlight does not cause an increase in nocturnal activity as it does in the laboratory.
- The effect of social interactions as measured by an increase in nocturnal activity in heterosexual couples is observed in various laboratory conditions but do not appear to be true in the wild.

Chapter 4: Results - Morning Behaviour

4.1 Introduction

Various molecular and behavioural properties of the circadian clock of *Drosophila* have been characterised using mutants that have been identified and cloned over the past four decades (reviewed in section 1.3). These mutants show defects in constant darkness (arrhythmicity in per^{01}, tim^{01}, $Clock^{jerk}$ and Pdf^{01}) or constant light (rhythmicity of cry^{b} and cry^{0}). However, they also exhibit altered behaviour in LD conditions. In particular, their behaviour in LD appears to occur as a response to the external stimulus of changes in light conditions. There is no anticipation of the transition of lights on – off or *vice versa*.

It is believed that the two components of bimodal activity patterns observed in standard laboratory conditions of LD12:12 and constant temperatures of 25°C are controlled by two independent oscillators. The clock neurons of *Drosophila* have been classified on this basis into morning and evening oscillators (M and E cells) (Grima *et al.*, 2004; Stoleru *et. al.*, 2004). Specifically, the morning cells comprising of the PDF-expressing small and large ventral lateral neurons have been shown, in the laboratory, to control the anticipation of lights-on while the evening cells (dorsal lateral neurons, LNds) are responsible for the anticipation of lights-off.

In natural conditions the flies experience a dynamic environment wherein the solar light intensity and ambient temperature are constantly changing. For this reason, it is difficult to ascertain whether or not there is any 'anticipation' in the wild. There is no lab-like transition of lights-off to lights-on or *vice versa* which is currently taken to be the window of time during which anticipation occurs. In nature, there is a gradual increase and decrease in light intensity during dawn and dusk. In fact, according to

geophysical definitions, the transition from night to day involves three distinct phases – astronomical, nautical and civil twilight which occur before sunrise. Astronomical twilight starts at the time in the night when the sun is situated 18° below the horizon and the average illumination at this time is 0.0006 lux. Nautical twilight occurs when the sun is at an angle of 12° below the horizon and has an illumination of 0.06 lux. Civil twilight is the brightest part of the night occurring just before sunrise when the ambient illumination is as high as 6 lux but the sun is still 6° below the horizon (Association of Internationale de Signalisation Maritime). The transition time between twilights (just as with day length) depends on the latitude and seasons. In fact, during the peak of summer, astronomical twilight in Leicester lasts for the entire night. For this reason, and for the fact that the illumination is a reasonable 0.06 lux, nautical twilight was chosen as the anchor for morning behaviour.

All the analysis in this chapter has been performed using the number of minutes before or after nautical twilight when the morning burst of locomotor activity occurs (see section 4.2.1). This parameter has been analysed in detail for various mutants for canonical clock genes and compared to wild-type to investigate the effect of these mutations on morning behaviour in natural conditions.

4.2 Materials and Methods

The locomotor activity profiles of wild-type and mutant flies were analysed in detail in order to describe the morning behaviour of these flies in natural conditions. In all further analysis, the data from Leicester and Padova has been assumed to be equivalent and comparable. Also, all data points in the analysis have been given equal weight as long as the number of flies is greater than or equal to 4. The strains of flies

discussed in this chapter are listed in table 4.1. Some details about these flies can be found in section 2.1.

Table 4.1: List of strains of flies analysed to investigate the morning component of locomotor behaviour in natural conditions.

S.No.	Genotype
1.	WTALA
2.	HU
3.	per^{01}
4.	tim^{01}
5.	$Clock^{jerk}$
6.	$per^{01}tim^{01}$
7.	Pdf^{01}
8.	cry^{0}
9.	*ywuashid,uasreaper;pdfgal4/+;+/+*
10.	*yw;pdfgal4/uascycΔ103;+/+*
11.	*yw;timgal4/uascycΔ103;+/+*

4.2.1 Morning onset, peak and offset

For all the genotypes studied, the time of onset, peak and offset of the morning burst of activity was determined fly-by-fly for each day of every experiment. The following criteria were kept in mind when determining these points, especially the morning onset:

- If a burst of activity occurred after a long period of rest during the dark phase of a day it was considered to be the morning burst.
- The morning burst of activity had to be composed of continuous movement with no more than half an hour (one bin) without any activity.
- Whenever possible, activity bursts with a steady increase and decrease in activity levels showing a clear peak were counted as the morning burst.
- Activity bouts solely in the middle of the night (not leading continuously into the morning) were considered to be nocturnal bursts.

- In general, the window of time that was allowed to contain the morning burst was 3 h before or after the bin during which the light intensity was 1 lux.
- If there was no bout of activity consistent with the parameters described above in the window of time, it was noted that there was no morning peak for that fly on that day. Finally, only days in which 4 or more flies of one genotype exhibited a morning burst of activity were analysed further as subsequent analyses depended on means ($n \geq 4$).

Two examples of single male WTALA flies are shown in figure 4.1 (A and B) and the selected morning bursts are indicated by arrows.

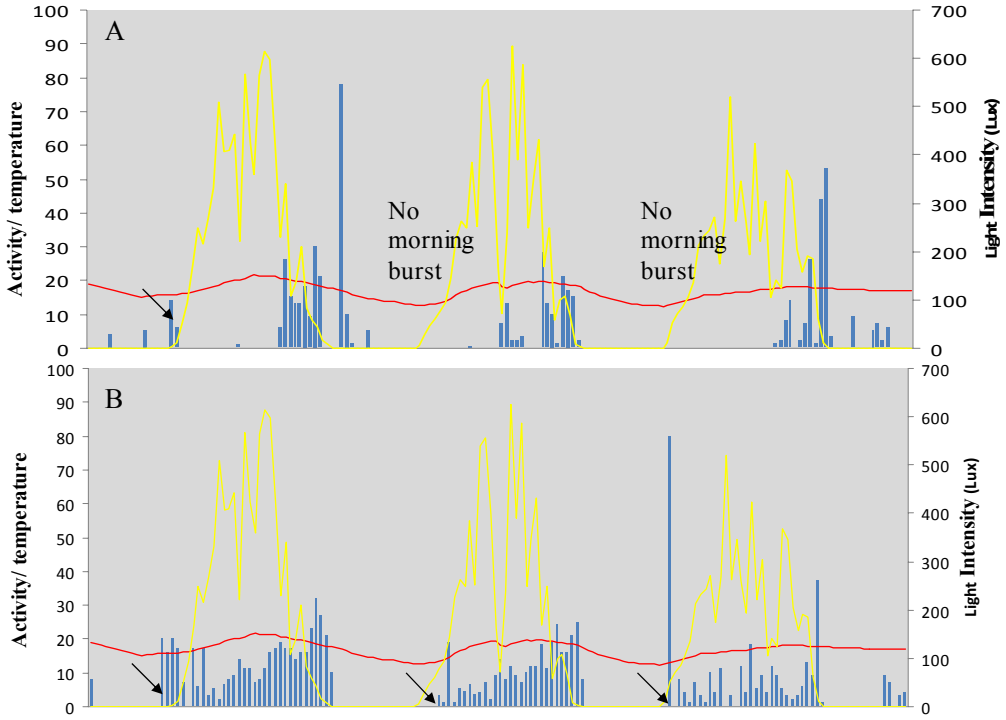


Figure 4.1: Representative activity profiles of single male WTALA flies to show the application of the criteria used to determine the morning onset of behaviour. (A) Fly1 shows a morning burst on the first day but not on the following two days. (B) Fly2 has morning activity on all three days (arrows).

The average time of the onset of the morning activity burst for a genotype was computed for each day and time of nautical twilight for that day was subtracted from

this. Thus the morning onset has been described as the number of minutes before (negative values) or after (positive values) nautical twilight and has been referred to in all subsequent figures as 'M-on – Nautical Twilight'.

4.2.2 Environmental parameters

- The average night temperature was calculated as the average temperature during the time that the light intensity was zero (dark phase) as recorded by the environment monitor which was located adjacent to the activity monitors (see section 2.2.2).
- The photoperiod was calculated as the number of hours during which the light intensity was greater than 0 lux as recorded by the environment monitor.
- The time of Nautical Twilight for the dates of the experiment was obtained from the official United States Naval Observatory (USNO) Astronomy Application Department Online Database (http://aa.usno.navy.mil/). The geographical co-ordinates used for Leicester were 52°40'N 1°0'W with time zone 0 while those for the recording station in Padova were 45°41'N 12°15'E with time zone 1.

4.2.3 GAL4 works at 10°C

There is a lot of discussion about the efficiency of the GAL4-UAS system at low temperatures because GAL4 is a yeast protein, so it works best at high temperatures of approximately 30°C and its efficiency reduces with a decrease in temperature (reviewed by Duffy, 2002). In order to test whether the GAL4-UAS constructs that have been used in natural conditions are able to express GAL4 at low temperatures, they were tested in the laboratory in LD12:12 and constant temperature of 10°C (which is lower than the average temperature in any of the natural studies described in this thesis). Figure 4.2 shows the activity profiles of the flies *yw;timgal4/uascycΔ103;+/+* and

yw;uascycΔ103/+;+/+ respectively. As can be seen, in LD12:12, control *yw;uascycΔ103/+;+/+* flies show a distinct evening peak (indicated by arrows in figure 4.2) while *yw;timgal4/uascycΔ103;+/+* lose this evening behaviour. This shows that GAL4 is able to drive the expression of CYCΔ which in turn stops the functioning of the circadian clock in temperatures as low as 10°C. Some experiments using the UAS-GAL4 system at temperatures like 10 and 18°C have shown that the functioning of the system is not compromised at low temperatures (Hong *et. al.*, 2006; Liu *et. al.*, 2003). Thus, the low temperatures on cold days in natural conditions were not expected to interfere with the functioning of the GAL4-UAS system.

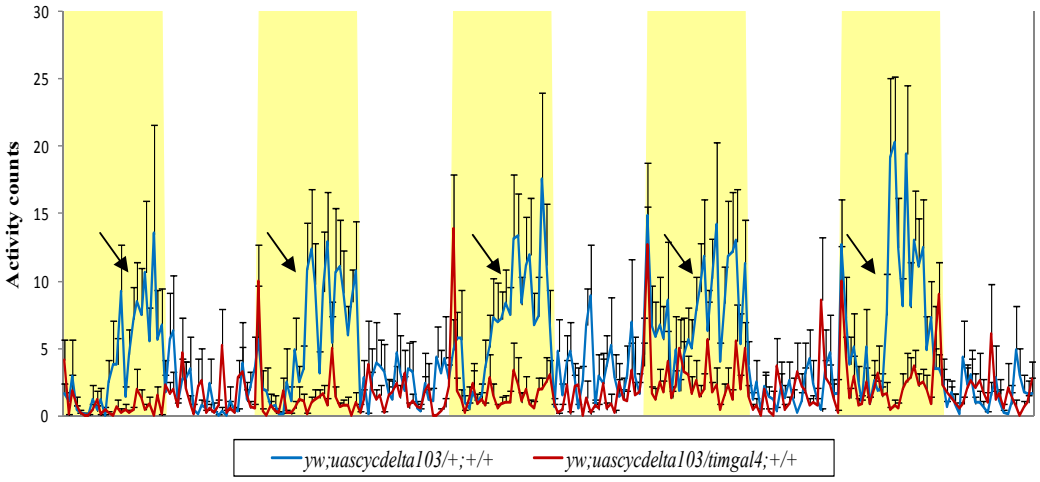


Figure 4.2: Mean activity profiles (± SEM) of *yw;timgal4/uascycΔ103;+/+* (red, n=30) and control *yw;uascycΔ103/+;+/+* (blue, n=26) in LD12:12 and constant temperature of 10°C. Control flies (blue) have an evening peak anticipating the lights on-off transition (arrows) while those with GAL4 driving the expression of CYCΔ (a dominant negative that stops the circadian clock) do not exhibit this evening peak.

4.3 Results

4.3.1 Locomotor activity of wild-type and mutants in natural conditions

Locomotor activity data for wild-type and mutant flies was collected over two seasons – 2007 and 2008. The data obtained from all natural recordings was analysed as described in section 2.2.3. In natural conditions, even though there is a light-dark cycle,

the transition between lights-off to on and *vice versa* is very smooth and does not cause a startle effect as is observed in the laboratory. Thus, a clear circadian rhythm detected by the CLEAN and autocorrelation analysis provides an indication of the ability of the fly to entrain to natural conditions. All flies which did not have a circadian rhythm as detected by this analysis were excluded from all analyses described ahead. Table 4.2 provides a summary of the percentage of flies of each genotype which were unable to entrain to natural conditions (all the experiments for each genotype were pooled).

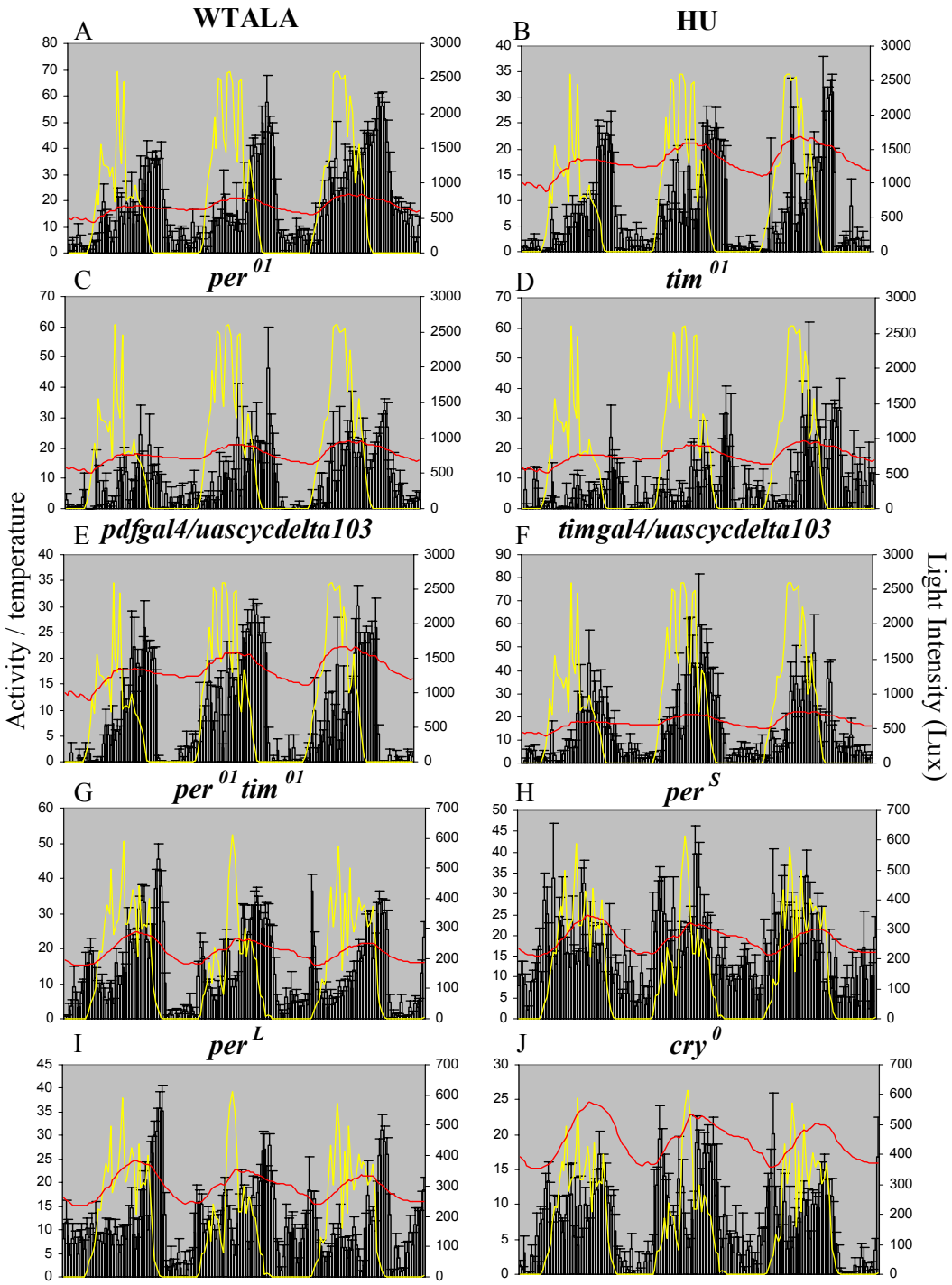
Table 4.2: Percentage of flies of each genotype which did not entrain to natural conditions as determined by CLEAN and autocorrelation analysis.

Genotype	N	Un-entrained (%)
WTALA	300	0.666667
HU	137	2.18
per^{01}	278	5.03
tim^{01}	145	21.37
$Clock^{jerk}$	226	50
$per^{01}tim^{01}$	100	5
per^{S}	75	8
per^{L}	71	19.71
Pdf^{01}	77	5.05
cry^{0}	99	3.89
$norpA^{p41}$	36	2.77
ywuashid,uasreaper;pdfgal4/+;+/+	53	11.32
yw;pdfgal4/uascycΔ103;+/+	81	0
yw;timgal4/uascycΔ103;+/+	143	14.68

It can be seen in table 4.2 that the genotype with the largest number of un-entrained flies was found to be $Clock^{jerk}$ (50%). tim^{01} and per^{L} follow with ~ 20% and *yw;timgal4/uascycΔ103;+/+* and *ywuashiduasreaper;pdfgal4/+;+/+* with 10-15% of flies not entraining to natural conditions. For all other mutants, more than 90% of flies showed a 24-h rhythm on the CLEAN-autocorrelation analysis. Almost all wild-type flies were found to entrain to natural conditions. Thus, apart from $Clock^{jerk}$, mutants for

clock genes do not appear to have a large effect on the entrainment of flies to natural conditions.

An example profile for each genotype over three consecutive days in natural conditions is shown in figure 4.3. Due to equipment limitations, the examples are taken from different natural experiments performed in Leicester. The activity is represented in bars, light intensity in yellow and temperature in red. It can be seen that superficially, the activity profiles of wild-type and mutants look similar, although it cannot be determined from this figure if the mutations affect characteristics such as the position of the onset or peak of the morning or evening components of locomotor behaviour. For this reason, a detailed analysis was performed on the position of the morning and evening peaks.


WTALA
HU
A
B
C
per 01
D
tim 01
E pdfgal4/uascycdelta103
F timgal4/uascycdelta103
G
per 01 tim 01
H
per s
I
per L
J
cry 0
Activity / temperature
Light Intensity (Lux)

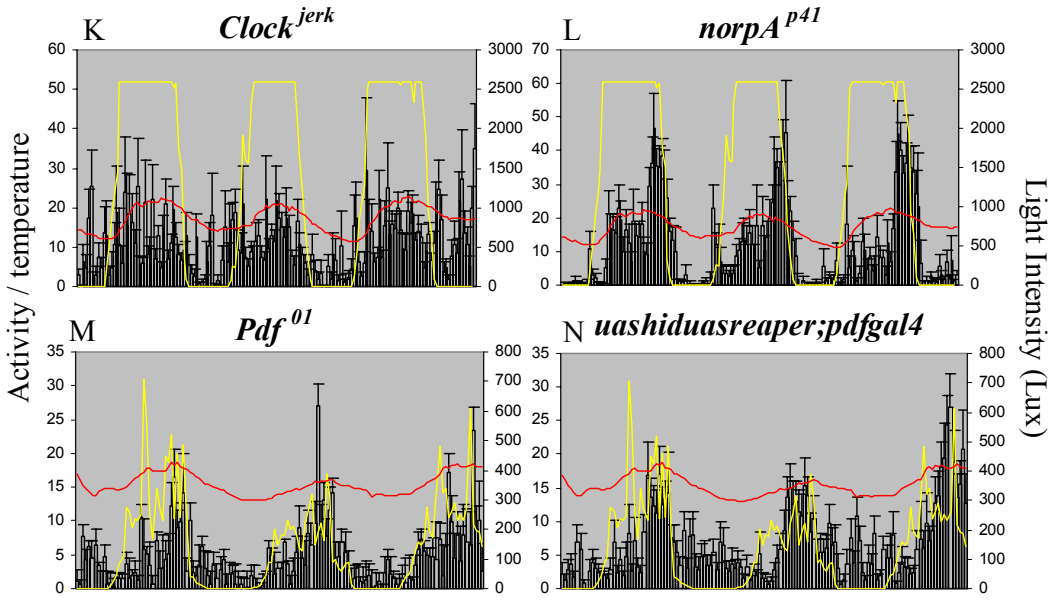


Figure 4.3: Examples of locomotor activity profiles of wild-type and mutants in natural conditions. Mean activity (± SEMS) is in bars, temperature in red and light intensity in yellow. (A) WTALA (n=19), (B) HU (n=27), (C) *per*^{01} (n=13), (D) *tim*^{01} (n=8), (E) *yw;pdfgal4/uascycΔ103;+/+* (n=17), (F) *yw;timgal4/uascycΔ103;+/+* (n=35), (G) *per*^{01}*tim*^{01} (n=25), (H) *per*^{S} (n=24), (I) *per*^{L} (n=19), (J) *cry*^{0} (n=23), (K) *Clock* ^{jerk} (n=14), (L) *norpA*^{p41} (n=18), (M) *Pdf* ^{01} (n=22), (N) *ywuashiduasreaper;pdfgal4/+;+/+* (n=26). The examples are taken from different natural experiments performed in Leicester: A-F: 4-6th Sept 2007, G-J: 30th July-1st Aug 2008, K-L: 1st-3rd Aug 2007, M-N: 16-18th July 2008.

Of the genotypes shown above, the following were only analysed for their evening component and have not been discussed further in this chapter - *per*^{S}, *per*^{L} and *norpA*^{p41}. For all the others, the characteristics of morning behaviour are described in detail below.

4.3.2 Seasonal profile of morning onset of locomotor activity of WTALA

Figure 4.4 shows the position of the morning onset represented as the number of minutes before or after nautical twilight across the seasons in both Padova (orange) and Leicester (blue). The recording period for Padova lasts much longer, from the end of

March till early November, due to better weather conditions while that in Leicester only lasts from May to early October.

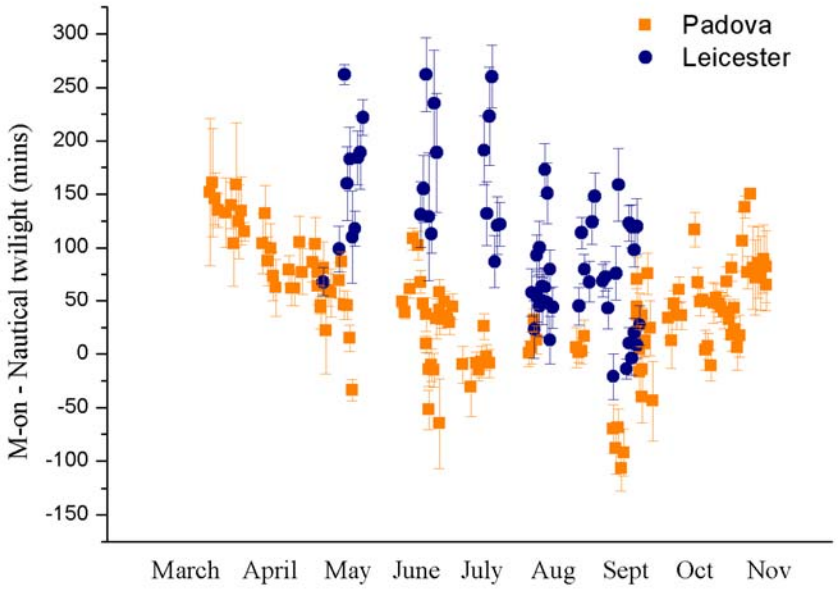


Figure 4.4: Change in the position of morning activity (mins ± SEMS) with seasons in Leicester (blue) and Padova (orange). The U-shaped graph in Padova shows the advance of morning onset in high temperatures and long photoperiods of summer.

The shorter recording season referred to above is evident in figure 4.4. The characteristic U-shaped graph in Padova shows that the morning onset occurs earlier in summer than in spring and autumn. This trend is also observed in Leicester, but cannot continue due to the shorter recording season. The advance in the position of the onset of morning locomotor activity could be due to the warmer temperatures or longer photoperiods which are characteristic of summers in Europe.

4.3.3 Morning onset is highly temperature sensitive

The relative time of morning onset of behaviour shows a strong correlation with temperature (Leicester-blue, Padova-orange). This correlation exists for wild-type flies

WTALA and HU (figure 4.5 A and B) and also for all the strains of mutant flies that have been analysed. Figure 4.5 also shows that this relationship is retained in flies that are mutant for core clock genes such as per^{01} (C), tim^{01} (D) and $Clock^{jerk}$ (E), cry^{0} (F) and also the double-mutant $per^{01}tim^{01}$ (G). The one mutant that shows a relatively weak correlation is tim^{01}.

I then investigated whether specifically targeting the clock neurons that have been attributed to controlling morning behaviour in laboratory studies (M-cells, refer to section 1.6.1.3) also affects morning behaviour in natural conditions. Figure 4.5H clearly demonstrates that expressing the dominant negative CYCΔ using a driver under the control of the *Pdf* promoter (which is only expressed in M-cells) and thereby stopping the clock in these neurons, does not effect this correlation between the position of morning onset and the average temperature at night. This is confirmed by the observation that Pdf^{01} flies also retain this correlation (figure 4.5I). The M-cells were then killed using a construct driving the expression of *head involution defective* (*hid*) and *reaper* genes under the control of the same *Pdf*-promoter driver. The morning onset of these flies too retains a significant correlation with temperature (figure 4.5J). Flies with an even more severe combination, that of CYCΔ expressed in all clock cells using the *tim* driver also exhibit this effect of average night temperature on the onset of morning behaviour (figure 4.5K).

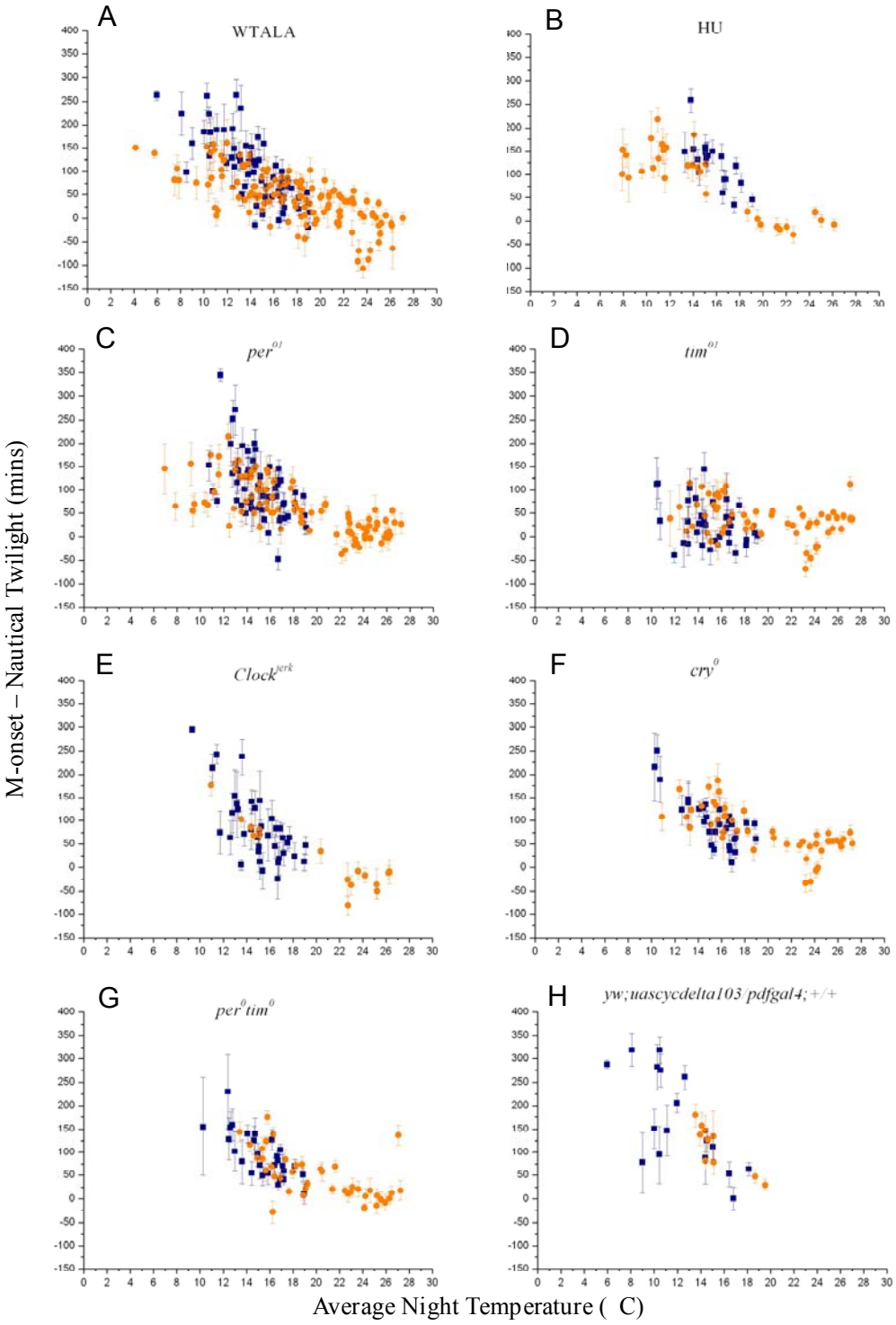


Figure 4.5: Position of morning onset (mins ± SEM) of locomotor activity of wild-type and mutant flies. Leicester-blue, Padova-orange.

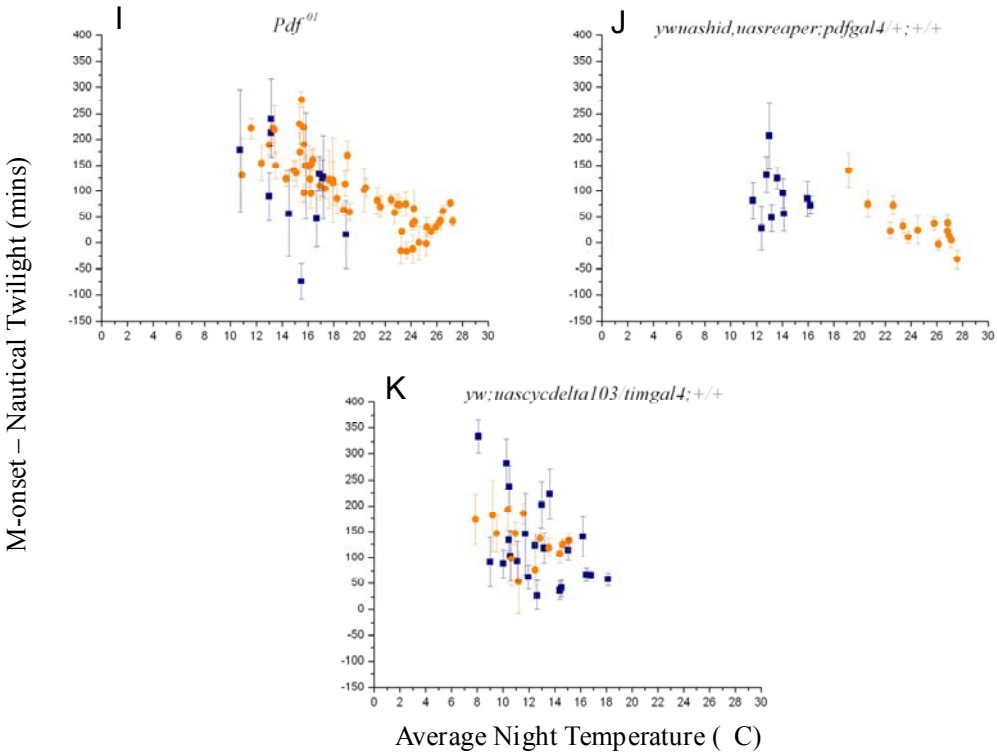


Figure 4.5 (continued)

A summary of the linear regression analysis performed on all the data reported above (figure 4.5) can be found in table 4.3. All the regression lines of the pooled data from Leicester and Padova have a significant correlation.

Table 4.3: Statistical overview of the correlation between M-on – Nautical Twilight and average night temperature in wild-types and mutants.

Genotype	R	p-value	N
WTALA	-0.7149	<0.0001	192
HU	-0.7451	<0.0001	49
per^{01}	-0.6680	<0.0001	157
tim^{01}	-0.2204	0.0275	99
$per^{01}tim^{01}$	-0.7026	<0.0001	71
$Clock^{jerk}$	-0.7668	<0.0001	53
cry^{0}	-0.6842	<0.0001	76
Pdf^{01}	-0.7151	<0.0001	72
ywuashid,uasreaper;pdfgal4/+;+/+	-0.6905	<0.0001	24
yw;pdfgal4/uascycΔ103;+/+	-0.7430	<0.0001	27
yw;timgal4/uascycΔ103;+/+	-0.4532	0.0043	37

4.3.4 Comparison between mutants and wild-type

A comparison of the two wild-type strains using an Analysis of Covariance (ANCOVA) with temperature as covariate, revealed that the morning onset of HU (collected in northern Europe) is delayed compared to WTALA (collected in southern Europe) (see table 4.4). Thus it would appear that this particular phenotype may be sensitive to the genetic background of the fly. For this reason, the regressions of morning activity on temperature of all the genotypes described above were compared to both WTALA and HU in order to find any significant differences between wild-type and mutants with respect to their morning behaviour. A result was considered significantly different if it fell outside the 'wild-type range' provided by HU and WTALA. A graphical representation of all the regression lines can be seen in figure 4.6. Clearly, some of the mutants are more similar to the wild-types than others. The slopes of these lines were compared using t-tests to determine the temperature sensitivity of their morning behaviour. The data obtained from mutants was also compared to wild-types by using an ANCOVA with temperature as a covariate. This analysis helped determine if, at the same temperature, mutants started their morning activity later (delay, D) or earlier

(advance, A) than wild-type. The strains for which the slopes of their regression lines differ from wild-type have been excluded from the ANCOVA analysis. Also, the minimum significance level has been reduced to 0.002 (0.05/number of comparisons) in order to control for multiple-testing. These results are summarised in table 4.4.

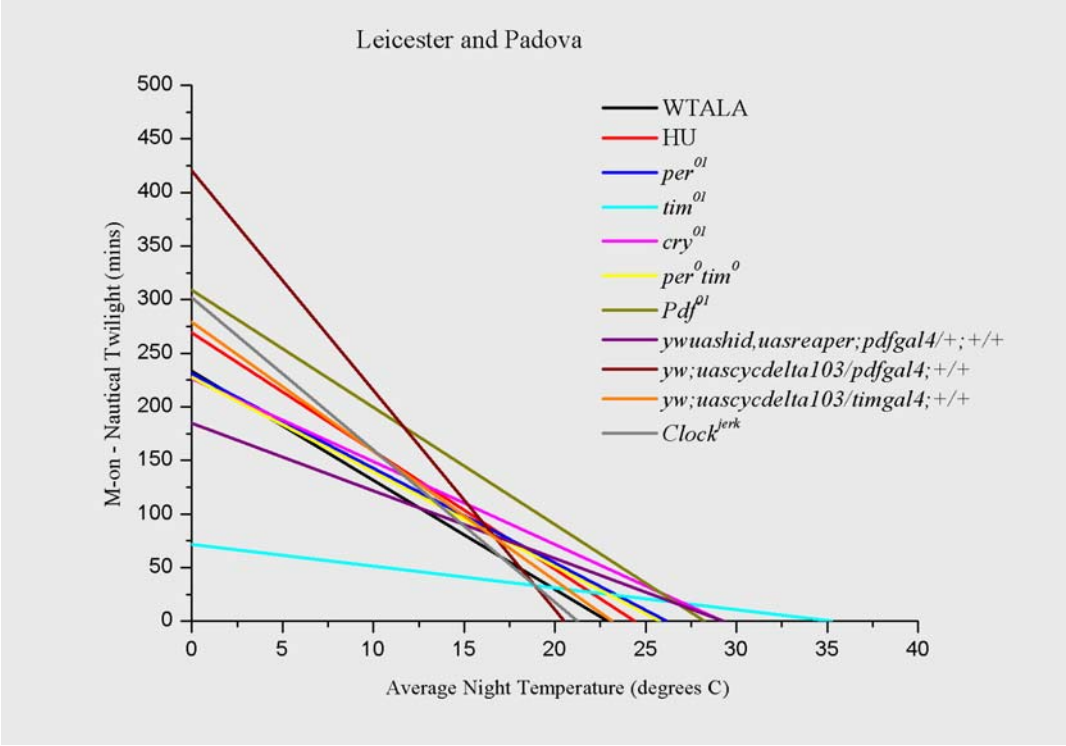


Figure 4.6: Comparison of regression lines for the morning onset versus average night temperature.

Of all the strains of flies analysed only tim^{01} shows a significant deviation when the slope of its regression line is compared to both WTALA and HU. The other canonical clock mutants – per^{01}, $Clock^{jerk}$, $per^{01}tim^{01}$, cry^{0} and Pdf^{01} do not show any difference from either wild-type. Along with these, flies in which the clock has been stopped in some or all clock neurons (*yw;pdfgal4/uascycΔ103;+/+* and *yw;timgal4/uascycΔ103;+/+*) and those in which the morning cells have been killed (*ywuashid,uasreaper;pdfgal4/+;+/+*), are also statistically similar to wild-type in their

morning behaviour. Therefore the temperature sensitivity of morning behaviour in natural conditions is only different for *tim*^{01}.

Table 4.4: Summary of statistical analysis of the position of morning onset as a function of night temperature in wild-types and mutants. Student t-tests were used to compare the slopes of regression lines of mutants to WTALA and HU. All the data were also compared using Analysis of Covariance (ANCOVA). Data significantly different from both ($p<0.002$) are indicated by *. (D, delay, A, advance)

Genotype	**Slopes (p-value)**		**ANCOVA**	
	V/S WTALA	V/S HU	V/S WTALA	V/S HU
WTALA	---	0.6059	---	$F_{(1,238)}$=9.327 p=0.0025-D
HU	0.6059	---	$F_{(1,238)}$=9.327 p=0.0025-D	---
per^{01}	0.2085	0.1819	$F_{(1,346)}$=16.792 p=0.0000-D	$F_{(1,203)}$=0.187 p=0.6657
tim^{01}	<0.0001*	<0.0001*	---	---
per^{01}*tim*^{01}	0.3161	0.2160	$F_{(1,260)}$=8.975 p=0.0030-D	$F_{(1,117)}$=0.338 p=0.5616
Clock^{jerk}	0.0143	0.1532	$F_{(1,242)}$=0.0527 p=0.8186	$F_{(1,99)}$=3.607 p=0.0604
cry^{0}	0.0609	0.0511	$F_{(1,265)}$=35.565 p=0.0000-D	$F_{(1,122)}$=2.031 p=0.1566
Pdf^{01}	0.5938	0.9703	$F_{(1,261)}$=85.377 p=0.0000-D*	$F_{(1,118)}$=19.203 p=0.0000-D*
ywuashid,uasreaper; pdfgal4/+;+/+	0.0664	0.0425	$F_{(1,213)}$=6.926 p=0.0091-D	$F_{(1,70)}$=0.0002 p=0.9866
yw;pdfgal4/uascycΔ103; +/+	<0.0001	0.0060	$F_{(1,216)}$=20.492 p=0.0000-D	$F_{(1,73)}$=2.487 p=0.1190
yw;timgal4/uascycΔ103; +/+	0.4369	0.7878	$F_{(1,226)}$=5.655 p=0.0182-D	$F_{(1,83)}$=0.073 p=0.7871

The ANCOVA analysis reveals that only *Pdf* ^{01} shows a significant deviation from wild-type. This is a conservative analysis for this genotype because ANCOVA uses the pooled slopes of the two genotypes to calculate the corrected mean value for morning onset. By this comparison, the morning onset of *Pdf* ^{01} is delayed in comparison to WTALA and HU. All the other strains are not significantly different from either one or

the other wild-type and so it is considered that they fall within the wild-type range for this particular phenotype. Thus, in natural conditions, most mutants for the input pathway to the clock and the core oscillator itself initiate their morning behaviour at the same time as wild-type.

4.3.5 Morning activity burst in darkness

Even when light intensity detected by the environment monitor is less than 0 lux, low intensity light is present at night. This is in the form of astronomical and nautical twilights which range from 0.0006 to 0.06 lux (see section 4.1). It is possible that morning behaviour in natural conditions occurs as a response to such a low intensity external light stimulus. In order to investigate this possibility, flies were exposed to natural conditions for three days and then covered with a black screen in the night to prevent them from experiencing twilight. The covers were not removed till late the next morning (after 8 AM) so the flies did not experience sunrise. A representative activity profile of a single male WTALA fly in one such experiment is shown in figure 4.7. As can be seen, the fly starts to move before the covers are removed, when it is still in darkness. A similar profile was observed for all the genotypes analysed (see below).

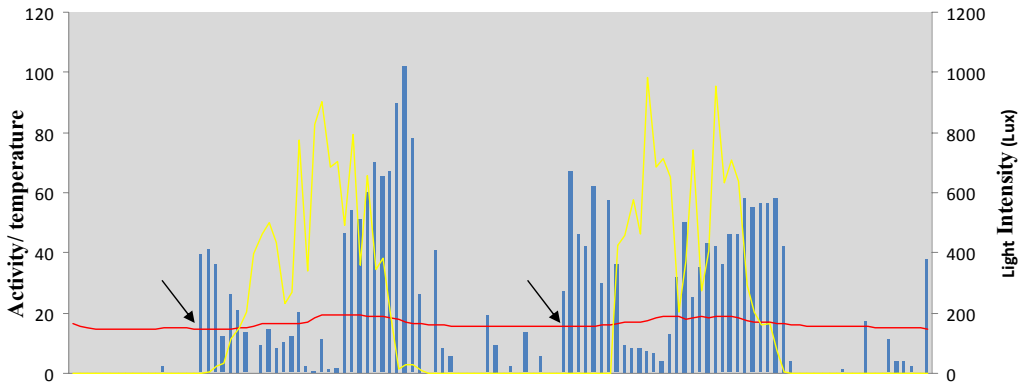


Figure 4.7: Activity profile of a single male WTALA fly showing locomotor activity in natural conditions followed by a morning in darkness. The morning onsets are indicated by an arrow. As can be seen, the fly started its morning behaviour before exposure to light (when the covers were removed).

In another experiment, flies were exposed to natural conditions for three days after which half of them were covered for some days with the black screen used above while the other half were maintained in natural conditions. This experiment was designed to investigate the effects of many days of natural temperature cycles, in the absence of light, on the morning and evening behaviour of wild-type and mutant flies. The analysis of the morning component of behaviour is discussed here while that for the evening component is presented in chapter 5. The time of morning onset of flies in darkness was then compared to that of the same strain maintained in natural conditions on the same day. Figure 4.8 shows the activity profile of single male WTALA flies in order to illustrate the morning onset in natural conditions (A) and darkness (B) as indicated by the arrows. In darkness, the fly begins its morning activity at the same time that light intensity starts to increase outside (as represented by the dotted yellow line in figure 4.8B). A similar profile was observed for all the genotypes analysed.

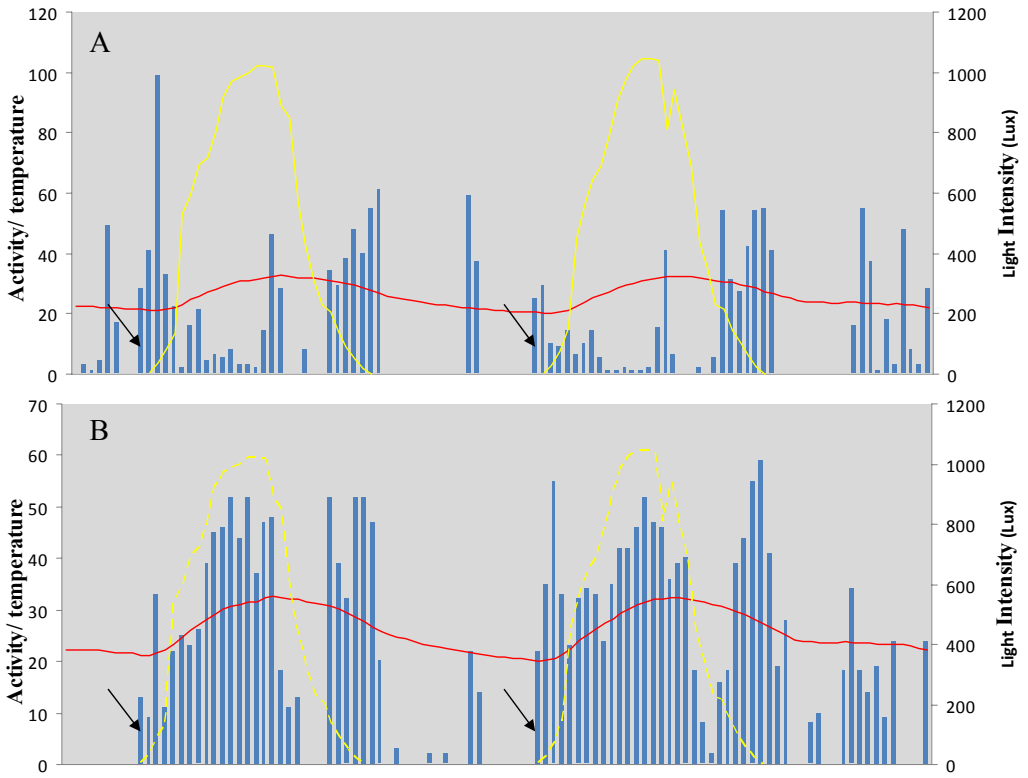


Figure 4.8: Activity profile of a single male WTALA fly showing locomotor activity in (A) natural conditions and (B) darkness. The morning onset on each day is indicated by an arrow. The temperature is in red while the external light intensity is represented in yellow (the dotted yellow line in B is the change in external light).

Since flies maintained in darkness show a burst of activity in the morning even in the absence of light, an attempt was made to determine whether this burst occurs at the same time as those in natural conditions. In order to do this, the time of morning onset of activity in darkness was compared either to the day before (figure 4.7) or to the same day (figure 4.8) in natural conditions for WTALA (A), per^{01} (B) and tim^{01} (C) $Clock^{jerk}$ (D), $per^{01}tim^{01}$ (E), cry^{0} (F) and Pdf^{01} (G) (see figure 4.9).

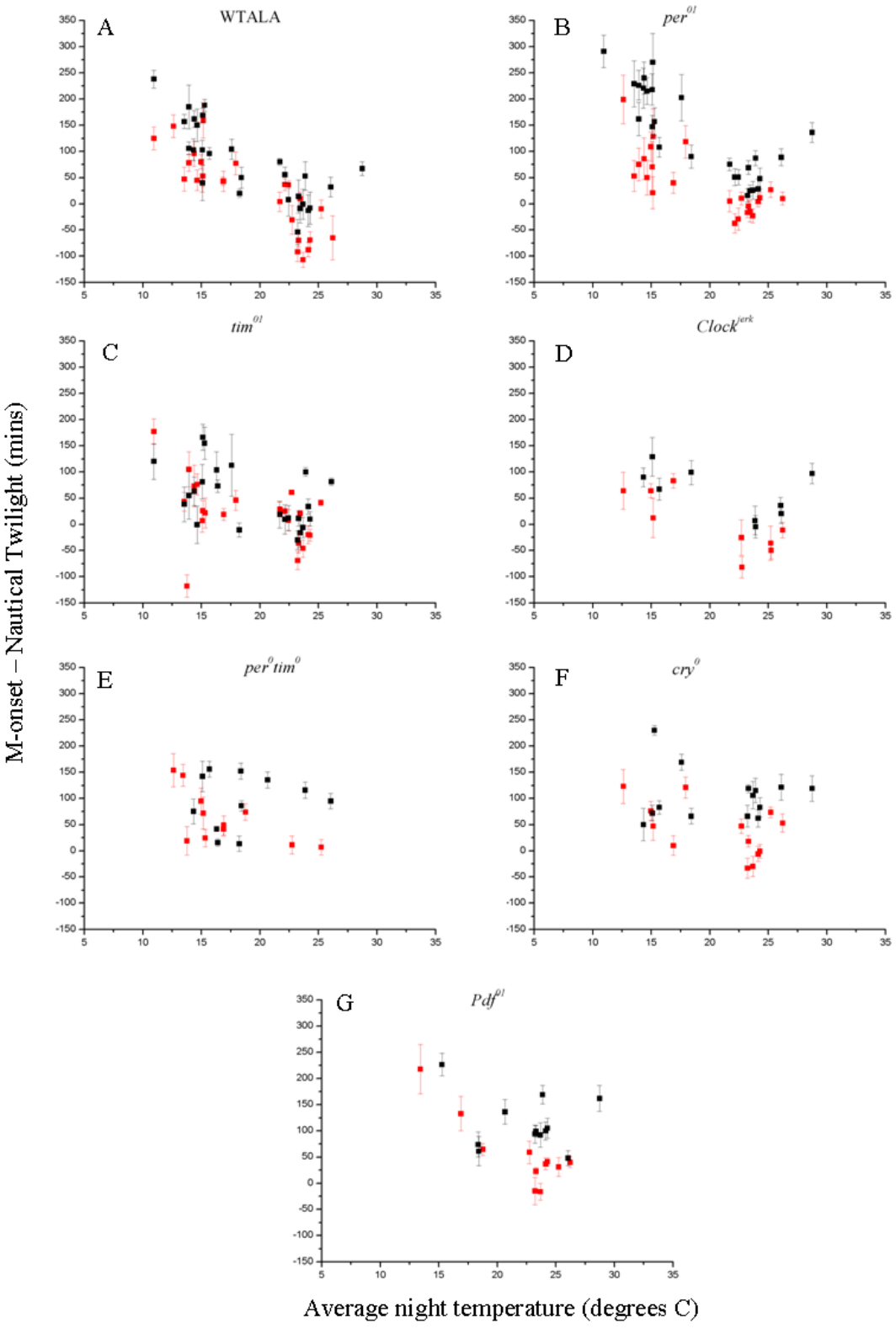


Figure 4.9: Comparison of the position of morning onset of activity plotted against the average night temperature when the flies are maintained in darkness (black) or natural conditions (red).

The data obtained from both these sets of experiments were pooled in order to perform the statistical analysis described below. The position of the morning onset of flies in darkness was compared to that in natural conditions by an ANCOVA using the average night temperature as a covariate, the results of which are summarised in table 4.5. Table 4.5 also shows the correlations and p-values of the regression lines obtained for the subset of data used in this comparison. It was found that at the same temperature, all the strains of flies, including WTALA and mutants, initiate movement later in darkness than in natural conditions. This observation indicates that, in the wild, flies respond to extremely low levels of ambient light.

Table 4.5: Comparison of morning onset against average night temperature for flies maintained in natural conditions compared to those in darkness. The data were compared using an ANCOVA with temperature as a covariate. (D, Delay)

Genotype	R	p-value	N	ANCOVA
WTALA natural	-0.8409	<0.0001	25	$F_{(1,810)}$=43.015 p~ 0 – D
WTALA darkness	-0.7828	<0.0001	27	
per^{01} natural	-0.7588	<0.0001	23	$F_{(1,754)}$=165.75 p~ 0 – D
per^{01} darkness	-0.8131	<0.0001	26	
tim^{01} natural	-0.4432	0.0342	22	$F_{(1,641)}$=5.789 p=0.0164 – D
tim^{01} darkness	-0.4990	0.0131	23	
$Clock^{jerk}$ natural	-0.7962	0.0059	9	$F_{(1,159)}$=31.172 p~ 0 – D
$Clock^{jerk}$ darkness	-0.5419	0.1056	9	
$per^{01}tim^{01}$ natural	-0.6439	0.0238	11	$F_{(1,326)}$=12.126 p=0.0005 – D
$per^{01}tim^{01}$ darkness	0.1277	0.6925	11	
cry^{0} natural	-0.5464	0.0432	13	$F_{(1,405)}$=50.913 p~ 0 – D
cry^{0} darkness	-0.0650	0.8180	14	
Pdf^{01} natural	-0.8646	0.0003	11	$F_{(1,312)}$=59.747 p~ 0 – D
Pdf^{01} darkness	-0.2043	0.5032	12	

`

The fact that all the strains show a morning burst of activity in darkness indicates that they are either responding to temperature changes, the other environmental cue in natural conditions, or that this burst of activity is endogenous, even in clock mutants. In

order to differentiate between these two possibilities, the position of morning onset in darkness (same data as reported above) was anchored to the time of day at which temperature is minimum (M-onset - T-min). Thus, positive and negative values indicate that the activity started before or after the temperature increase began. This was then plotted along with the average night temperature. Figure 4.10A shows that in WTALA in darkness, the morning onset clearly depends on temperature (table 4.6). A similar relationship is observed for per^{01} and tim^{01} (less evident) (figures 4.10B and C respectively). On cold days it occurs many hours after temperature increases. This could be because, on cold nights, the flies wait for a physiologically optimum temperature to initiate their activity. On the other hand, on hot nights (average night temperature > 17°C) morning onset occurs as many as 3 h before temperature starts to increase. Such a clear relationship between position of morning onset and temperature was not observed for the other mutants (fig 4.10) - $Clock^{jerk}$ (D), $per^{01}tim^{01}$ (E), cry^{0} (F) and Pdf^{01} (G) but very few observations were made at <15°C for these genotypes. Nevertheless on many days, for these strains too, the morning onset does occur many hours before the minimum temperature of the day (when temperature is still decreasing). The correlations between M-onset - T-min and average night temperature for all strains are reported in table 4.6.

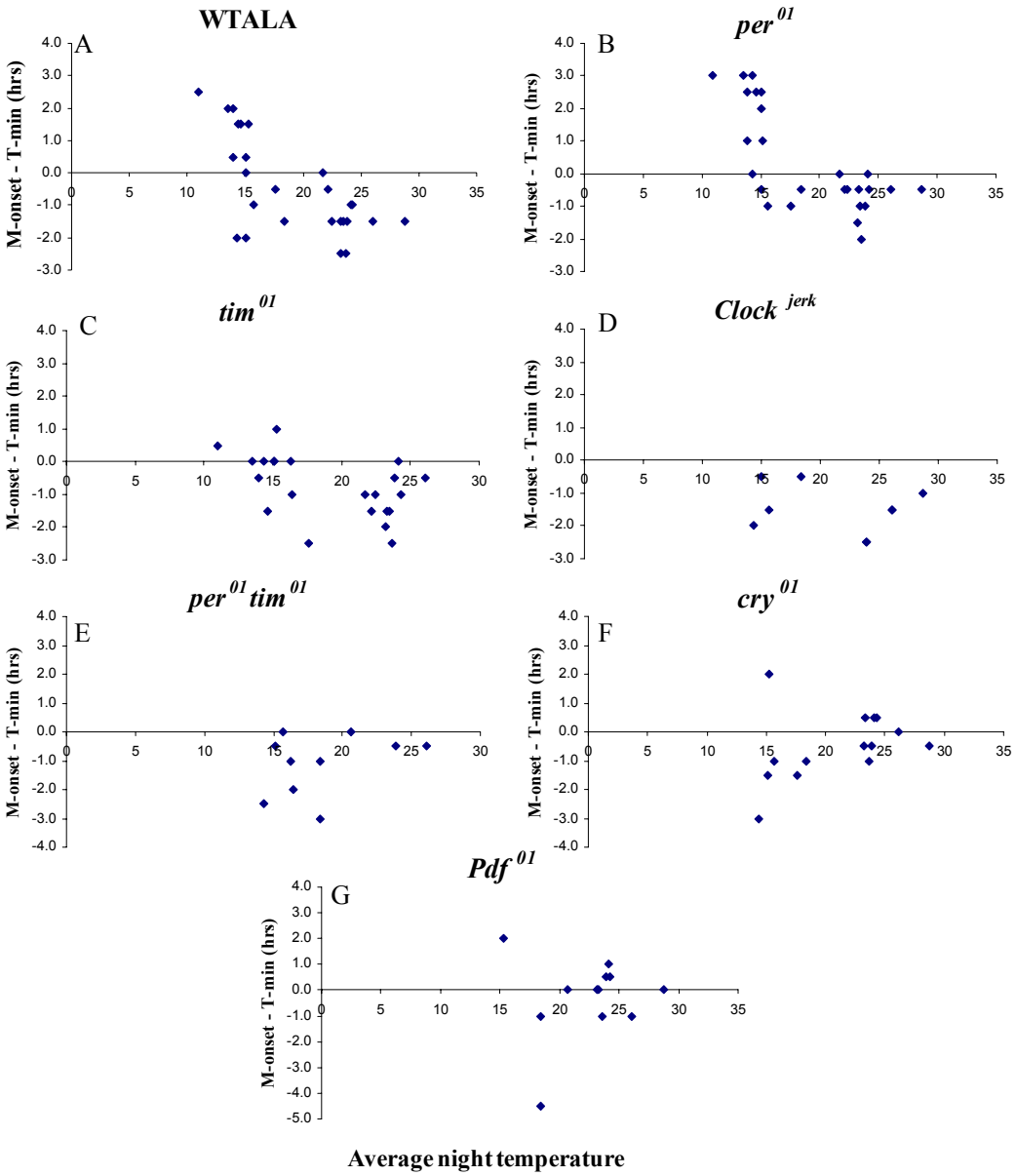


Figure 4.10: Position of morning onset compared to the time of day at which minimum temperature occurs. This has been plotted against the average night temperature on the X-axis.

Table 4.6: Correlation and p-values of the regression lines obtained when the M-onset - T-min in darkness is plotted against average night temperature.

Genotype	R	p-value
WTALA	-0.6872	<0.0001
per^{01}	-0.7204	<0.0001
tim^{01}	-0.4944	0.0141
$Clock^{jerk}$	-0.2173	0.5210
$per^{01}tim^{01}$	0.3448	0.2665
cry^{0}	0.3479	0.1867
Pdf^{01}	0.1016	0.7296

All the strains of flies show a morning onset in darkness that either occurs many hours before or after the time that temperature starts to increase. Thus, this initiation of activity does not appear to be driven by linear environmental changes. This implies that the activity which occurs in the morning in darkness is endogenous in nature and may be the manifestation of either the fully functional (WTALA) or residual circadian clock (mutants), or a clock independent behavioural programme.

4.4 Discussion

Morning behaviour in natural conditions appears to be extremely sensitive to environmental cues of light and temperature. This is evident from the seasonal profile of the position of the morning onset across the year in both Leicester and Padova (figure 4.4). The U-shaped curve shows that there is an advance in the morning onset when anchored to nautical twilight in the summer as compared to spring and autumn in Padova. This could be due to the warm nights that are associated with summers in Italy. This relationship can also be seen in Leicester albeit with the limitations of a shorter recording season. The shift of the curve to the right is because the months of May, June and July are still quite cold in Leicester while it is much warmer in Padova. The advance

in the position of the morning onset in the warm month of August confirms that this seasonal relationship is retained in Leicester.

Investigating the position of the morning onset in greater detail showed that there is a very strong negative correlation between the position of morning onset and average night temperature (figure 4.5). On warmer nights, the onset occurs earlier than on colder ones. This correlation was found to be highly significant for all the strains of flies analysed (table 4.3). However, a comparison of the morning onset of HU with WTALA revealed that the northern (HU) strain initiates morning activity later (thus at brighter light intensities) than the southern (WTALA) one (table 4.4). While this is of course due to 'genetic background', it is interesting that this result also accords with the view that flies in northern Europe have adapted to having a less light sensitive circadian clock than those in southern Europe) because of the exotic photoperiods experienced at higher latitudes (Pittendrigh *et al.*, 1989, Tauber *et. al*., 2007). The morning onset, as well as being temperature dependent, appears to be stimulated by very low levels of light, so the delay in morning onset of HU flies can be interpreted in this context. In addition it has been shown that flies measure day length as an indicator of winter and go into reproductive diapause (Saunders *et. al.*, 1989). In southern Europe, winter is characterised by short photoperiods and cold temperatures. In northern Europe, however, these cold temperatures occur at relatively longer photoperiods. Thus, one way for the flies to adapt to the northern latitudes and go into diapause at the right time may be by reducing the light sensitivity of their circadian clock (Tauber *et. al*., 2007). This is supported by the fact that the less light sensitive allele of *tim*, *ls-tim,* which is more abundant in southern latitudes since it originated there, is spreading to the north probably by directional selection (Sandrelli *et. al.*, 2007).

Irrespective of the reasons for HU's delay in morning onset, the difference between morning onset of HU and WTALA reveals that this phenotype is sensitive to the genetic background of the fly. Knowing that many of the mutants are on cantonised backgrounds (see section 2.1) the regression lines (figure 4.6) of all the mutant strains were compared to both WTALA and HU. It was found that tim^{01} deviates from wild-type in the slope of its regression line (table 4.4). This indicates that the temperature sensitivity of the position of morning onset in these flies is different from wild-type. From the regression lines (figure 4.6), it is evident that this sensitivity is compromised in tim^{01}. It has already been shown that apart from the synchronisation of the clock to light via its interaction with CRY, TIM also plays a role in entrainment to temperature (Glaser and Stanewsky, 2007). Temperature has been shown to affect TIM because short heat pulses result in a rapid degradation of the protein (Sidote *et. al.*, 1998). It has recently been discovered that the splicing of *tim* is also temperature sensitive (Boothroyd *et. al.*, 2007). The lack of TIM in tim^{01} flies could thus explain the reduction of temperature sensitivity of their morning behaviour, but we should expect similar phenotypes for the other clock mutants. Another (but unlikely) possible explanation for this result could be that tim^{01} flies respond to very low intensities of light irrespective of temperature while those in which the clock has been stopped in the morning cells, respond only to higher intensities of light.

A comparison of the position of the morning onset of various strains revealed that Pdf^{01} is delayed with respect to onset of morning activity when compared to wild-types (table 4.4). This effect of Pdf^{01} on the morning peak is not surprising as it has been shown in the laboratory to control morning anticipation (Renn *et al.*, 1999). Strangely, the morning behaviour of mutants per^{01}, $per^{01}tim^{01}$, $Clock^{jerk}$ and cry^{0} are not significantly different from the two wild-type strains. Manipulations of the clock neurons

by either killing some cells or stopping the clock in them does not cause any change in the position of the onset of morning activity.

An interesting observation from both these sets of analyses (comparing slopes and ANCOVAs) of morning behaviour has been that the behaviour of the double-mutant $per^{01}tim^{01}$ is similar to per^{01} instead of tim^{01}. It appears that the removal of both PER and TIM from the clock network results in wild-type behaviour. This is confirmed by the fact that $Clock^{jerk}$ flies, which lack the transcription of both PER and TIM, also shows morning behaviour indistinguishable from wild-type. Some TIM-independent properties of PER, such as its ability to repress CLK-CYC transcription, have already been characterised (Rothenfluh *et al.*, 2000). More recently, it has been demonstrated that PER is able to homodimerise and that this dimer has circadian functions (Landskron *et. al.*, 2009). Thus, it is possible that these, or some other unknown TIM-independent property of PER is responsible for the extreme (temperature independent) low light sensitivity of tim^{01} in natural conditions. This would also explain why this phenomenon is lost in $per^{01}tim^{01}$ mutants.

A conclusion from the above discussion would be that morning behaviour in natural conditions occurs only as a response to changing light intensity. However, when the flies were covered in darkness either only for the morning or for a few days in succession, it was found that they still exhibited a clear morning peak even in darkness (figure 4.7 and 4.8). This shows that morning behaviour does occur in all the strains, even without the environmental cue of increase in light intensity. However, since the onset of this activity in darkness is delayed when compared to natural conditions for all the strains (figure 4.9; table 4.5), the endogenous morning peak appears to be masked by the low levels of illumination during twilight. This morning activity does not appear to be a response to increasing temperature since on hot days the morning onset of all the

strains occurs much earlier than the time at which temperature begins to rise (i.e. temperature minimum) (figure 4.10). On the other hand, on cold days, the morning activity begins much later than the daily temperature rise. Thus, morning activity onset appears to be programmed both in wild-type as well as clock mutants and this is masked in natural conditions by the low levels of illumination during twilight. The morning onset shows a temperature-sensitive phase relationship with the daily increase in temperature that is also largely intact in the mutants. So, either a physiologically based clock independent programme of morning behaviour is released under these conditions, or clock mutants have residual oscillator activity that allows them to time these temperature-sensitive events.

The sensitivity of flies to low intensities of light has been investigated in the laboratory. It has been shown that flies prefer dim light and are more active at lower light intensities (~ 10 lux) than higher ones (~ 100 - 1000 lux). It has also been shown that when twilight is simulated in the laboratory (by logarithmically increasing the light intensity from 0-max lux in 1.5h), flies perform bulk of their activity at ~ 7.5 lux corresponding to artificial dawn and dusk (Reiger *et. al.*, 2007). Here, we report that on hot days in natural conditions, the morning activity of flies occurs much earlier than nautical twilight and is most probably initiated by light intensities as low as 0.0006 lux (astronomical twilight). In the laboratory, it has been shown that hamsters use twilight to entrain their circadian rhythms to days both longer and shorter than 24h (Boulos *et al.*, 1996; 2002). When this study was placed in a natural context by mimicking natural conditions of summer and winter solstice and equinox in the laboratory and providing extremely low intensities of twilight (0.001 lux), it was observed that twilights increased the strength of light:dark cycles as a *zeitgeber* by facilitating entrainment (Boulos *et al.*, 2005). It is possible that natural twilight plays a similar role in entrainment of the

circadian clock of *Drosophila* and this has been uncovered in this study. In fact, the in-depth analysis of morning behaviour of wild-type and mutant flies in natural conditions described in this chapter has demonstrated that the dynamic environment of the morning does have a significant influence on the locomotor activity behaviour of flies. In fact, it appears that even with the lack of components of the clock network, the strong environmental cues of light and temperature cycles appear to be sufficient to modulate morning behaviour of wild-type and mutant flies. A few subtle differences have been observed between wild-type and clock mutants but the presence of endogenous morning behaviour has been observed in all the strains of flies that have been analysed. This study has thus uncovered some novel aspects of circadian biology and this has been extended to the evening component of circadian locomotor activity in the next chapter.

4.5 Conclusions

- The morning onset of behaviour occurs earlier in summer than autumn and spring in both Leicester and Padova.
- There is a strong negative correlation between the position of morning onset and average temperature during the night such that it occurs earlier on hotter nights than on cold ones.
- tim^{01} mutants appear to be compromised in the temperature sensitivity of their morning onset of behaviour.
- Morning behaviour in hot natural conditions is initiated by extremely low intensities of light which occur during twilight.

Chapter 5: Results – Evening behaviour

5.1 Introduction

As described before, in LD conditions of the laboratory, the fly exhibits bimodal activity patterns with a morning and evening peak which are believed to be controlled by separate sets of neurons in the brain (Grima *et al.*, 2004; Stoleru *et al.*, 2004). The dorsal lateral neurons constitute the evening oscillators (E-cells) and drive the anticipation of evening (lights-off transition). In the previous chapter, the morning component of locomotor activity of flies in natural conditions has been described in detail and some subtle differences between wild-type and mutants in these conditions have been uncovered. The same method of analysis has now been applied to the evening component of behaviour to provide a detailed description for wild-type and mutant flies compromised in different components of their circadian clock.

5.2 Materials and methods

The strains of flies analysed for the evening component of their locomotor behaviour are listed in table 5.1. In all further analysis, the data from Leicester and Padova has been assumed to be equivalent and comparable. Also, all data points in the analysis have been given equal weight as long as the number of flies is greater than or equal to 4. Examples of activity profiles for each genotype in natural conditions can be found in section 4.3.1.

Table 5.1: List of strains of flies analysed for their evening peak in natural conditions.

S.No.	Genotype
1.	WTALA
2.	HU
3.	per^{01}
4.	tim^{01}
5.	$Clock^{jerk}$
6.	$per^{01}tim^{01}$
7.	Pdf^{01}
8.	cry^{0}
9.	per^{S}
10.	per^{L}
11.	$norpA^{p41}$
12.	*ywuashid,uasreaper;pdfgal4/+;+/+*
13.	*yw;pdfgal4/uascycΔ103;+/+*
14.	*yw;timgal4/uascycΔ103;+/+*

5.2.1 Evening onset, peak and offset

For all the genotypes studied, the time of onset, peak and offset of the evening burst of activity was determined fly-by-fly for each day of every experiment. The following criteria were kept in mind when determining these points:

- If a burst of activity occurred after a long period of rest during the day it was considered to be the evening burst.
- If a burst of activity occurred in the middle of the afternoon on very hot days, with another clear bout after it, the second one was considered to be the evening peak and the first as an 'afternoon peak' (see section 3.2.4).
- The evening burst of activity had to be composed of continuous movement with no more than half an hour (one bin) without any activity.
- Whenever possible, activity bursts with a steady increase and decrease in activity levels and subsequently a clear peak were chosen to identify the evening peak.
- If there was no bout of activity consistent with the parameters described above, it was noted that there was no evening peak for that fly on that day. Finally, only days

in which 4 or more flies exhibited an evening burst of activity were analysed further as means were used ($n \geq 4$).

- The number of days analysed for the evening peak is less than that for the morning peak because the presence of a high number of spurious counts in the activity monitors rendered some of the data unusable (see section 2.2.1).

Two examples of single male WTALA flies are shown in figure 5.1 (A and B) and the selected evening bursts are indicated by arrows.

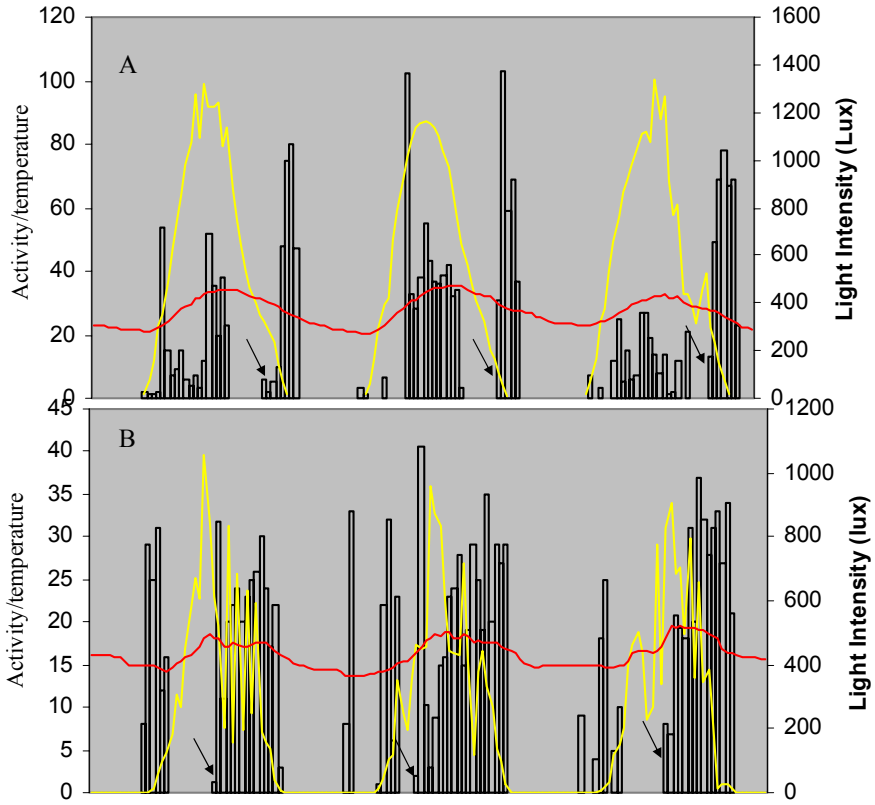


Figure 5.1: Representative activity profiles of single male WTALA flies to show the application of the criteria used to determine the evening component of behaviour (arrows).

5.2.2 Environmental parameters

The average day temperature was calculated as the average temperature during the time when the light intensity was greater than zero (light phase), as recorded by the environment monitor located next to the activity monitors (see section 2.2.2).

5.3 Results

5.3.1 Seasonal profile of evening onset in natural conditions

The evening component of locomotor behaviour was analysed for the same data set that was studied for the morning onset in chapter 4. In particular, the position of the evening onset with respect to lights-on (E-onset – Lights-on), i.e., the time when light intensity starts to increase (lux ≥1), has been analysed for the wild-type strain WTALA. In Padova, the position of E-onset – Lights-on changes with season such that the evening peak occurs later in the day during summer as compared to spring and autumn (figure 5.2). This could be attributed to the long photoperiods and hot days that are characteristic of summer in Italy. A similar trend can be observed in Leicester.

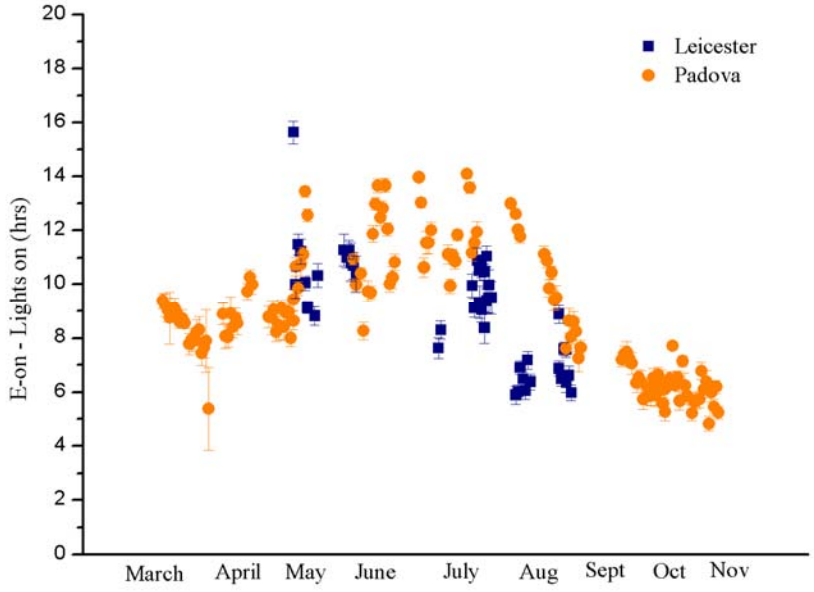


Figure 5.2: Seasonal profile of E-onset – Lights-on (hrs ± SEM) in Leicester and Padova. The position of the evening onset changes with season as it occurs later in summer than spring and autumn. This relationship can be seen in both Leicester (blue) and Padova (orange).

In order to determine the main cue that controls the position of evening onset, it was compared to photoperiod and also to the average temperature of the day. As can be seen in figure 5.3A, the position of the evening onset depends on photoperiod. However, at the same photoperiod, it occurs earlier in Leicester than in Padova. This could be due to fact that the same photoperiods are accompanied by very different temperatures at the two locations, as it is colder in Leicester compared to Padova. This would imply that temperature is the factor that is more important in determining the position of the evening onset. In figure 5.3B, E-onset – Lights on has been plotted against average temperature of the day. Here the differences between the onsets of evening activity in Padova and Leicester appear to be reduced. This also indicates that temperature is the principle environmental cue that is responsible for the position of

evening onset of locomotor activity. For this reason, all further analysis was done by comparing the relative time of evening behaviour to average temperature of the day.

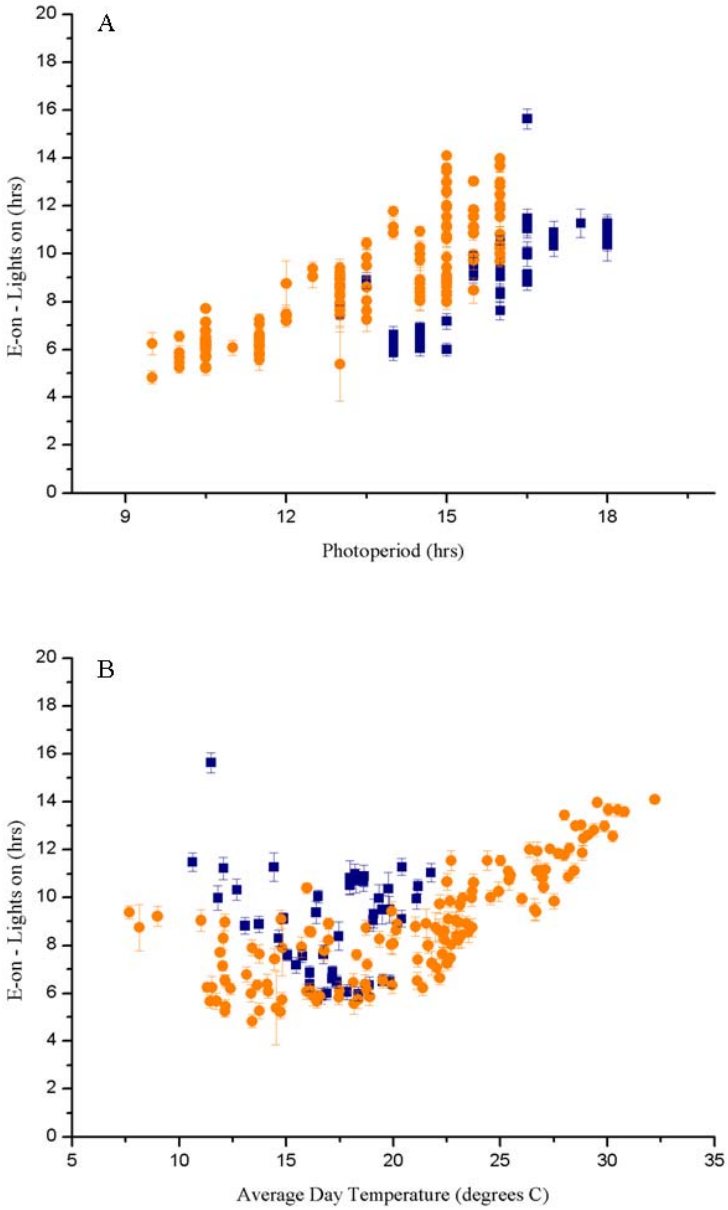


Figure 5.3: (A) E-onset – Lights on (hrs ± SEM) compared to photoperiod (B) E-onset – Lights on (hrs ± SEM) compared to average day temperature. At the same photoperiod, evening activity starts earlier in Leicester (blue) than in Padova (orange) (A). This difference between Leicester and Padova is minimised when the position of evening onset is plotted along with the average day temperature (B). This indicates that temperature is the main environmental cue for determining the position of evening onset.

5.3.2 Evening activity as a function of decreasing temperature or light

An attempt has been made to determine whether or not evening activity occurs as a response to environmental changes such as decreasing light intensity or temperature. In nature, the temperature peak usually occurs within two hours of the peak of light intensity. Thus, these two environmental cues are very highly correlated. For this reason, and the fact that temperature has been shown to be a more important determinant for the positioning of evening onset of activity, all the data reported in this chapter has been compared to the time of the daily temperature peak. This has then been plotted against the average temperature of the day. Both the onset and peak of evening activity are reported.

Figures 5.4 and 5.5 show that the positions of E-onset – T-peak and E-Peak – T-peak have a significant correlation with the average day temperature in WTALA and HU (A and B respectively). The data for mutant strains is reported in figure 5.4 (C-N) for the evening onset and in figure 5.5 (C-N) for the evening peak.

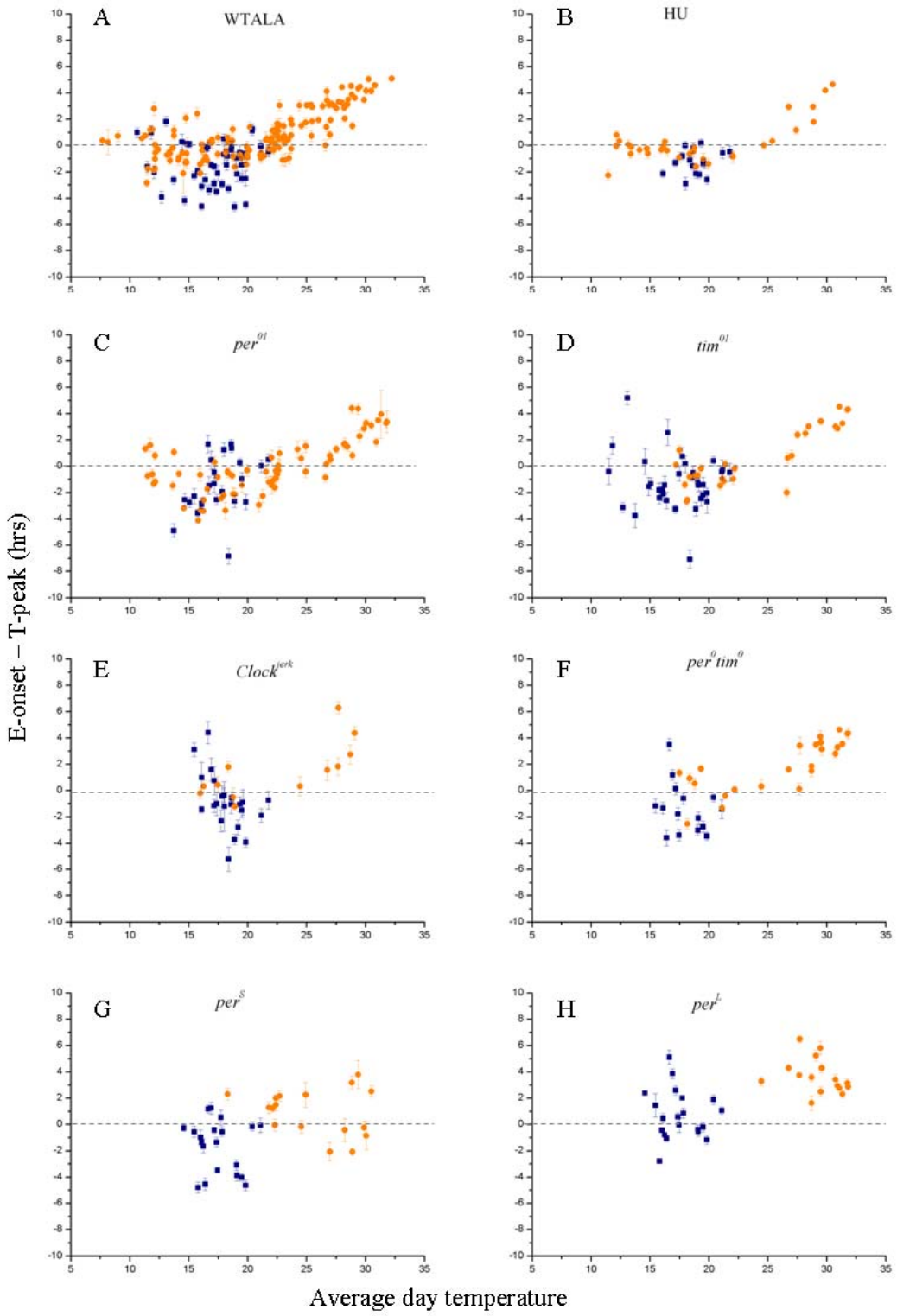


Figure 5.4: E-onset – T-peak (hrs ± SEM) plotted against average day temperature (°C). Leicester – blue, Padova – orange.

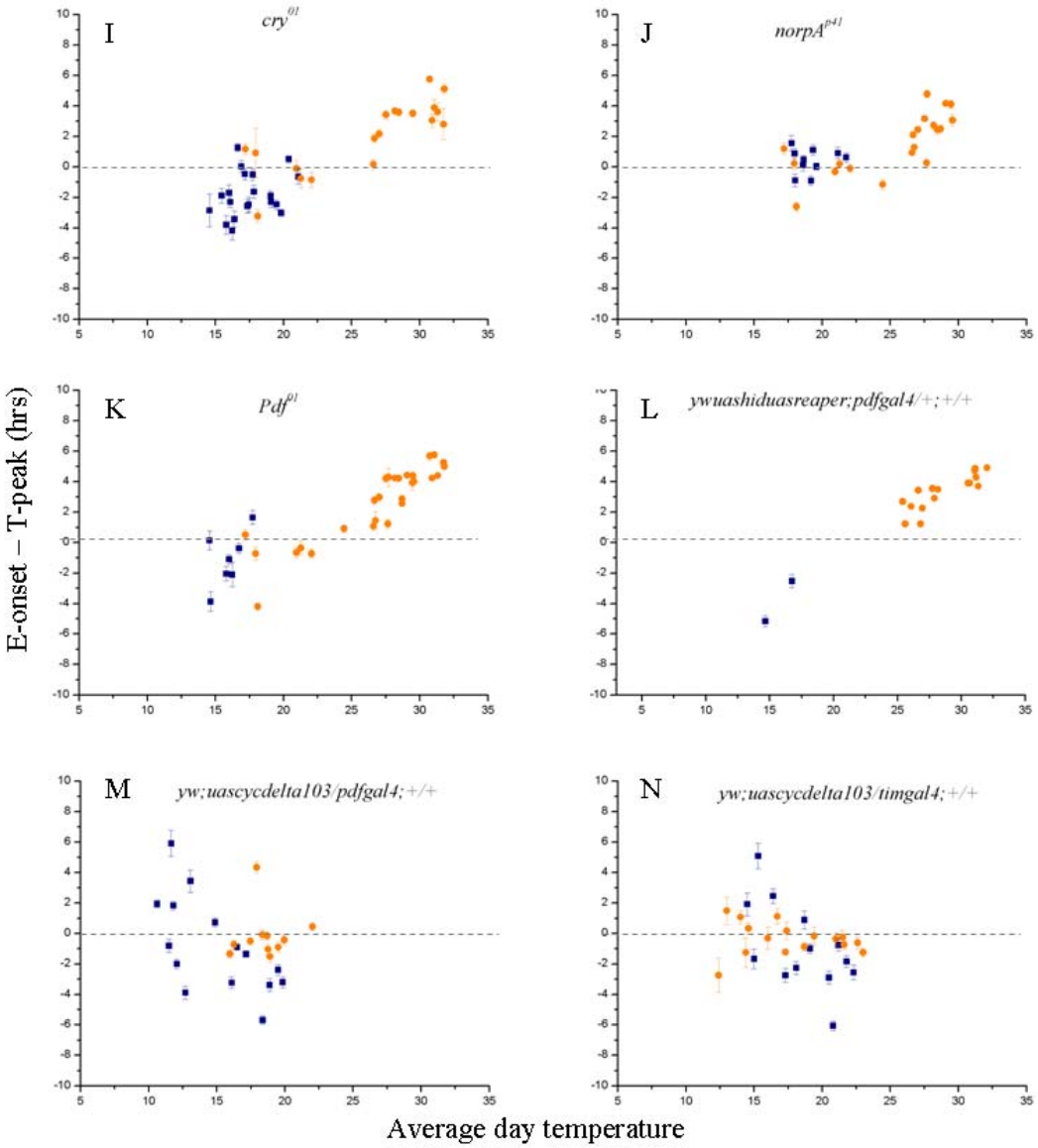


Figure 5.4 (continued)

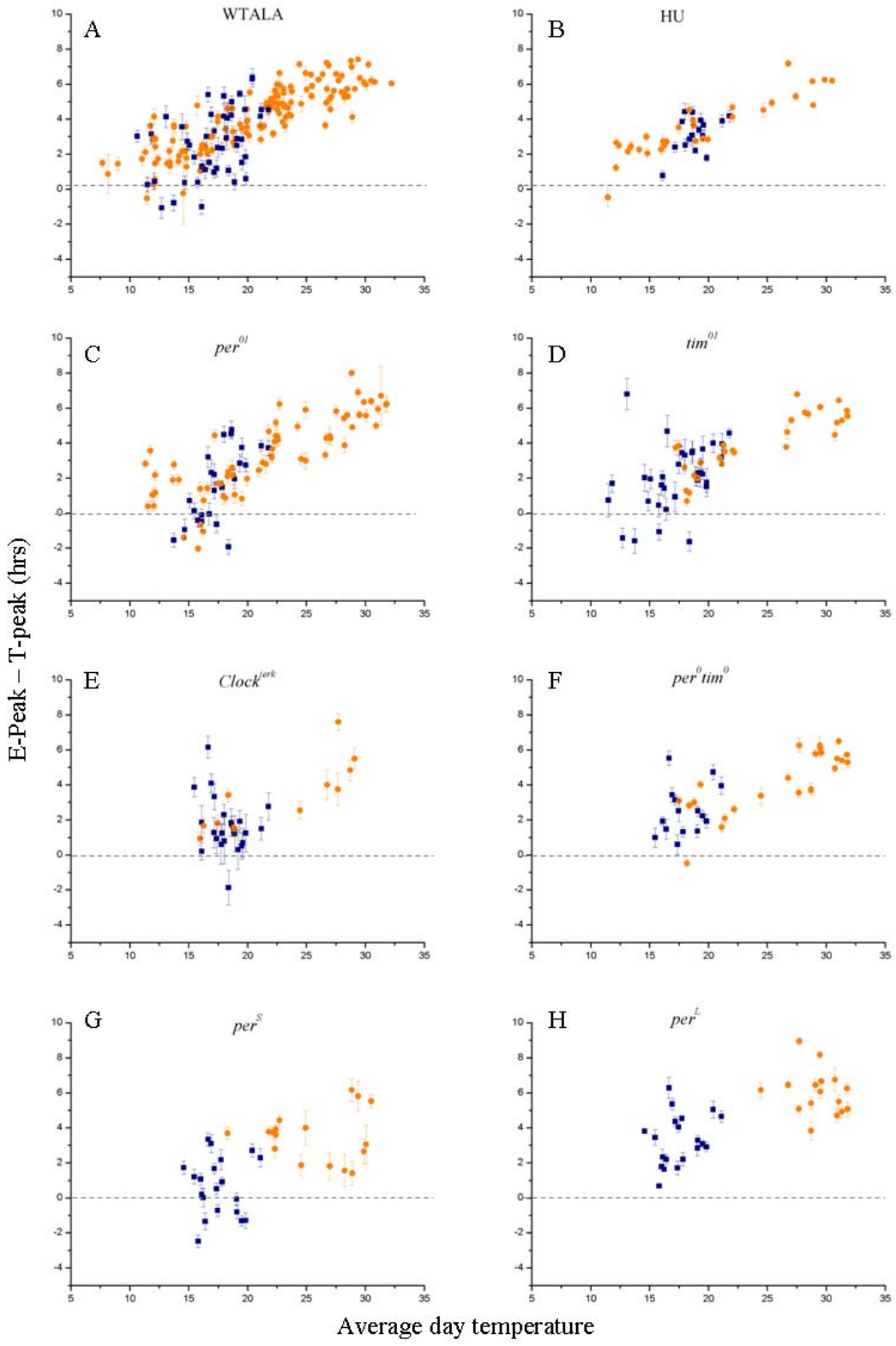


Figure 5.5: E-Peak – T-peak (hrs ± SEM) plotted against average day temperature (°C). Leicester – blue, Padova - orange.

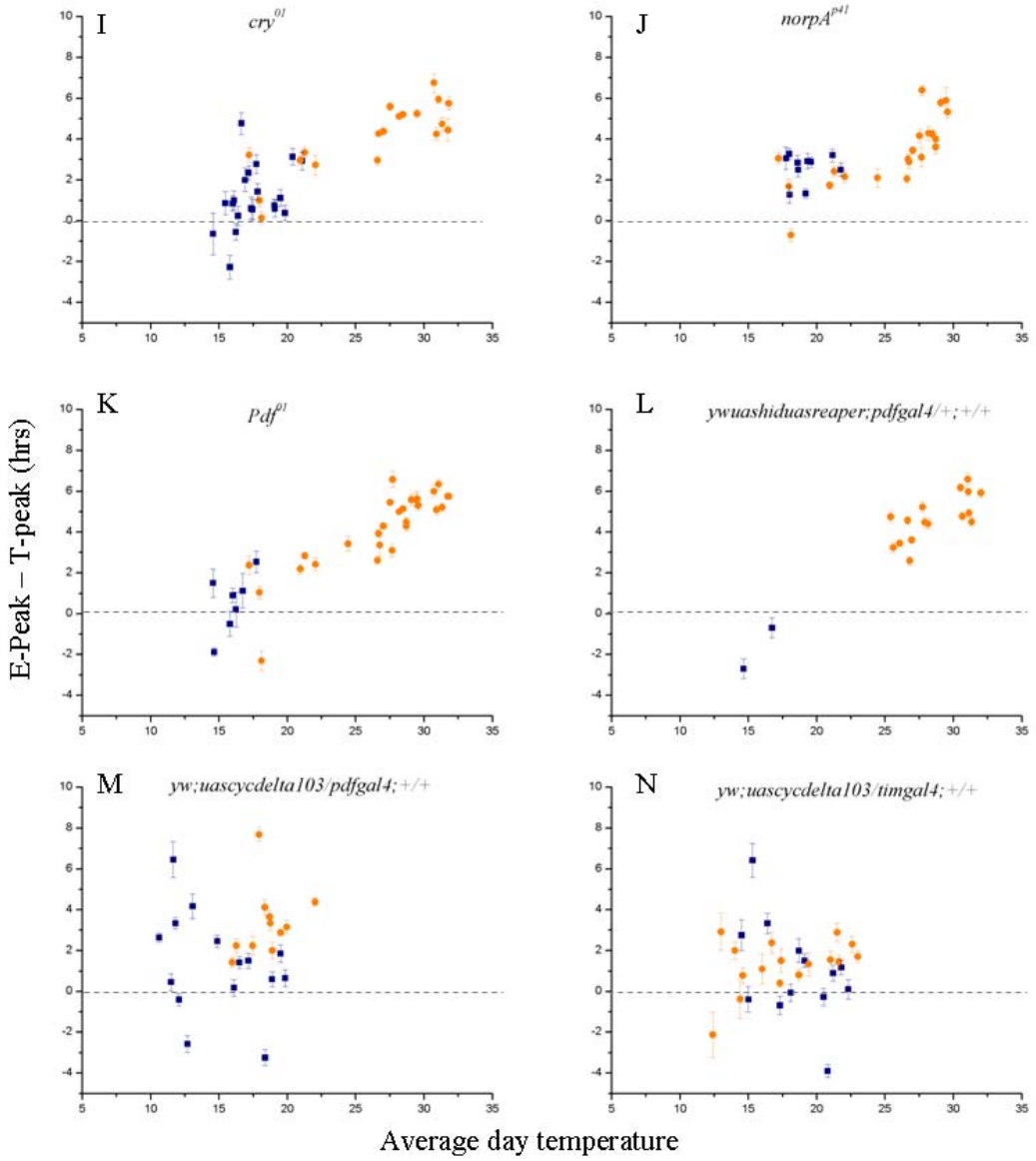


Figure 5.5 (continued)

The correlations and p-values of the data reported in figures 5.4 and 5.5 can be seen in table 5.2. Almost all the strains of flies show a significant correlation between the position of the evening onset and evening peak with temperature. Strangely, none of the mutants for the core clock genes (per^{01}, tim^{01}, $per^{01}tim^{01}$, $Clock^{jerk}$, per^{S} and per^{L}) or those responsible for visual and thermal inputs (cry^{0} and $norpA^{p41}$) disrupt the correlation between the position of evening onset or peak and temperature. Pdf^{01}

mutants, or flies in which the morning cells have been killed (*ywuashid,uasreaper;pdfgal4/+;+/+*), also retain this correlation. Flies in which the clock has been stopped either in all clock cells or only in the morning cells (*yw;timgal4/uascycΔ103;+/+* and *yw;pdfgal4/uascycΔ103;+/+* respectively) show a loss of correlation between onset and temperature. *yw;timgal4/uascycΔ103;+/+* also seems to disrupt the correlation between evening peak and temperature. Interestingly, in both strains (*yw;timgal4/uascycΔ103;+/+* and *yw;pdfgal4/uascycΔ103;+/+*) the correlation between evening onset and temperature is negative while that for wild-types and all other mutants is significantly positive. However, the amount of data for these strains of flies is limited, especially for high temperatures.

Table 5.2: Correlation between E-onset – T-peak and E-peak – T-peak and average day temperature. The R and p values are reported for all the strains of flies.

Genotype	**E-onset – T-Peak**		**E-Peak – T-Peak**		**N**
	R	**p-value**	**R**	**p-value**	
WTALA	0.6344	<0.0001	0.7875	<0.0001	185
HU	0.5870	<0.0001	0.8439	<0.0001	47
per^{01}	0.6357	<0.0001	0.7816	<0.0001	91
tim^{01}	0.5824	<0.0001	0.7162	<0.0001	62
$per^{01}tim^{01}$	0.7549	<0.0001	0.7628	<0.0001	39
$Clock^{jerk}$	0.4175	0.0113	0.5391	0.0007	35
cry^{0}	0.8636	<0.0001	0.8347	<0.0001	39
Pdf^{01}	0.8891	<0.0001	0.8942	<0.0001	35
per^{L}	0.6019	<0.0001	0.6884	<0.0001	36
per^{S}	0.3930	0.0146	0.5739	0.0002	37
$norpA^{p41}$	0.7177	<0.0001	0.6838	<0.0001	31
ywuashid,uasreaper;pdfgal4/+;+/+	0.9647	<0.0001	0.9381	<0.0001	18
yw;pdfgal4/uascycΔ103;+/+	-0.3492	0.0742	0.0911	0.6513	26
yw;timgal4/uascycΔ103;+/+	-0.3821	0.0371	-0.0691	0.7167	29

Since, as mentioned above, the amount of data available for *yw;pdfgal4/uascycΔ103;+/+* and *yw;timgal4/uascycΔ103;+/+* is limited, an attempt

was made to substantiate the differences between their evening onset and peak and that of wild-types. The onset and peak were thus anchored to the time of Lights-on as well. This was done to confirm the negative correlation seen above (table 5.2). The position of the evening onset and peak plotted against average day temperature can be seen in figures 5.6 and 5.7 respectively. The data reported here is only for WTALA, HU, *yw;pdfgal4/uascycΔ103;+/+* and *yw;timgal4/uascycΔ103;+/+* but that for the rest of the strains can be seen in appendix 2.

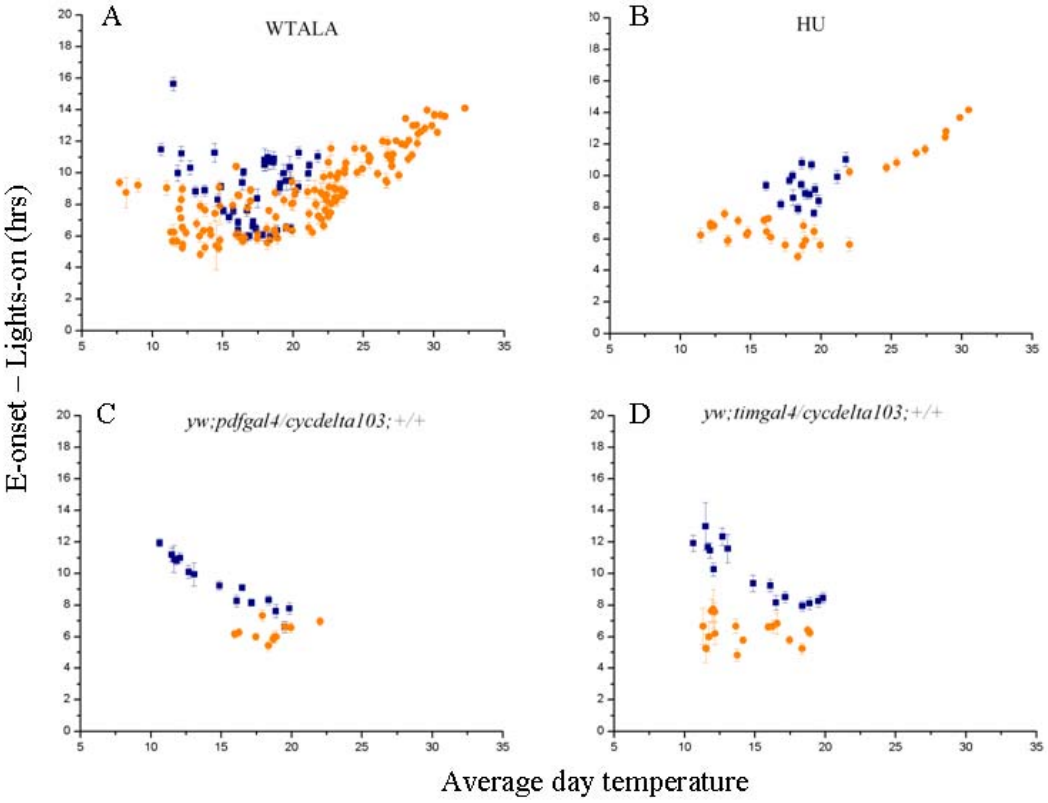


Figure 5.6: E-onset – Lights-on (hrs ± SEM) plotted against average day temperature (°C). Leicester – blue, Padova - orange.

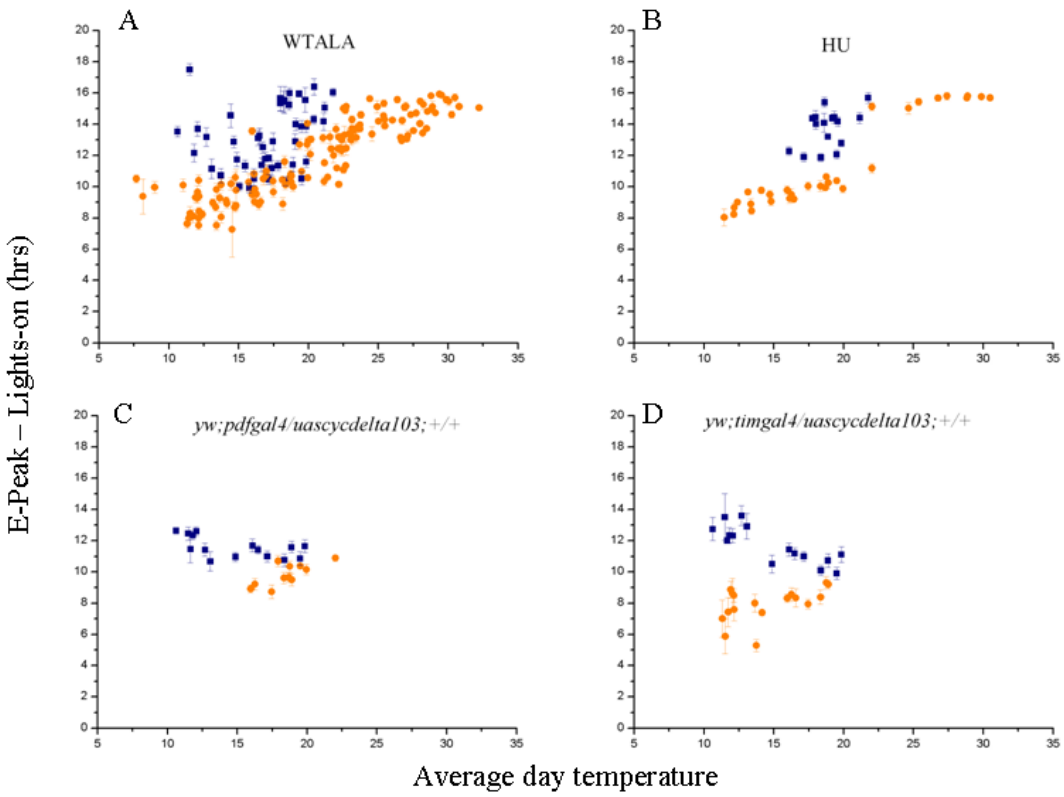


Figure 5.7: E-Peak – Lights-on (hrs ± SEM) plotted against average day temperature (°C). Leicester – blue, Padova - orange.

The correlation and p values of the regression lines of the data reported in figures 5.6 and 5.7 are listed in table 5.3. It can be seen that the correlation of both the wild-types WTALA and HU are positive while those of *yw;pdfgal4/uascycΔ103;+/+* and *yw;timgal4/uascycΔ103;+/+* are negative. This comparison confirms the previous observation that, for the limited data reported here, stopping the clock in all or some of the clock cells not only results in a disruption of the correlations between evening onset and peak with temperature but inverts the relationship. This observation needs to be confirmed by adding data collected on hot days.

Table 5.3: Correlation between E-onset – Lights-on and E-peak – Lights-on and average day temperature.

Genotype	E-onset – T-Peak		E-Peak – T-Peak		N
	R	p-value	R	p-value	
WTALA	0.6179	<0.0001	0.7006	<0.0001	185
HU	0.7769	<0.0001	0.8099	<0.0001	47
yw;pdfgal4/uascycΔ103;+/+	-0.8544	<0.0001	-0.5367	0.0039	26
yw;timgal4/uascycΔ103;+/+	-0.3821	0.0372	-0.0691	0.7167	29

5.3.3 Comparing evening activity of mutants to wild-types

A graphic representation of the regression lines obtained from the comparisons of position of evening onset and peak with time of temperature peak and average day temperature (figs 5.4 and 5.5) is shown in figure 5.8A and B respectively. From these figures, the negative correlation of the regression lines for *yw;pdfgal4/uascycΔ103;+/+* and *yw;timgal4/uascycΔ103;+/+* mentioned in section 5.3.2 are evident.

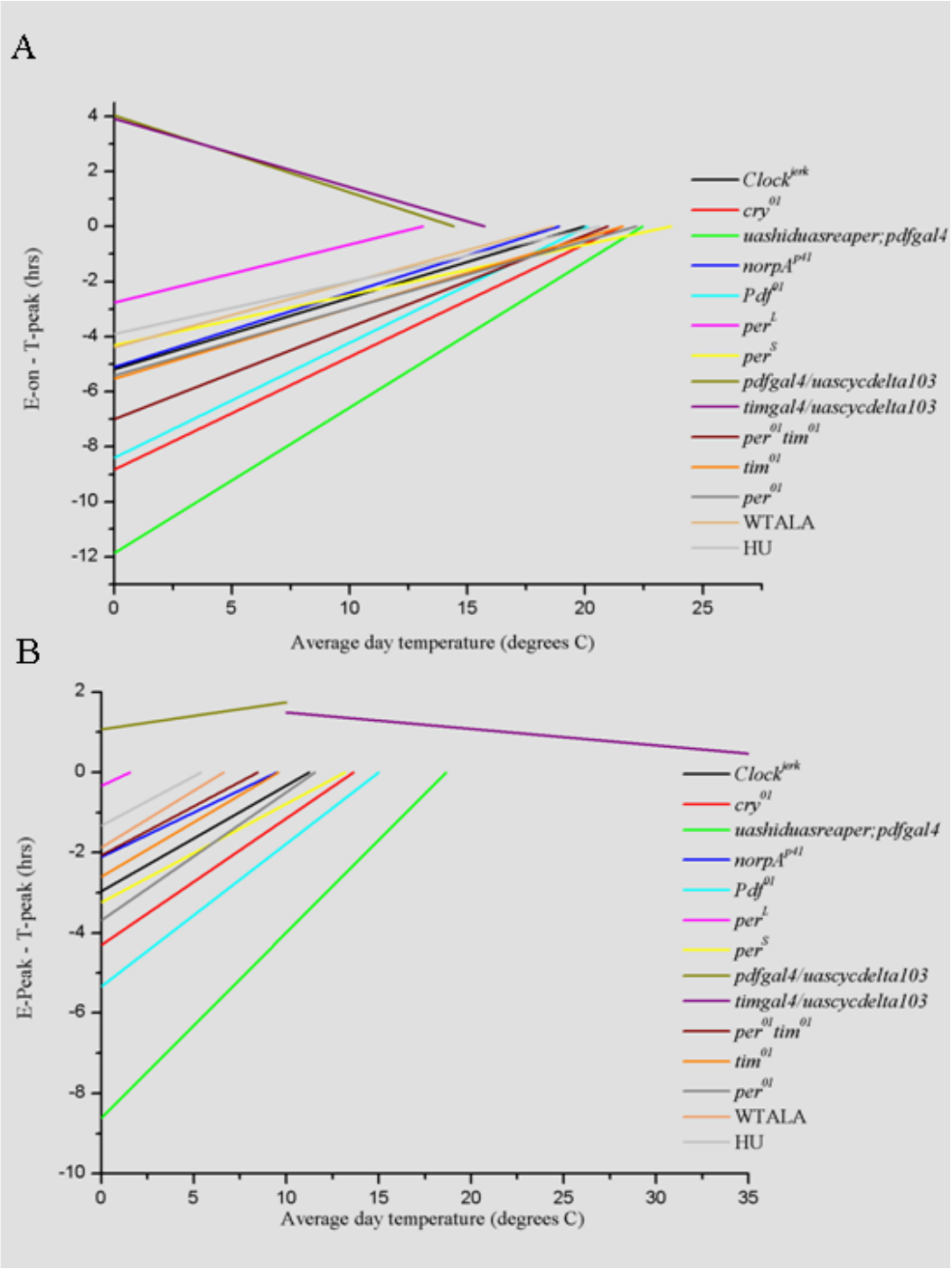


Figure 5.8: A graphic representation of regression lines for evening activity compared to average day temperature for all the genotypes analysed. (A) E-onset – T-peak (B) E-Peak – T-peak.

The slopes of the regression lines of all the strains were compared to those of WTALA and HU using Student t-tests to determine which genotypes differ from both wild-type strains (as in chapter 4). The minimum significance level has been reduced to 0.002 (0.05/number of comparisons) in order to control for multiple-testing. These statistics are summarised in table 5.4. The slopes of WTALA and HU are not significantly different from each other. It can also be seen that killing the morning cells affects the temperature sensitivity of the position of both evening onset and peak while cry^{0} and Pdf^{01} mutants and flies in which the clock has been stopped in some or all clock neurons specifically affect the temperature sensitivity of the evening onset and not the peak.

Table 5.4: Comparison of slopes of the regression lines of E-onset – T-peak and E-Peak – T-peak and average day temperature of mutants compared to wild-types (WTALA and HU) using Student t-tests. Data significantly different from both ($p<0.002$) are indicated by *.

Genotype	**E-onset – T-peak slope (p)**		**E-Peak – T-peak slope (p)**	
	V/S WTALA	V/S HU	V/S WTALA	V/S HU
WTALA	---	0.3164	---	0.4326
HU	0.3164	---	0.4326	---
per^{01}	0.8253	0.2916	0.2277	0.1270
tim^{01}	0.6665	0.2938	0.7483	0.7170
$per^{01}tim^{01}$	0.0566	0.0197	0.3367	0.8035
$Clock^{jerk}$	0.7052	0.4637	0.6909	0.9077
cry^{0}	0.0006*	0.0002*	0.3820	0.1383
Pdf^{01}	0.0005*	<0.0001*	0.0677	0.0112
per^{L}	0.6408	0.7189	0.0904	0.3370
per^{S}	0.3468	0.9249	0.4138	0.8710
$norpA^{p41}$	0.5411	0.1973	0.7449	0.4888
ywuashid,uasreaper;pdfgal4/+;+/+	<0.0001*	<0.0001*	0.0011*	<0.0001*
yw;pdfgal4/uascycΔ103;+/+	<0.0001*	0.0003*	0.0026	0.1080
yw;timgal4/uascycΔ103;+/+	<0.0001*	<0.0001*	<0.0001	0.0023

The data for all the strains of flies were also compared to both wild-types using ANCOVA (temperature as covariate) to see if at the same temperature, mutants move earlier or later than wild-type (figures comparing WTALA individually to each mutant can be seen in appendix 3) (see table 5.5). The minimum significance level has been reduced to 0.002 (0.05/number of comparisons) in order to control for multiple-testing. Some strains, including *yw;pdfgal4/uascycΔ103;+/+* and *yw;timgal4/uascycΔ103;+/+* were excluded from the ANCOVA analysis because the slope of their regression lines are significantly different from wild-type. In this comparison too, WTALA and HU are not significantly different. All strains of flies (except per^L) show an advance in the position of their evening peak as compared to WTALA and HU. Conversely, the evening onset appears to be unaffected by all clock mutants except per^L. It is interesting to note that the evening parameters of onset and peak are affected by *per* mutants such that per^L delays the position of the evening onset while per^S and per^{01} advance that of the evening peak as compared to wild-type.

Table 5.5: Comparison of E-onset – T-peak and E-Peak – T-peak between mutants and wild-type (WTALA and HU) using ANCOVA with average day temperature as the covariate. Data significantly different from both ($p<0.002$) are indicated by *. (A, Advance; D, Delay)

Genotype	**E-onset – Tpeak ANCOVA**		**E-Peak – Tpeak ANCOVA**	
	V/S WTALA	V/S HU	V/S WTALA	V/S HU
WTALA	---	$F_{(1,229)}=2.583$ p=0.1093	---	$F_{(1,229)}=0.49$ p=0.4842
HU	$F_{(1,229)}=2.583$ p=0.1093	---	$F_{(1,229)}=0.49$ p=0.4842	---
per^{01}	$F_{(1,273)}=16.91$ p~ 0	$F_{(1,135)}=2.444$ p=0.1202	$F_{(1,273)}=42.23$ p~ 0 A*	$F_{(1,135)}=17.79$ p~ 0 A*
tim^{01}	$F_{(1,244)}=8.969$ p=0.0030	$F_{(1,106)}=1.002$ p=0.3189	$F_{(1,244)}=26.12$ p~ 0 A*	$F_{(1,106)}=11.92$ p=0.0007A*
per^{01}*tim*^{01}	$F_{(1,221)}=1.976$ p=0.1611	$F_{(1,83)}=0.042$ p=0.8368	$F_{(1,221)}=22.68$ p~ 0 A*	$F_{(1,83)}=12.306$ p=0.0007A*
Clock^{jerk}	$F_{(1,217)}=1.041$ p=0.3087	$F_{(1,79)}=0.056$ p=0.8128	$F_{(1,217)}=39.245$ p~ 0 A*	$F_{(1,79)}=24.418$ p~ 0 A*
per^{S}	$F_{(1,219)}=11.79$ p=0.0007	$F_{(1,81)}=2.08$ p=0.1530	$F_{(1,219)}=81.63$ p~ 0 A*	$F_{(1,81)}=44.87$ p~ 0 A*
per^{L}	$F_{(1,218)}=13.21$ p=0.0003D*	$F_{(1,80)}=20.42$ p~ 0 D*	$F_{(1,218)}=0$ p=1	$F_{(1,80)}=1.124$ p=0.2922
cry^{0}	---	---	$F_{(1,221)}=63.173$ p~ 0 A*	$F_{(1,83)}=48.61$ p~ 0 A*
norpA^{p41}	$F_{(1,213)}=0.075$ p=0.7844	$F_{(1,75)}=3.135$ p=0.0806	$F_{(1,213)}=45.92$ p~ 0 A*	$F_{(1,75)}=32.79$ p~ 0 A*
Pdf^{01}	---	---	$F_{(1,217)}=55.27$ p~ 0 A*	$F_{(1,79)}=48.52$ p~ 0 A*
ywuashiduasreaper; pdfgal4/+;+/+	---	---	---	---

5.3.4 Evening activity in darkness

The experiment described in section 4.3.5, where flies were exposed to natural conditions for 3 days and then half of them were covered up with a black screen for a few days and the other half were maintained in natural conditions, was also analysed for its effect on the evening peak. Figure 5.9 shows the activity profile of WTALA (A), per^{01} (B), tim^{01} (C) and cry^{0} (D) in natural conditions (blue) and complete darkness (green) on the same days. The light intensity outside is represented in yellow and the temperature is in red. It can be seen that in darkness, WTALA, tim^{01} and cry^{0} show a clear morning and evening peak but the morning peak is much broader in darkness that in natural conditions (except cry^{0}). The evening peak is preceded by a dramatic decline of activity in both conditions. The bimodal behaviour of per^{01}, on the other hand, is completely lost in darkness and instead, it appears that their locomotor activity simply tracks temperature changes.

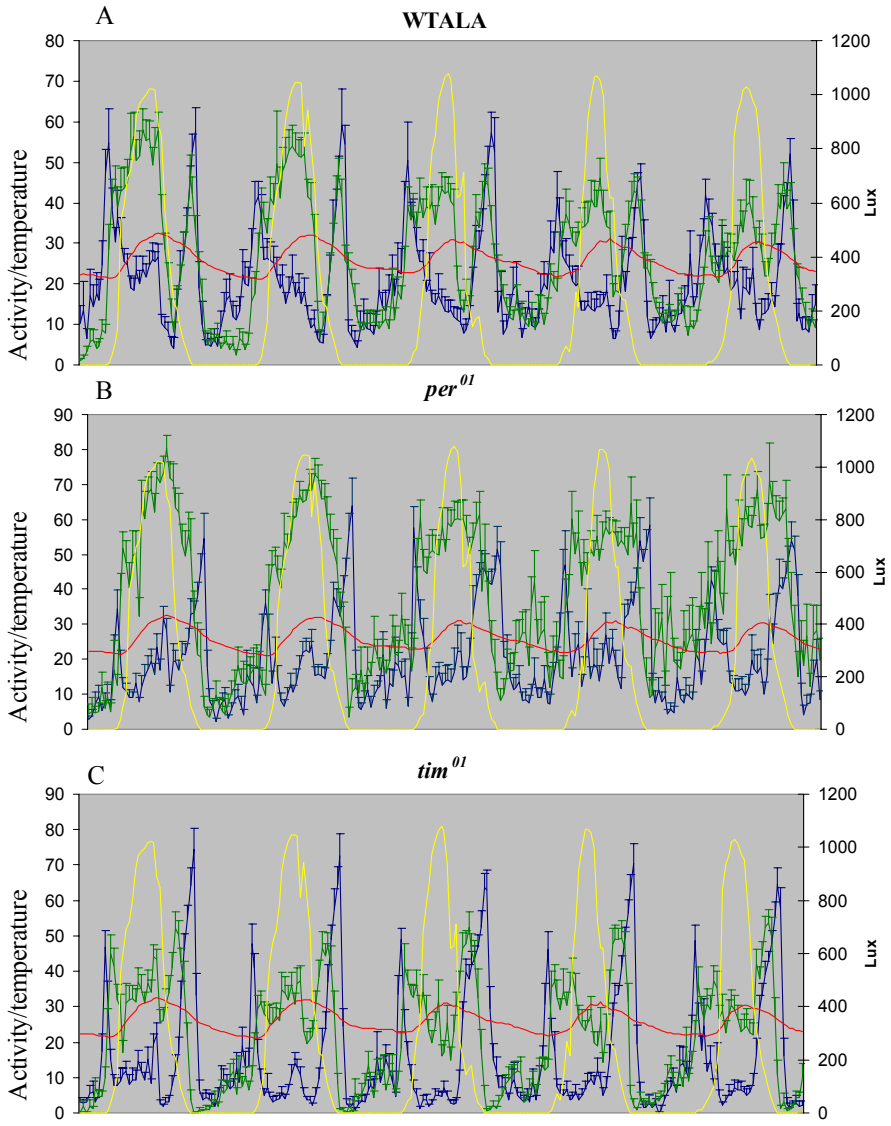


Figure 5.9: Mean activity (+SEM) profile of flies maintained in natural conditions (blue) compared to those in darkness (green). The external light intensity is represented in yellow and temperature in red. All strains except *per*^{01} retain the evening peak.

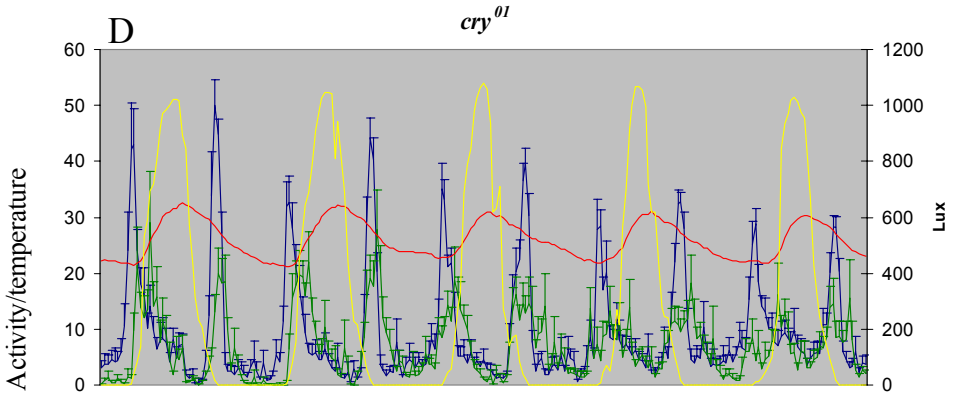


Figure 5.9 (continued)

This experiment was analysed on a fly-by-fly basis to investigate whether the position of the evening peak changes between natural conditions and darkness for WTALA, tim^{01} and cry^{0}. per^{01} was excluded from this analysis because there was no detectable evening peak in darkness. An ANCOVA was used to compare the data because the temperature was different between the 5 days of the experiment and had to be used as a covariate. The ANCOVA revealed that the position of evening onset of WTALA does not differ between natural conditions and darkness, whereas the evening peak does, occurring earlier in darkness. In tim^{01} the position of both the onset and peak is advanced in darkness compared to natural conditions (see table 5.6). cry^{0} mutants do not show any difference between the position of the evening peak in natural conditions and darkness (open and filled blue circles in figure 5.10). In fact, their evening peak in natural conditions and darkness occurs at the same time as that of WTALA in darkness (filled black squares in figure 5.10) (p=0.3288). Thus, natural light does not delay the evening peak of cry^{0} as it does for the other strains.

Table 5.6: Summary of statistics (ANCOVA) used to analyse the difference between the position of evening onset and peak in natural conditions and darkness.

Genotype	E-onset – T-peak	E-Peak – T-peak
WTALA	$F_{(1,225)}$=2.079 p=0.1506	$F_{(1,225)}$=21.95 p~ 0
tim^{01}	$F_{(1,242)}$=22.128 p~ 0	$F_{(1,242)}$=193.69 p~ 0
cry^{0}	$F_{(1,175)}$=1.633 p=0.2028	$F_{(1,175)}$=0.243 p=0.6225

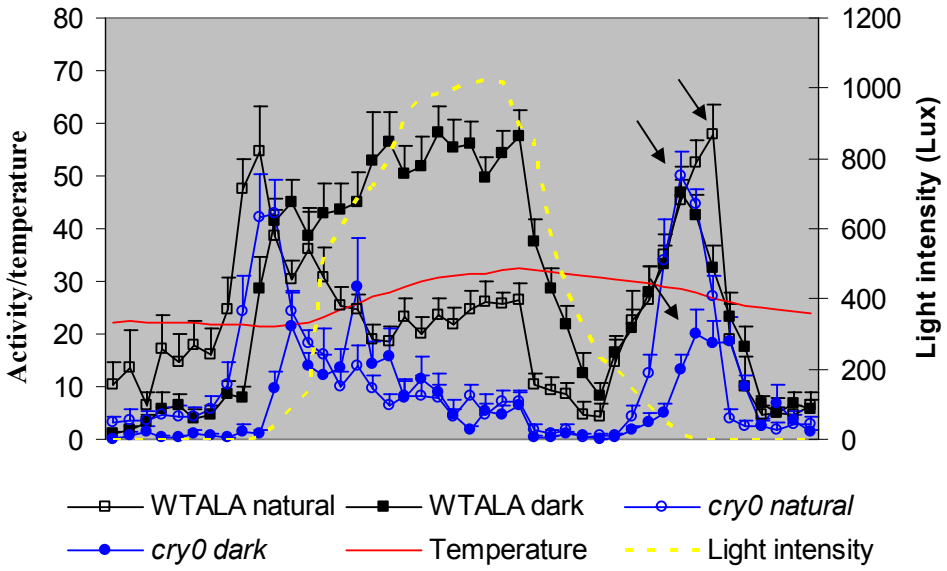


Figure 5.10: Mean activity profiles (+SEM) of WTALA and *cry*^{0} on one representative day in natural conditions and darkness. The evening peaks of WTALA and *cry*^{0} in darkness (filled squares and circles respectively) coincide with that of *cry*^{0} in natural conditions (open circles) (p=0.3288). WTALA in natural conditions (open squares) peaks later than all others. Light intensity - yellow, temperature - red.

5.4 Discussion

The position of the evening peak, like that of the morning, appears to be sensitive to the environmental cues of temperature and light. The evening onset and peak of flies in natural conditions were compared to the time of day that temperature starts to decline. This was done to explore whether the increase in activity occurs just as

a response to this or any other environmental cue (such as decreasing light intensity). In WTALA and HU, on cold days, the evening onset does occur close to the time of temperature peak and on some days it even occurs a few hours earlier (figure 5.4A and B). However, on hot days, the evening onset occurs as many as 6-8 hours after the temperature peak. The trend in position of the evening peak, of course, closely follows that of the onset (figure 5.5A and B). Thus, evening behaviour could not be a direct response to any environmental change. In the laboratory too, it has been shown that the position of the evening behaviour depends on temperature such that on cold days it occurs earlier than on hot days. The molecular mechanism believed to be responsible for this is the alternative splicing of the 3'UTR of *per* which is enhanced at cold temperatures and results in an early upswing of the protein and a subsequent increase in locomotor activity (Majercack *et al.*, 1999). However this molecular mechanism cannot be solely responsible for this phenotype because the correlation between evening behaviour and temperature is retained in per^{01} and $per^{01}tim^{01}$ mutants (figure 5.4B and D, figure 5.5B and D, table 5.2).

Furthermore, the correlation between evening behaviour and temperature is compromised in *yw;pdfgal4/uascycΔ103;+/+* and *yw;timgal4/uascycΔ103;+/+* strains to such an extent that it changes from the normal positive relationship to an inverse one. (table 5.2). This is confirmed by comparing evening behaviour to Lights-on and obtaining a similar result (table 5.3). A newly emerging view is that the evening cells are dominant in light and morning cells in darkness (Murad *et al.*, 2007; Picot *et al.*, 2007; Stoleru *et al.*, 2007). This property is believed to be responsible for the seasonal responses of the circadian clock such that the M-cells control activity in winter with short photoperiods and the E-cells control locomotor activity in summer with relatively long photoperiods. This is in agreement with the observation that light activates output

from E-cells and inhibits M-cells (Picot *et al.*, 2007). In natural conditions, however, it can be seen that even at photoperiods longer than 12 hours, killing the morning cells (*ywuashiduasreaper; pdfgal4/+;+/+*) or using Pdf^{01} gives a significant enhancement of the temperature-sensitivity of the evening onset (compare slopes in figure 5.8) whereas stopping the clock in M or M and E cells (*yw;pdfgal4/uascycΔ103;+/+* and *yw;timgal4/uascycΔ103;+/+)* reverses the temperature-evening onset relationship (table 5.4, fig 5.8). These observations are based on a limited data set with few points in high temperatures and are being confirmed by collaborators. Even so, such temperature effects on evening behaviour, particularly by the morning PDF cell manipulations in LD conditions are novel. Until relatively recently, PDF was thought to only affect the anticipation of morning in LD conditions (Lin *et al.*, 2004). However, recent work has shown that, unlike wild-type, the evening peak of Pdf^{01} flies do not track photoperiod (Yoshii *et al.*, 2009).

When compared to wild-type, cry^{0} also affects the temperature-sensitivity of the evening onset (but not the peak) (table 5.4). It has already been demonstrated in laboratory studies that along with photoreception, CRY also plays a role in temperature responses of the circadian clock (Kaushik *et al.*, 2007). In addition Rosato *et al.* (2001) showed that CRY interactions in the yeast-two hybrid system are temperature-sensitive. The work described in natural conditions adds further support to the view that CRY is a remarkably versatile molecule with both photoreceptive and thermal functions for circadian behaviour.

In the laboratory, it has been shown that, in LD conditions, the evening peak of per^{S} is advanced and that of per^{L} is delayed compared to wild-type (Hardin *et al.*, 1992). The same effect is observed when DD is accompanied by temperature cycles (Busza *et al.*, 2007). In natural conditions, at the same temperature, the evening onset

occurs later in per^L whereas the peak occurs earlier in per^S when compared to wild-type (table 5.5). Therefore, it is observed that, in natural conditions too, the period altering alleles, per^S and per^L, retain their effects on the position of evening behaviour. It has been argued that loss of *per* function (as in per^{01}) does not make flies arrhythmic in DD, but instead they manifest significant ultradian rhythmicity (Dowse *et al.*, 1987; Helfrich and Engelmann, 1987; Helfrich-Forster, 2000). In natural conditions, per^{01} flies have a clear evening peak and at the same temperature, this peak occurs earlier than that of wild-type. By analogy to the per^s result, this work further supports the view of the presence of shorter rhythms in per^{01} under natural light-dark and temperature cycles.

The effect of darkness on evening behaviour in moderately hot conditions has also been evaluated in this chapter (figure 5.9). Not only WTALA, but also tim^{01} and cry^0 maintain a robust evening peak in darkness, as in natural conditions. The presence of an evening peak in tim^{01} could be explained as a physiological response to temperature changes. If this were true, the same might be observed in per^{01}. However, tim^{01} clearly has a different profile to per^{01}, whose evening peak is completely lost, and whose behaviour appears to track changes in temperature (figure 5.9B). In fact, this dramatic effect of per^{01} on the evening peak is even more remarkable since there was little effect of per^{01} on morning behaviour (chapter 4). These observations imply that, in natural conditions, *per* mutants preferentially affect the evening component of locomotor activity behaviour. On the other hand, it seems extraordinary that an apparently clockless mutant such as tim^{01}, in contrast to per^{01}, should show M and E components in natural temperature cycles at all (figure 5.9C). The inescapable conclusion would seem to be that a residual clock in tim^{01} flies is still timing M and E behaviour in natural temperature cycles.

A detailed analysis and comparison of the position of the evening onset and peak in darkness and natural conditions (table 5.6) revealed that the evening onset occurs at the same time in WTALA while the peak is delayed in natural light. The evening onset and peak of tim^{01} are both delayed in natural lighting when compared to darkness. In cry^{0}, light does not delay the position of the evening peak and in fact, this occurs at the same time at that of WTALA in darkness (figure 5.10). This implies that the presence of light does not affect the timing of the evening behaviour of cry^{0}. This is in accordance with the fact that cry^{0} is known to be compromised in its properties of circadian photoreception (Dolezelova *et al.*, 2007). However, this effect of CRY on the timing of evening behaviour is probably through a TIM-independent pathway as tim^{01} flies behave similarly to wild-type.

All these observations enforce the clock-like nature of the evening peak in natural conditions, even in some classic 'clockless' mutants. However, its position is modulated by seasonally changing environmental parameters such as temperature and photoperiod.

5.5 Conclusions

- The timing of the position of evening onset and peak is sensitive to the average temperature of the day such that they occur earlier on cold days than hot ones.
- The correlation between position of evening onset and peak and temperature remains uncompromised in most clock mutants.
- Killing the M cells or stopping the functioning of the clock in M or both M and E cells appears to disrupt the correlation between position of evening behaviour and temperature. However, since these results are based on a limited temperature range, they need to be confirmed by increasing the data-set.

- In natural conditions, as in the laboratory, per^{S} and per^{01} advance morning behaviour while per^{L} delays it.
- In darkness, per^{01}, but not tim^{01}, mutants lose the prominent evening peak which is present in corresponding natural conditions and appear to track temperature changes.
- Light seems to delay evening behaviour because the onset and peak of wild-type flies occur earlier in darkness than on the same days in natural conditions. This effect seems to be a TIM-independent effect of CRY.

Chapter 6: Results – Cycling of TIM in natural conditions

6.1 Introduction

The circadian locomotor activity profile of *Drosophila* has been extensively investigated in the laboratory. A large amount of information about the molecular basis of the clock has been accumulated over the years. Various clock genes have been identified and their function has been elucidated (see section 1.3). Most of the genes that constitute the core of the clock cycle in the abundance of their products (RNA and protein) over the course of a day. This cycling of core clock gene products is the molecular basis for the circadian activity profile of flies in the laboratory.

The activity profile of wild-type and mutants in natural conditions has already been described in great detail in previous chapters. At the level of the molecular clock, a preliminary investigation into the cycling of TIM in the peripheral clock of the eyes and the master clock of the brain of WTALA has been initiated. Since it is believed that CRY-mediated TIM degradation is an important link between the environment and the endogenous clock (Ceriani *et al.*, 1999), TIM was chosen as the protein of interest in the work reported below.

6.2 Results

6.2.1 Cycling of TIM in the peripheral clock of the eyes – western blot

WTALA flies were entrained to natural conditions in Leicester for 3 days and then 8 collections were made at 3 hour intervals from midnight on 5th September 2006. The photoperiod during this time was 15 h and the temperature cycled between 17°C at night till 25°C during the day. Western blots were performed (ά -TIM in rat, 1:1,000) on heads of flies to check for cycling of TIM (two replicates) in the peripheral clock of

their eyes (figure 6.1A). A western blot was also performed in laboratory conditions of LD 15:9 and constant temperature of 25°C (figure 6.1B). This was done to check if, at the same photoperiod, the natural temperature cycle has an effect on TIM cycling. In order to correct for loading errors, the same membrane was probed for Heat Shock Protein (HSP70) which is constitutively expressed at all times. The intensity of the band corresponding to TIM was then normalised to HSP70 in the same lane. Also, tim^{01} was used as a negative control. The relative abundance of TIM in natural and laboratory (control) conditions is reported in Figure 6.1C. It can be seen that the cycling of TIM in natural conditions closely resembles that in the laboratory control with a peak at ZT 21, indicating that in natural conditions, the effect of light on TIM cycling dominates over any effect of temperature cycles.

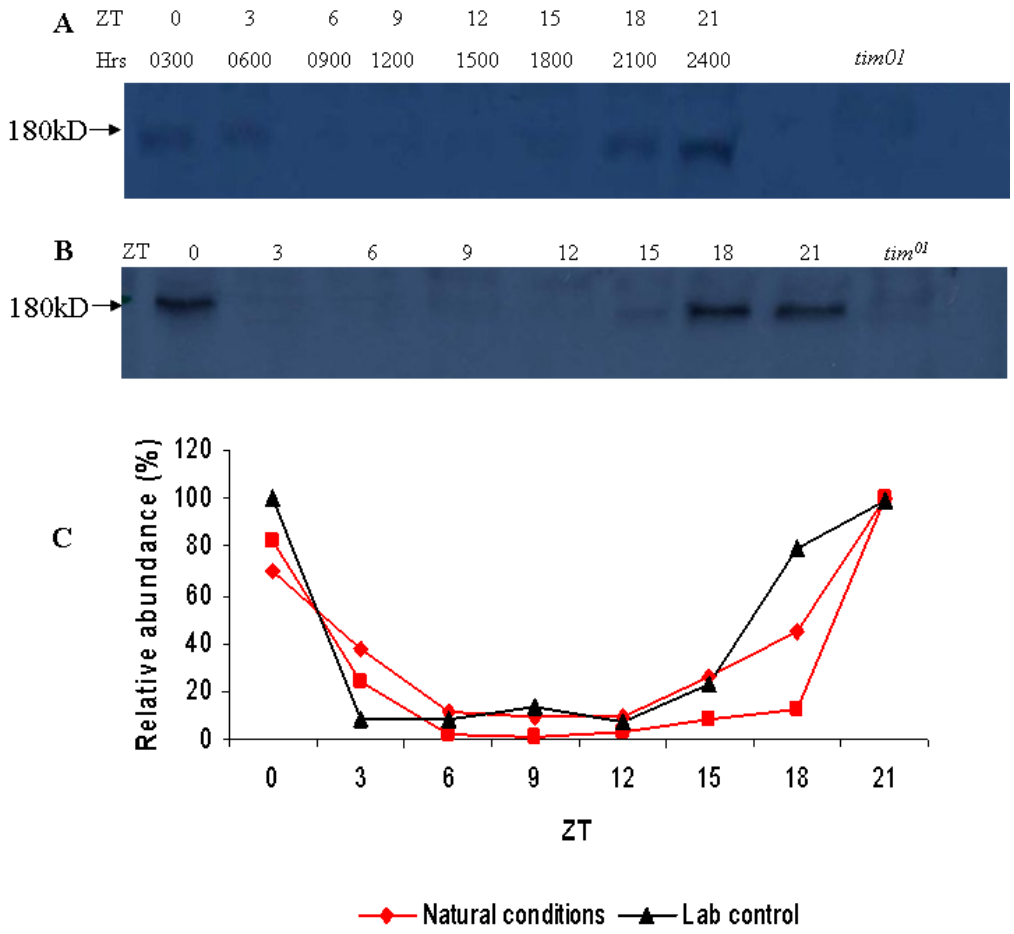


Figure 6.1: Western blot for TIM on *Drosophila* heads. (A) Natural conditions (collected on 5th September 2006; LD 15:9, temperature 25:17°C), (B) Laboratory control (LD 15:9, temperature 25°C) and (C) Cycling profile. Two replicates were performed for the collection in natural conditions and these are individually represented in red in C. tim^{01} was used as a negative control and does not show a band corresponding to TIM (~ 180kD). The profiles in natural conditions resemble the laboratory control with a peak at ZT 21.

6.2.2 Cycling of TIM in clock neurons – immunocytochemistry

In order to check the integrity of the antibody used for ICC experiments, a control experiment was performed at ZT 21 on brains of WTALA flies entrained to LD 12:12 and constant temperature 25°C (standard laboratory conditions). Figure 6.2 shows the lateral (A) and dorsal (B) neurons stained for PDF and TIM and also the merge of the two. In figure 6.2A, it can be seen that the sLNvs and lLNvs are stained for both

PDF and TIM. However, the LNds (stars) that do not express PDF are only stained for TIM. Also, the 5th sLNv, which is PDF^{-}, can be clearly seen (arrows in figure 6.2A). The dorsal neurons, in particular the DN1s and DN3s can also be clearly distinguished in the TIM staining and they lie close to the dorsal projections that originate from sLNvs (see PDF staining in figure 6.2B). The DN2s cannot be clearly distinguished in this brain. These observations indicate that the anti-TIM antibody is specific and can be used for further experiments.

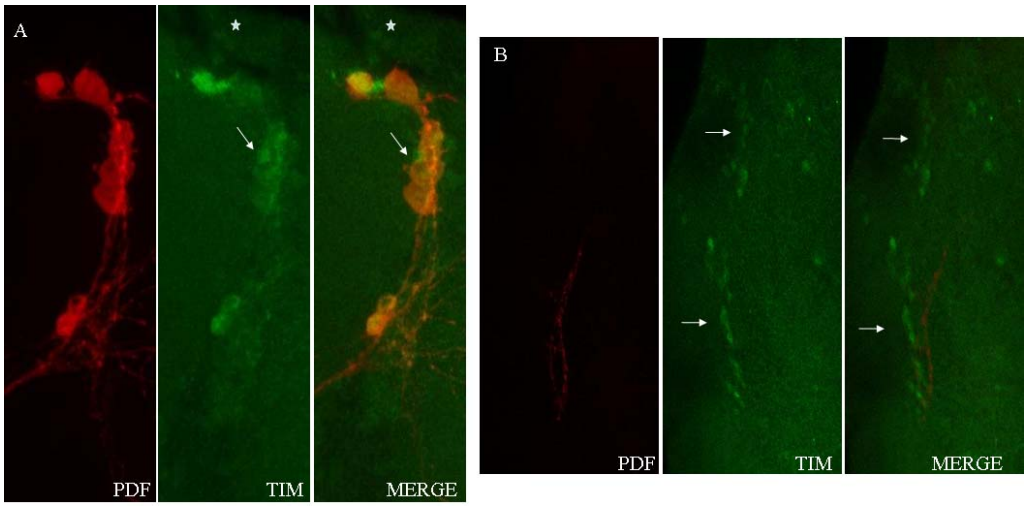


Figure 6.2: TIM ICC on flies collected at ZT 21 after entrainment to LD 12:12 and temperature 25°C. (A) Lateral neurons – the sLNvs and l-LNvs are seen to be stained for both PDF and TIM but the 5th sLNv (PDF^{-}) is only stained for TIM (arrows). The LNds are also faintly visible in the staining for TIM (stars). (B) Dorsal neurons – the DN1s and DN3s (arrows) are clearly stained for TIM and lie close to the dorsal projections from the lateral neurons (stained by PDF). The DN2s cannot be clearly distinguished in this brain.

WTALA flies were entrained for 3 days to natural conditions in Padova and collected every 3 hours from 3PM on 20th May 2009 till 3PM the next day. The locomotor activity profile for WTALA on these days can be seen in appendix 4-1. It can be seen in figure 6.3 (A, B and C) that in all the groups of neurons, the TIM staining is only visible during the night hours from midnight till 6 AM (environment monitor 0 lux

at 9 PM and >1 lux at 6AM). There is no signal at any point during the light hours of the day. It is interesting to note that the Lateral Posterior Neurons (LPNs) are visible in this experiment, even though they were not observed in the laboratory control. This could be due to the high temperature range (31:21°C) during the day of collection.

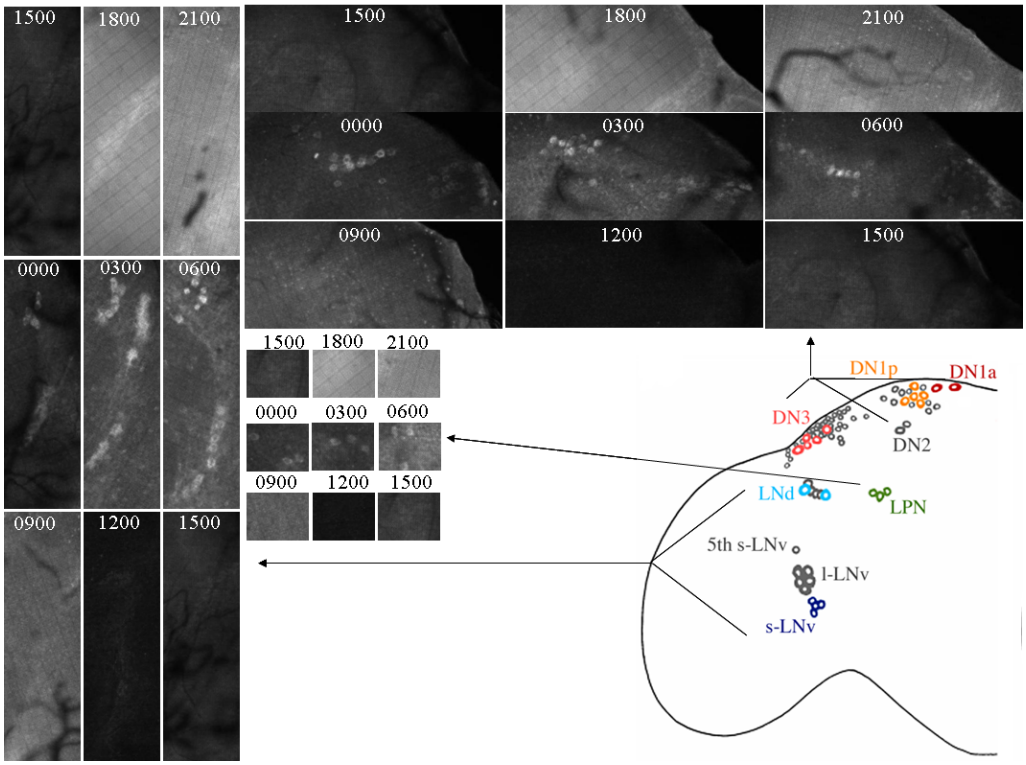


Figure 6.3: TIM ICC on flies collected in natural conditions in Padova on 20-21st May 2009. TIM staining was only observed in 3 time-points between midnight and 6 AM (darkness). (A) Lateral neurons, (B) Dorsal neurons and (C) Lateral Posterior neurons. (Figure of brain taken from Shafer *et. al.*, 2006).

The amount of TIM signal in each set of clock neurons has been quantified (using ImageJ 1.42q) for all the time-points and the cycling profile is reported in figure 6.4. TIM expression in the various groups of lateral neurons (figure 6.4A) including 5th PDF$^-$ sLNv, lLNvs and LNds peaks at 6AM. Due to the broad peak of PDF$^+$ sLNvs, it is not possible to determine the exact time of the peak of TIM abundance. The dorsal neurons (figure 6.4B), i.e. DN1s and DN2s (DN3s were not quantified), appear to peak

between midnight and 3AM, which is earlier than the lateral neurons. It was not possible to determine the time of peak of the lateral posterior neurons (LPNs). A summary of the ANOVAs performed on each group of neurons is provided in table 6.1.

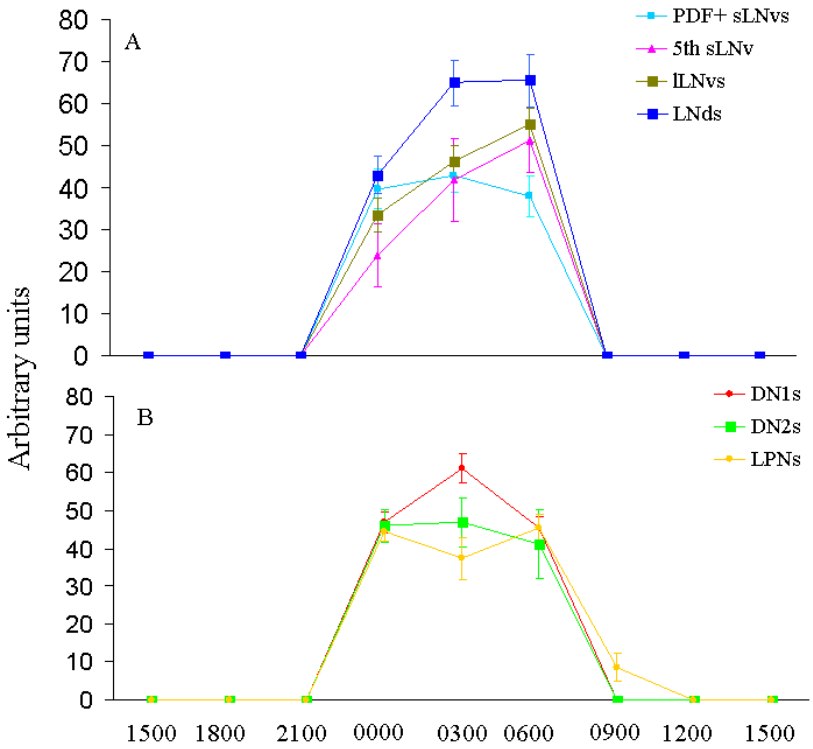


Figure 6.4: Cycling profile of TIM abundance (staining intensity ± SEM) in clock neurons of flies collected in natural conditions in Padova on 20-21st May 2009. (A) Lateral neurons and (B) Dorsal neurons and LPNs. TIM abundance appears to peak earlier in dorsal neurons (~ 3AM) than lateral neurons (~ 6AM).

Table 6.1: Time of peak of TIM expression in the various groups of clock neurons in natural conditions of Padova on 20-21st May 2009. ANOVAs are used to show any significant time differences.

Neuron group	Peak	Peak (p_{ANOVA})
PDF^+ sLNvs	NA	0.8010
5^{th} PDF^- sLNv	3-6AM	0.0582
lLNvs	6AM	0.0005 *
LNds	3-6AM	0.0053 *
LPNs	NA	0.3092
DN1s	3AM	0.0013 *
DN2s	0-3AM	0.8158

In another ICC done on TIM on a cold day (11-12th September 2008) in Leicester, there was a very weak signal for TIM (representative brain at 3AM in figure 6.5). The activity profile of WTALA for these days can be found in appendix 4-2. The temperature range during this collection was from 11°C at night till 18°C in the day. The cold temperatures at night could be responsible for the lack of TIM staining during this time.

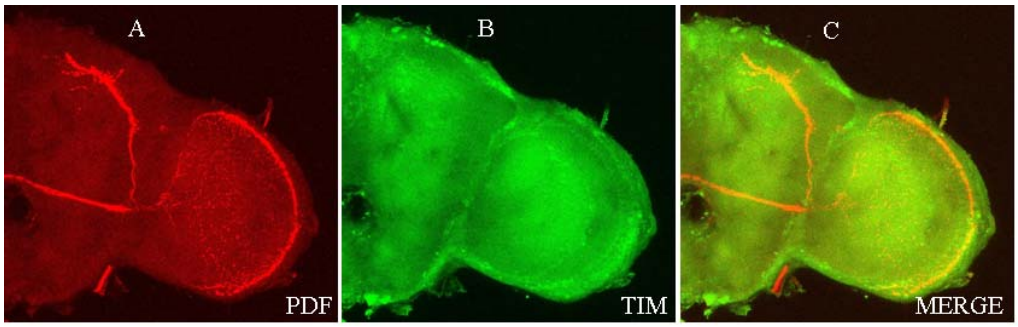


Figure 6.5: Representative brain at 3AM showing the low signal obtained for TIM in flies collected in natural conditions in Leicester on 11-12th September 2008.

6.2.3 Does the clock in the evening cells run with a shorter period?

Some experiments were conducted in the laboratory to check the effect of eliminating the PDF-expressing morning cells (*ywuashiduasreaper;pdfgal4/+;+/+*) or stopping the clock in these neurons (*yw;pdfgal4/uascycΔ103;+/+*). These flies were

entrained to LD 12:12 for 3 days at constant temperature 25°C and then released in DD for at least 5 days in order to determine the free-running period of locomotor activity. It was found that when the sLNvs and lLNvs are removed from the network of clock neurons, 35-55% of the flies are rhythmic whereas according to the current model, the loss of these pacemaker cells should result in arrhythmicity in DD. The rhythmic flies exhibit a shortening of their period by approximately 3 h compared to controls (table 6.2). An ANOVA (Neuman-Keuls Posthoc test) was used to compare the test flies with both their appropriate controls (GAL4 driver and UAS responder) and was found to be highly significant.

Table 6.2: Rhythmicity and period of various strains specifically disrupting the morning cells and their respective controls. Data were compared using an ANOVA and found to be highly significant.

Strain	**N**	**Rhythmic flies (%)**	**Period (±SEM)**	**F-ratio**	**p value**
ywuashiduasreaper;+/+;+/+	31	28 (90)	24.04 (±0.12)		
ywuashiduasreaper;pdfgal4/+;+/+	29	10 (34.5)	21.57 (±0.21)	93.095	~ 0
yw;pdfgal4/+;+/+	21	21 (100)	24.24 (±0.08)		
yw;uascycΔ103/+;+/+	24	23 (95.8)	23.26 (±0.23)		
yw;pdfgal4/uascycΔ103;+/+	34	19 (55.8)	21.31 (±0.39)	39.648	~ 0

6.3 Discussion

It is known from laboratory studies that TIM is degraded in the presence of light (Hunter-Ensor *et al.*, 1996; Lee *et al.*, 1996; Young *et al.*, 1996; Zeng *et al.*, 1996). It has also been shown that TIM production is stimulated at high temperatures as compared to low (Majercak *et. al.,* 1999). In nature, the period of daylight is always accompanied by higher temperature than that at night. Thus light and temperature constitute two opposite forces acting on TIM accumulation. Western blot has revealed that in the peripheral clock of the eyes TIM does seem to cycle in a manner similar to that of the laboratory control (figure 6.1). Since the laboratory control was performed at

the same photoperiod as the day of 'natural collection' but without a temperature cycle, this indicates that, at the molecular level, the effect of photoperiod dominates over that of temperature. Also, it can clearly be seen that, irrespective of the natural temperature cycle, TIM is degraded during the day and accumulates during the night.

A preliminary investigation into the cycling of TIM in clock neurons has revealed that the dorsal and lateral group of neurons may differ in their timing of TIM accumulation (figure 6.3). For the experiment reported in section 6.2.2, in the case of the neuronal groups, sLNvs, LPNs and DN2s, it is not possible to distinguish the peak of TIM abundance (table 6.1). This could be due to the fact that TIM could only be detected in 3 of the 9 time points (due to a long photoperiod on day of collection). Since collections every 3 hours did not provide enough resolution of the cycle of TIM abundance, the experimental design should be revised such that more collections are done during the night and less during the day. However, even with the experiment reported above, it can be seen that the dorsal neurons peak earlier (~ 3 hrs) than the lateral neurons (figure 6.4 and table 6.1). A similar observation, that of a difference in the cycling of TIM in lateral and dorsal neurons, has been made in the laboratory by Miyasako *et al.* (2007). They found that when LD and temperature cycles are applied 6 hrs out of phase with the thermocycle preceding the light-dark cycle, the dorsal neurons and LPNs peak earlier than the lateral neurons in TIM abundance. Thus, at a molecular level the DNs and LPNs were in phase with the temperature cycle while the lateral neurons continued to follow the phase of the light cycle. In nature, the peak and trough of temperature and light cycles are indeed out of phase by a couple of hours albeit in the opposite direction from the paper discussed above. However, the phase difference itself could be enough to de-couple the two groups of neurons at the molecular level as has been observed in the differential cycling of TIM in different groups of neurons in

natural conditions. The same trend is also found in the cycling of PER consistently across the different seasons in natural conditions in Padova (P. Menegazzi and R. Costa, personal communication). This work is being continued by our collaborators in order to confirm the preliminary findings reported here.

The observation that dorsal neurons peak earlier in TIM accumulation than the other groups could be due to their location in the fly brain. Since they are situated close to the ocelli, it is possible that they receive environmental information first and then transmit this to the other groups of neurons in the brain. It is already known that neuronal projections from the lateral to the dorsal neurons exist (Lin *et al.*, 2004; Mertens *et al.*, 2005). It is possible that there are some as-yet unidentified neuronal connections in the other direction which allow the feedback of the dorsal neurons on the lateral ones.

Another (and more likely) possibility to explain the discrepancy between the peak of TIM accumulation in dorsal and lateral neurons could be that the dorsal neurons are comprised of a clock which runs faster than the other neuronal groups. This possibility is enforced by our observation that eliminating all PDF-expressing cells (sLNvs and lLNvs) results in 35-55% behavioural rhythmicity and these flies have a short period compared to wild-type (table 6.2). Similarly, when another apoptotic gene (*bax*) was expressed in these cells, the rhythmic flies (17%) had a short period of 22.5 h compared to wild-type (Blanchardon *et al.*, 2001). Several other papers have referred to the short period behavioural rhythms that are manifested in flies lacking the PDF-expressing ventral lateral neurons. For example, removal of these cells from the circadian network by the lack of PDF in Pdf^{01} flies results in 24% rhythmicity in constant darkness and these flies have a short period (22.9 h) (Renn *et al.*, 1999). *disco* mutants, which lack the lateral neurons manifest ultradian or short circadian rhythms

(Dushay *et al.*, 1989; Dowse *et al.*, 1989). All these works suggest that clock neurons that do not express PDF have an endogenously shorter circadian rhythm than those that do.

This study of the cycling of TIM in natural conditions has shown that the main effect of the environment on the protein occurs through light in both the peripheral (eyes) and central clock. However, there is an effect of temperature cycles as seen by the fact that the neurons that are known to be particularly temperature-responsive (dorsal neurons) peak earlier in TIM abundance than the other clock neurons in the fly brain.

6.4 Conclusions

- In the eyes of flies collected in natural conditions, the effect of light on TIM dominates over that of temperature such that it is degraded during the day and accumulates during the night.
- In the brain of flies entrained to natural conditions, the accumulation of TIM peaks 3 hours earlier in DNs and LPNs as compared to LNvs and LNds. This enforces the possible role of DNs and LPNs as temperature-sensitive neurons.
- It is hypothesised that the clock in the dorsal neurons runs faster than that in the ventral neurons due to a number of observations reported above and also in the literature.

Chapter 7: Generation of an α-CRY Antibody

7.1 Introduction

The synchronisation of the endogenous circadian clock to the external environment is a topic of immense interest. One of the mechanisms of entrainment to light described in chapter 1 involves Cryptochrome, the dedicated blue-light circadian photoreceptor (Stanewsky *et al.*, 1998) which transmits light information to the core of the circadian clock via interaction with core clock proteins - TIM and PER (see section 1.6.1). It is believed to play a role in temperature entrainment though this has not yet been well elucidated (Kaushik *et al.*, 2007). CRY is also known to be a transcriptional repressor in mammals and other insects (Yuan *et al.*, 2007) and also may play a core clock function in the periphery of *Drosophila* (Krishnan *et al.*, 2001, Levine *et al.*, 2002, Collins *et al.*, 2006, Ito *et al.*, 2008). The exact mechanism by which CRY performs all these diverse roles is not known.

Most techniques which provide insights into the function of proteins, such as western blots, immunocytochemistry (ICC) and co-immunoprecipitation require the use of antibodies specific to the protein of interest. Since antibodies for most clock genes are not yet commercially available, an attempt was made to generate an α-CRY antibody. In order to do this, CRY-GST fusion protein was over-expressed in bacteria, purified and used as an antigen to produce an antibody specific to CRY. The protocol for purification of the protein was subjected to considerable modification and optimisation. The final protocol used has been described in the results section of this chapter. The antibody was produced in guinea pigs and then tested on western blots and ICC.

7.2 Results

7.2.1 Confirmation of plasmids

Two plasmids containing the coding sequence of full length (FL-CRY) or the first 188 amino acids at the N-terminal (NT-CRY) fused to the coding sequence for a *Glutathione S-transferase* (GST) tag were obtained from Ralf Stanewsky (Emery *et al.*, 1998). The integrity of the plasmid was confirmed by restriction digestion. Figure 7.1 (A and B) shows agarose gel images of the restriction digestion of pGEX-6p plasmid with *EcoRI* and *XhoI*. Two bands of DNA fragments were obtained in both cases, one corresponding to the vector (4900 base pairs) and the other either to the full-length coding sequence of 1.6 Kb (figure 7.1A) or the N-terminal of the coding sequence of approximately 500 bp (figure 7.1B). The two inserts in the vector were also confirmed by sequencing. The sequence of the insert that was finally used to generate the antibody (NT-CRY) has been aligned to the sequence of *cry* and can be found in appendix 5. The appendix also contains plasmid maps for the vector with the two inserts.

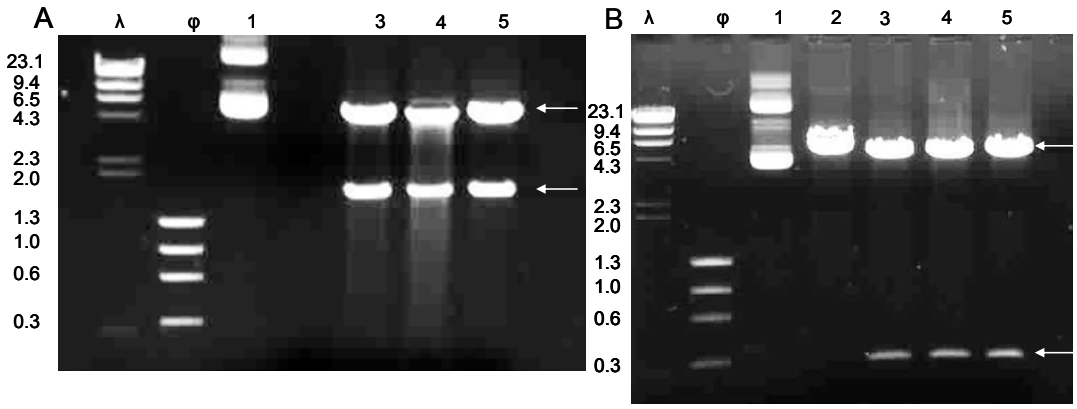


Figure 7.1: Gel image of the restriction digestion of pGEX-6p containing the inserts. (A) Full-length *cry* (B) N-terminal *cry* (amino acids 1-188). λ and φ markers containing DNA fragments of known sizes (in Kilo bases) are seen on the left. Lane 1 contains the uncut control plasmid while lanes 3, 4 and 5 contain the restriction digestion products. The vector is seen as a band of 4.9Kb and the inserts are either 1.6Kb (A) or 0.5Kb (B) (see arrows). Lane 2 in B contains the linearised form of the plasmid obtained after digestion with *EcoR1*.

7.2.2 Protein purification

Three different strains of *E. coli*, BM25.8, BL21 and XL1Blue, were transformed with the plasmids described above. Bacterial cultures were allowed to grow in standard conditions (see section 2.4.1) till they reached an optical density (OD) of 0.5. This OD represents the log phase of bacterial growth and is optimal for induction of fusion protein expression. Expression was induced by inoculating the various bacterial cultures with Isopropyl-beta-thio galactopyranoside (IPTG). The cultures were then harvested every hour to check the optimal time after induction at which the maximum fusion protein expression occurred. As expected, the amount of fusion-protein expressed increased with the time after induction with IPTG.

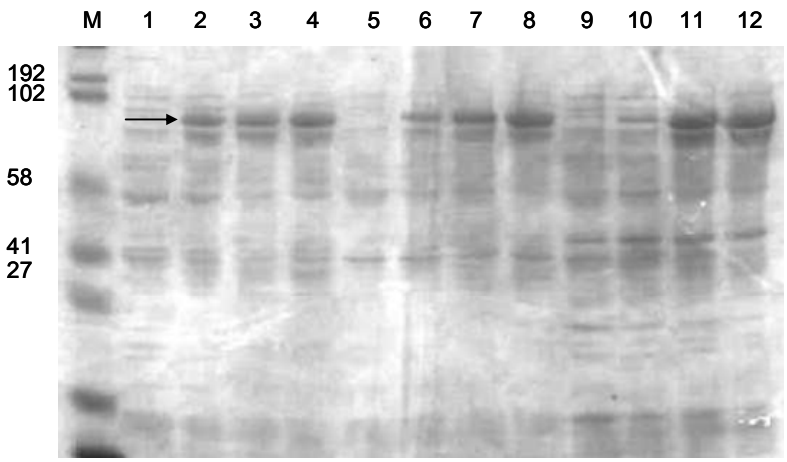


Figure 7.2: Acrylamide gel stained with coomassie-blue showing the over-expression of FL-CRY fusion protein (arrow). Protein samples from un-induced controls (lanes 1, 5 and 9) are run along with collections made 1 (lanes 2, 6 and 10), 2 (lanes 3, 7 and 11) and 3 (lanes 4, 8 and 12) hours after induction of protein expression with IPTG. The gel contains these extracts from BM25.8 (lanes 1-4), XL1Blue (lanes 5-8) and BL21 (lanes 9-12) strain of *E. coli*.

Based on figure 7.2, the bacterial strain chosen for all further work was BL21 and the cells were collected three hours after induction with IPTG. They were pelleted and re-suspended in ice-cold PBS. They were then sonicated and the soluble and insoluble fragments were separated by centrifugation. The insoluble pellet was again re-suspended in PBS and a sample of it, along with a sample of the soluble fraction, was run on an SDS-PAGE gel to check which fraction contained the protein of interest (see figure 7.3 A and B). Both FL-CRY-GST and NT-CRY-GST were found to be insoluble irrespective of the number of hours of induction.

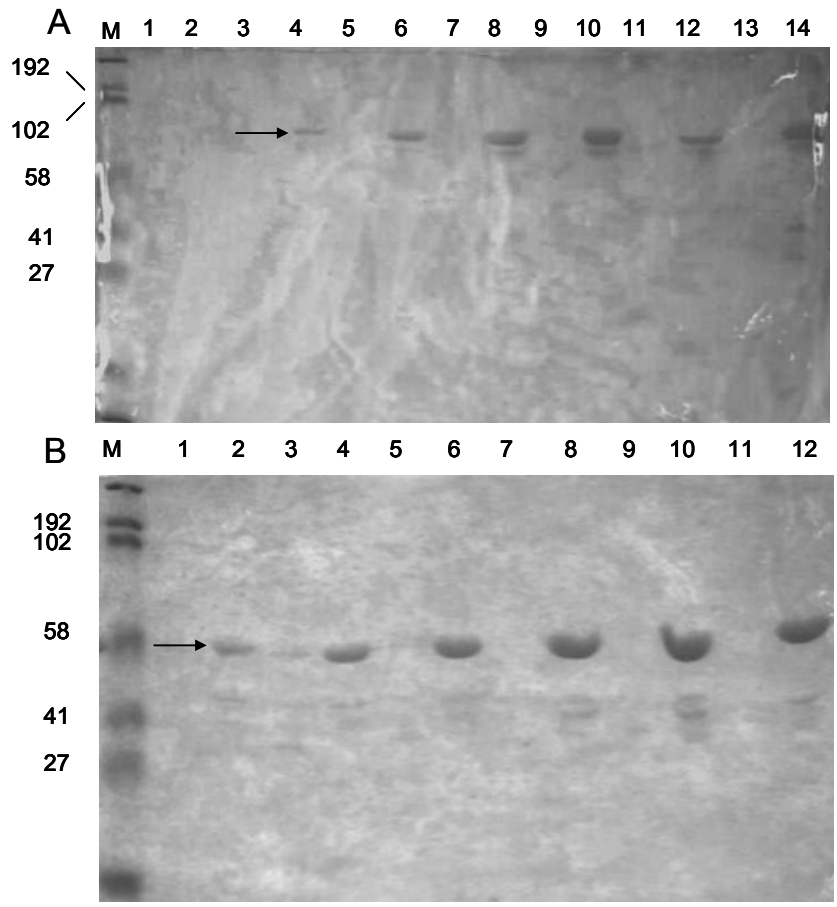


Figure 7.3: Acrylamide gel showing the soluble (odd lanes) and insoluble (even lanes) fractions of the bacterial cell protein extracts. (A) FL-CRY (B) NT-CRY. The fusion protein expression was induced by IPTG and cells were harvested 1 (lanes 1, 2), 2 (lanes 3, 4), 3 (lanes 5, 6), 4 (lanes 7, 8), 5 (lanes 9, 10), 6 (lanes 11, 12) hours and 16 (lanes 13, 14) hours after induction. Under all conditions, both FL-CRY and NT-CRY were insoluble (see arrows).

Several attempts were made to solubilise the GST-fusion protein in order to allow its binding to Glutathione beads by following the protocol described in GST Gene Fusion System handbook (GE Healthcare). However, even though the protocols designed to make the protein soluble worked (lane 2 of figure 7.4), the attempt to bind it to the beads failed as all the protein was found to be contained in the first flow-through fraction (lane 4 of figure 7.4) and none in the pull-down itself (lane 7 of figure 7.4).

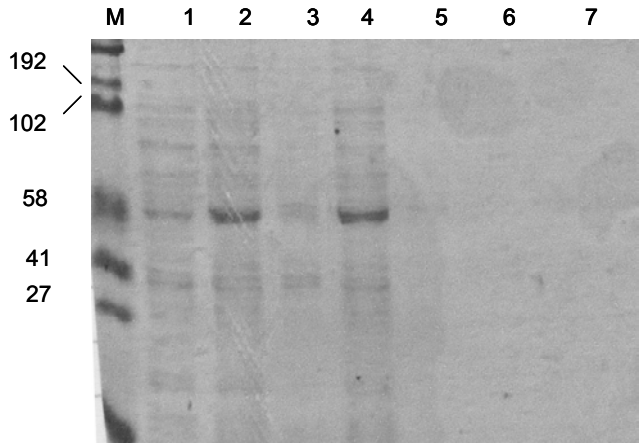


Figure 7.4: Acrylamide gel showing the affinity pull-down of GST-fusion protein. Lane 1 contains the un-induced bacterial culture as a control, lane 2 and 3 are the soluble and insoluble fractions of cultures collected 3 hours after induction with IPTG. Lanes 4, 5 and 6 contain samples of the first, second and third flow-through collected after attempting to bind the fusion protein to the Glutathione beads. Lane 7 contains the elution of the protein, if any, which is bound to the beads. All the protein is seen in the first flow-through and none in the pull-down.

Thus, in order to purify enough protein to inject into an animal for the generation of the antibody, an attempt was made to elute the protein directly from the polyacrylamide gel. For this, the insoluble fraction was run on the gel in large amounts along with a protein marker of known molecular weights. A part of the gel, containing the marker and some of the insoluble bacterial protein extract, was cut and stained with coomassie blue. It was then aligned along with the unstained portion of the gel and the area of the gel corresponding to the stained CRY-GST protein band (both FL and NT) was cut. The protein contained in the gel fragment was eluted either in water or an elution buffer containing 50mM Tris pH 7.5, 150mM NaCl and 0.1mM EDTA. As can be seen in figure 7.5, NT-CRY was able to elute in both water and elution buffer while FL-CRY did not elute at all. This could be due to the large size of FL-CRY-GST protein which prevents its extraction from the gel matrix into solution.

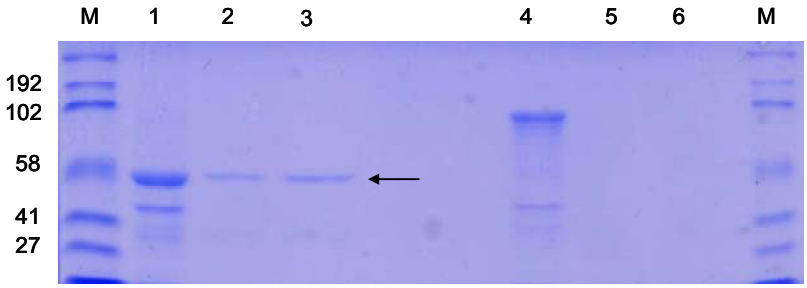


Figure 7.5: Acrylamide gel showing the fusion protein eluted from gel fragments. Lanes 1 and 4 contain a sample of over-expressed NT-CRY and FL-CRY fusion proteins respectively and serve as a control to confirm the eluted protein. Lanes 2 and 5 contain the fusion proteins eluted in elution buffer while the proteins in lanes 3 and 6 were eluted in water. NT-CRY eluted in both conditions while FL-CRY did not elute at all.

For this reason, the antibody was produced against the fragment corresponding to N-terminal CRY. The protein was eluted in water and quantified by running a sample of the eluted protein on an acrylamide gel along with known quantities of BSA (1µg, 2µg, 3µg etc.) and staining with coomassie blue. The intensity of the bands corresponding to the eluted protein samples was compared to those of BSA and in this manner the amount of protein was approximated (see figure 7.6). There appears to be at least 5µg of protein in the 5µl of sample run in the first four lanes of the gel.

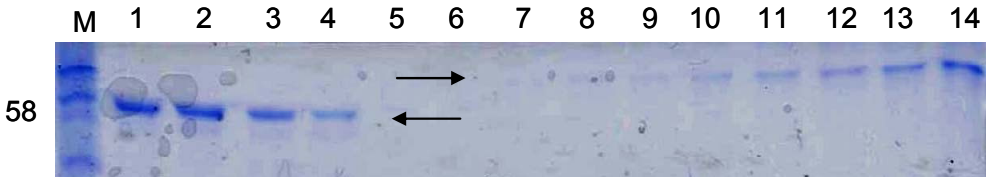


Figure 7.6: Acrylamide gel used for the quantification of eluted fusion protein to send for antibody generation. Lanes 1-4 contain samples of the eluted proteins while lanes 5-14 contain 0.1µg, 0.2µg, 0.4µg, 0.6µg, 0.8µg, 1µg, 2µg, 3µg, 4µg and 5µg of BSA respectively. There appears to be at least 5µg of eluted protein in each of the lanes 1-4.

Approximately 3.5mg of eluted protein was sent to Eurogentec to be injected into guinea pigs in order to purify an antibody against NT-CRY. The antibody obtained was then tested in western blot and ICC.

7.2.3 Testing the antibody

7.2.3.1 Western blot

The western blots done to test the α-CRY antibody was performed in the manner described in section 2.8.2. The antibody was first tested by using it at three different dilutions – 1:500, 1:2,000 and 1:5,000 against proteins extracted from heads of wild-type flies collected in darkness and also flies over-expressing CRY tagged with HA (*w;tingal4;uashacry*, see section 2.1.1). As expected, a large amount of CRY was detected in flies over-expressing the protein at all three dilutions (see figure 7.7). However, endogenous CRY was detected in the 1:500 and 1:2,000 dilution.

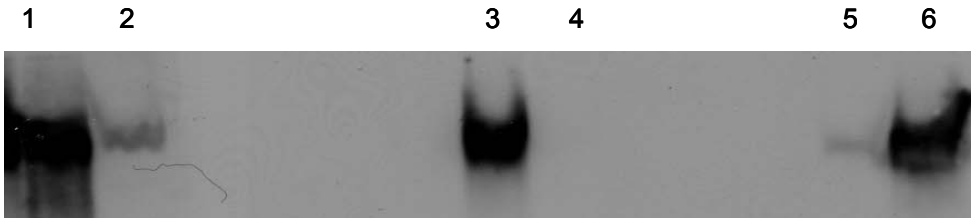


Figure 7.7: Western blot to test the functioning of α-CRY antibody. The antibody was used at three dilutions – 1:500 (lanes 1, 2), 1:2,000 (lanes 5, 6) and 1:5,000 (lanes 3, 4). Flies over-expressing HA-CRY were used as a positive control and CRY was detected in these flies in all dilutions (lanes 1, 3 and 6). Endogenous CRY obtained from wild-type flies (lanes 2, 4 and 6) was not detected in 1:5,000 dilution (lane 4).

The antibody dilution chosen for further work was 1:1,000. This dilution was used to check the specificity of the antibody by using various positive and negative controls. In figure 7.8, Lanes 1 and 3 contain head protein extracts from *timgal4;uashacry16.1* flies over-expressing CRY with a HA tag (see section 2.1). Lanes 2 and 4 contain CRY obtained from wild-type flies collected in darkness at ZT24. A part of the membrane (containing lanes 1 and 2) was probed with α-HA antibody while the rest of the membrane (lanes 2-7) was probed with the newly generated α-CRY antibody. Endogenous CRY was only detected upon exposure to α-CRY antibody (lane 4) while the positive control of HA-CRY was detected in both conditions (lanes 1 and

3) thus confirming that the band is the correct one. Lane 5 contains proteins extracted from cry^0 flies and it lacks the band corresponding to CRY and thereby serves as the negative control. Proteins from heads of cry^b flies were run in lane 6 and a small amount of CRY was detected. This is due to fact that the missense mutation in cry^b is not a null mutation (Emery *et al.*, 2000) but instead probably only renders the protein unstable which results in most of the protein being degraded but a detectable amount is still visible in darkness. Lane 7 contains endogenous CRY from wild-type flies collected in the light phase (ZT12). Even though some protein has been detected, it is much less when compared to that in darkness (lane 4). Since it is known that CRY is degraded by light (Lin *et al.*, 2001), this again confirms that the antibody seems to be specific to CRY.

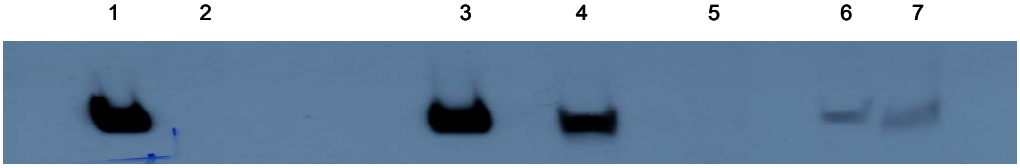


Figure 7.8: Western blot to test the specificity of α-CRY antibody. Lanes 1 and 2 were probed with α-HA to identify the specific band corresponding to CRY as detected in flies over-expressing UASHA-CRY under the control of a TIMGAL4 driver (lane 1) which is absent in wild-type flies with endogenous CRY (lane 2). Lanes 3-7 were probed with the newly generated α-CRY antibody. Both HA-CRY (lane 3) and endogenous CRY (lane 4) were detected and there was no band in extracts from cry^0 flies (lane 5). A small amount of CRY was detected in head extracts from cry^b (lane 6) and from wild-type flies collected at ZT11 (lane 7).

7.2.3.2 Immunocytochemistry (ICC)

Wild-type flies, along with those over-expressing HA-CRY (*w;timgal4;uashacry16.1*) were entrained to LD 12:12 and then left in darkness for 4 days. These flies were collected on the fourth day in darkness (in order to allow the accumulation of CRY protein in the neurons and thereby improve the chances of detection by the newly generated α-CRY antibody). The brains of these flies were dissected and an ICC was performed in the manner described in section 2.9. The α-CRY antibody was tested at a dilution of 1:500. The slides were visualised in the confocal microscope using the following settings:

	Laser 1: 488nm	Laser 2: 635nm
Laser Intensity	15%	19.9%
Laser Gain	659V	650V
Amplifier Gain	1	1
Amplifier Offset	1	1

For all the brains that were viewed, it was possible to see the staining of PDF in the small and large ventral lateral neurons (sLNvs and lLNvs) indicating that the experiment had worked. However, no staining was seen for the α-CRY antibody. A representative brain has been shown in figure 7.9.

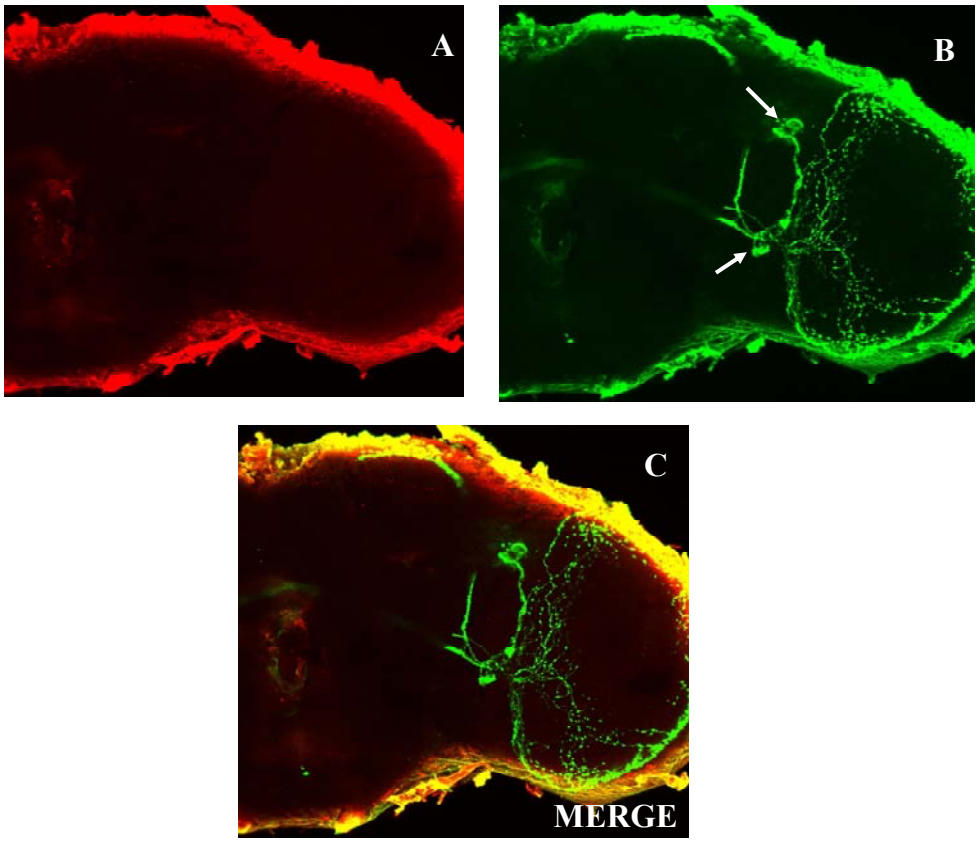


Figure 7.9: Immunocytochemistry to check labelling by CRY antibody. Wild-type flies were collected after 3 days in LD 12:12 and 4 days in DD. The brains were stained using α-CRY and α-PDF. No staining was observed for the α-CRY antibody (A) while the small and large ventral lateral neurons (see arrows) are seen when stained with α-PDF (B). C is the merged image of the two.

7.3 Discussion

Even though the over-expression of fusion protein was optimised for two different constructs of the circadian photoreceptor, CRY (containing the N-terminal or the full length coding sequence), the antibody was finally generated only against its N-terminal region (for reasons, see section 7.2.2). The animal chosen to produce the antibody was guinea pig, because most of the antibodies available in the laboratory against the other clock proteins PER and TIM have been generated in rats, rabbits or mice. Thus, generating an α-CRY antibody in guinea pigs would enable simultaneous double-staining for any two proteins. After the antibody was generated, it was tested on

western blots and ICC. As can be seen in figure 7.7 and 7.8, the antibody seems to work at a dilution of 1:1,000 in western blots and is extremely specific to CRY. However, when an attempt was made to use the antibody in immunocytochemistry experiments, even at a dilution of 1:500, it did not work as no neurons have been stained in figure 7.9A. This discrepancy between the antibody labelling in western blots and ICC could be explained by the way in which the antigen was purified. As has been described above in section 7.2.2, the fusion protein was over-expressed in bacteria, linearised by boiling and then run on an acrylamide gel from where it was extracted and used to inject into the animal in which it was produced. Thus, the final protein used as an antigen was denatured. In a western blot, the proteins loaded onto the acrylamide gel, and thereby those on the membrane to which the antibody is hybridised, are denatured. However, when performing an ICC experiment, the proteins that are fixed in the brain are preserved in their native state. It is possible that the antigenic motifs which resulted in the production of the antibody in guinea pigs are actually embedded in the secondary and tertiary structures of the protein when in its native state. Due to this reason, the antibody worked in western blots but not in immunocytochemistry.

The fact that the antibody works in western blots makes it useful for future experiments in the laboratory. It can be used to investigate various properties of the protein, for instance, its degradation by light and its cycling profile.

Chapter 8: Discussion

In this work, the circadian behaviour of *D. melanogaster* has been examined in detail over different seasons in natural conditions. An attempt has been made to determine the principle environmental cue that drives fly locomotor activity behaviour. Additionally, mutant strains have been used to dissect the role of circadian genes in entrainment to the complex natural environment. The general activity profile of wild-type flies and the analyses of their morning and evening components in natural conditions have been discussed in detail in chapters 3, 4 and 5 respectively. I would now like to place these results in a molecular and neuronal context and discuss their implications.

8.1 Behaviour of wild-type in natural conditions

Some conclusions from laboratory experiments hold true in the wild. However, due to the fact that simplified environmental conditions are used in the laboratory to dissect the circadian clock, at times, the conclusions from laboratory experiments are incomplete. For instance, the activity profile of wild-type flies is bimodal at warm and unimodal at cold temperatures. This effect of temperature on the activity profile is evident in natural conditions and also when these conditions are mimicked in the laboratory (section 3.2.2 and 3.2.3). However, on very hot days in natural conditions (average day temperature >29°C) apart from the morning and evening peaks, there is an additional 'afternoon peak' which occurs during the hottest part of the day. The absence of this peak in the laboratory has led to the belief that the 'siesta' at hot temperatures is a means for the fly to escape the heat of the afternoon (Majercak *et al.*, 1999). In natural conditions, however, flies increase their activity at this time of the day, possibly as a

stress-response to the hot temperatures (section 3.2.4). It is unlikely that the increase in activity was an attempt by flies to seek shade because immense care was taken to ensure that the entire recording apparatus was kept away from direct sunlight at both recording stations (section 2.2.1).

Another recent conclusion from laboratory experiments is that the presence of moonlight affects the circadian clock of *Drosophila* such that the morning and evening peaks shift into simulated dawn and dusk respectively (Bachleitner *et al.*, 2007). In fact, it is believed that the 5th non-PDF expressing sLNv is responsible for this phenomenon due to a shift in phase of PER-cycling in this neuron under these conditions. From the experiments presented in section 3.2.6, it appears that in natural conditions flies under full-moon are not any more nocturnal than flies under no-moon. It is has also been observed that the corresponding shift in phase of PER-cycling in the 5th sLNv in moonlight does not occur in the wild (P. Menegazzi and R. Costa, personal communication). In addition, work on the sexual behaviour of *Drosophila* implies that flies might be nocturnal (Fujii *et al.*, 2007). Again, my natural observations in natural conditions appear to refute these conclusions (section 3.2.7).

8.2 Role of natural light and temperature on the timing of morning and evening behaviour of wild-type and mutants

As an environmental cue, the role of light, especially in the timing of morning behaviour cannot be underestimated. This is evident from the fact that when covered in darkness, all strains of flies begin their morning activity later than in corresponding natural conditions (section 4.3.5). This implies that they respond to light intensities as low as the 0.0006-0.06 lux that accompanies twilights in nature. The particularly low light intensities that stimulate activity under warmer conditions seem quite

extraordinary as they are much lower than those tested in the laboratory (Reiger *et al.,* 2007). It is unlikely that this morning activity in natural conditions is clock regulated because all mutants (including photoreceptor mutants such as cry^0) show a similar response to these extremely low levels of light. Thus, it appears that morning behaviour of all flies is extremely sensitive to the low levels of light that occur during twilight.

Another argument in support of the view that morning behaviour is responsive to the environment is that between the two wild-type strains, at the same temperature, the morning onset of HU is later than that of WTALA implying that the northern strain is responding to higher light intensities than the southern one (section 4.3.4). No such difference between HU and WTALA was found in their evening behaviour (section 5.3.3). It is believed that one way for flies to adapt to the extreme photoperiods that are associated with northern Europe is by reducing their light sensitivity (Pittendrigh *et al.*, 1989; Tauber *et al.*, 2007). In nature, the obvious way for flies to do so would be to alter their activity in the morning which is when the dynamic changes occur in the environment, especially with respect to the intensity and quality of light.

Light also causes a subtle effect on timing of evening behaviour because in darkness, the evening peak of wild-type and mutant flies is delayed as compared to natural conditions (section 5.3.4). Any effect of light on the timing of evening activity probably occurs through circadian photoreceptors. This is because the evening peak of cry^0 occurs at the same time in natural lighting and in darkness. Not surprisingly, the position of this evening peak coincides with that of wild-type flies in darkness. Thus, the loss of the dedicated circadian photoreceptor affects the timing of evening activity but not the morning (Stanewsky *et al.*, 1998).

Apart from light, in natural conditions, temperature also plays a vital role as an environmental cue. The timing of morning and evening activity depends largely on the

ambient temperature as is evident from the highly significant correlations that are obtained in our analyses for both wild-type and mutant strains (section 4.3.3 and 5.3.2). It appears that the loss of genes that constitute the core oscillator as we know it today only marginally affects these correlations. One example of an effect of a clock gene on behaviour in natural conditions is the loss of temperature-dependence of the morning onset in tim^{01}. Recently, it has been shown that the splicing of *tim* RNA is dependent on temperature (Boothroyd *et al.*, 2007). TIM protein abundance also responds to high and low temperatures (Majercak *et al.*, 1999) as well as to temperature pulses (Sidote *et. al.*, 1998). Thus although TIM is justifiably recognised as the light-sensitive canonical clock protein (Hunter-Ensor *et al.*, 1996; Lee *et al.*, 1996; Young *et al.*, 1996; Zeng *et al.*, 1996), and plays the major role in light resetting (Ceriani *et al.*, 1999), the temperature-dependent light sensitivity of morning onset which is lost in tim^{01} argues for a thermal role for TIM. Indeed recent work on diapauses reveals that *tim* variants affect this phenotype, which is temperature and photoperiod dependent (Sandrelli *et al.*, 2007; Tauber *et al.*, 2007).

Another example of a known clock gene effecting timing of behaviour in natural conditions is that concerning the classic *per* alleles. It is known from the laboratory that *per* mutants preferentially affect the evening peak. In particular, the evening peak is advanced in per^S and delayed in per^L as compared to wild-type (Hardin *et al.*, 1992). In natural conditions too, it has been found that at the same temperature, the evening onset of per^L is delayed while the evening peak of per^S (and per^{01}) is advanced compared to wild-type. Also, when kept in darkness over a few days, per^{01} flies lose the evening component that is manifested in natural conditions, yet unexpectedly, other mutants (such as tim^{01}) retain it. In contrast, for morning behaviour as mentioned above, per^{01} appear quasi-normal, but tim^{01} mutants have the temperature insensitivity. Furthermore

tim mutants do not appear to differ in the timing of their evening behaviour (as does *per*^{01}*)* yet both are apparently equally 'clockless'. Thus, this work has uncovered the possibility for differing roles of PER and TIM (and other clock proteins) in morning and evening behaviour, which in turn means that their relevance may differ within the various neuronal subgroups that make up the circadian network.

In the laboratory, morning and evening behaviour are believed to be controlled by two independent sets of neurons (Grima *et al.*, 2004; Stoleru *et al.*, 2004). Also, different neuronal groups are believed to be dominant over others in darkness (M cells) and in light (E cells) (Murad *et al.*, 2007; Picot *et al.*, 2007; Stoleru *et al.*, 2007). In natural conditions, however, killing the PDF-expressing M cells (using *ywuashiduasreaper;pdgfal4;+/+*) or stopping the clock in these cells (using *yw;pdfgal4/uascycΔ103;+/+*) does not generate the expected dramatic phenotype. One could argue that perhaps in the wild, the GAL4 expression is not optimal due to low temperatures at night and this causes the wild-type-like behaviour of these strains. However, in a behavioural experiment in the laboratory (section 4.2.3) at constant temperature of 10°C, which is far lower than those in the vast majority of my observations, GAL4 seems to be able to drive UAS transgenes to disrupt clock function. Thus, it appears that the lack of functional M cells in this case is compensated by some means. Stopping the clock in PDF-expressing LNvs (M cells) as well as both M and E cells gave subtle effects in morning behaviour whereby the morning onset has a slightly enhanced temperature sensitivity. Strikingly, the same transgenes reverse the normal thermal relationship with evening onset. Thus, for the limited data-set discussed in this work, stopping the clock in M or M and E cells affects both morning and evening behaviour. Similarly, in *Pdf*^{01} mutants the temperature-sensitivity of evening behaviour is enhanced while morning activity is slightly delayed compared to wild-type at the

same temperature. This also shows that in the complex natural environment, the PDF-expressing LNvs not only control timing of morning behaviour but also (and more evidently so) have a major input into the timing of evening behaviour. The role of temperature in entrainment has only recently begun to be investigated in *Drosophila* chronobiology (Glaser and Stanewsky, 2005; Matsumoto *et al.*, 1998; Miyasako et al., 2007). Our results show that it is an extremely important cue which controls the timing of both morning and evening behaviour and warrants far more attention.

8.3 Alternative mechanisms of timing behaviour in clockless flies

The most surprising result of this work has been the *very presence* of these temporally regulated morning and evening peaks in flies which are mutant for core clock genes or those lacking a fully functional network of clock neurons. In the laboratory, flies genetically compromised in their circadian clock, react to changes in the environment rather than anticipate LD or temperature cycles, such that they become active in light/thermophase and not in darkness/cryophase (Wheeler *et al.*, 1993; Yoshii *et al.*, 2005). This does not hold true for natural conditions, whereby on most days when the temperature is not too low, these mutant flies manifest an activity peak in the morning and then reduce their activity to generate a siesta, then they rise for another independent activity peak in the evening. Both morning and evening peaks are modulated by environmental changes such as increasing or decreasing light intensity and temperature. However, these locomotor components are not a direct response to these changing stimuli, and thus appear to be temporally regulated.

There are three possible explanations for these observations on 'clockless' flies. It is possible that there is an underlying clock-independent physiological mechanism that results in 'residual' morning and evening activity components, which the clock will

enhance. The initiation of this residual mechanism by increasing light and temperature early in the day would generate a morning onset/peak. However, the same mechanism would have to be triggered by the *decreasing light and temperature* later in the day in order to enable mutant flies to manifest an evening peak. Thus a clockless fly 'knows' whether it is in the morning or evening because it must react in the same way to opposite environmental changes which come at different times of day. Thus it seems unlikely that the behaviour observed in clockless flies has an underlying programme that is kick-started by environmental changes.

Another explanation for timed behaviour in mutants could be the presence of an hourglass interval timer. An interval timer is a mechanism by which an individual measures a period of time from a stimulus and initiate a physiological event in response to it. An essential requirement of this is the daily resetting of the interval timer by external cues as it is not self-sustaining in constant conditions. An interval timer mechanism is already believed to underlie some molecular properties of the circadian clock such as *per* mRNA transcription *in vivo* (Qiu and Hardin, 1996) and more recently nuclear translocation of the PER and TIM proteins *in vitro* (Meyer *et al.*, 2006). It is possible that in natural conditions, at the behavioural level, an increase in locomotor activity is locked to an environmental cue and modulated by temperature. This underlying clock-independent interval timer could become dominant, especially in clockless flies, in the complex environment of natural conditions. This hypothesis can easily be tested in the laboratory by using the standard operating protocols relating to 'natural' photoperiods and temperature cycles described in this report. Analysing the phase of morning and evening peaks of mutants while lengthening or shortening the photoperiod at different temperatures would reveal if initiation of locomotor activity is locked to lights-off or lights-on and modulated by temperature.

The final inescapable possibility is that clockless mutants harbour a residual clock that is manifested in natural conditions in mutant flies. It is possible that in flies lacking the morning cells, for example, other neurons are able to take over their role in the clock network of the brain. Recent work has revealed that the anterior group of DN1s and newly characterised LPNs play a role in temperature entrainment (Miyasako *et al.*, 2007). It was demonstrated that when light and temperature cycles are applied out of phase in the laboratory, TIM abundance in these neurons cycles in phase with temperature while the other circadian neurons cycle in phase with the light-dark regime. In natural conditions, due to the presence of a daily temperature cycle, these cells could compensate for the lack of sLNvs and be responsible for the clear morning and evening peaks observed in these flies.

Also, for almost a decade PDF was the only established neuropeptide that was known to regulate circadian rhythms in flies (Renn *et al.*, 1999). Recently, however, the identification of IPNamide, a neuropeptide expressed in some DN1s has raised the possibility of other neuropeptides being involved in the functioning of the circadian clock (Shafer *et al.*, 2006). Additionally, it has recently been demonstrated that the PDF-expressing lLNvs are involved in light-mediated arousal and possibly transmit this light information to the circadian clock via the sLNvs (Shang *et al.*, 2008). These studies have uncovered a novel role of these circadian neurons and also linked the arousal and circadian circuits. It is also possible that there are some other neurons (maybe those that have been characterised for sleep and arousal), that do not express the canonical clock proteins but are able to drive timed behaviour in natural conditions.

It has been postulated that the circadian clock is comprised of two oscillators which together give rise to 24-h rhythms (Daan and Pittendrigh, 1959). Currently, it is believed that the M and E cells are the two oscillators in the brain which synchronise

circadian rhythms in flies. These cells have been identified by applying light-dark cycles in constant temperature (Grima *et al.*, 2004; Stoleru *et al.*, 2004). In nature, however, light-dark cycles are always accompanied by temperature cycles. In fact, a dual oscillator model proposed by Pittendrigh and Bruce had hypothesised one light-dependent and another temperature-dependent oscillator, both of which feedback on each other to manifest circadian rhythms (Pittendrigh and Bruce, 1959). The results presented in this thesis support this view because both light and temperature cycle with a slight delay in nature. Thus, it is possible that the LNvs and LNds (currently the M and E cells) together comprise a light-sensitive oscillator, and the DN1s and LPNs form a temperature-sensitive one. Clearly more experiments are required that attempt to dissect these functions of the different neuronal subgroups in the wild. This may clarify their interplay when challenged with natural temperature and light-dark cycles.

At the molecular level, numerous lines of research are reanalysing the current model of the negative feedback loops that constitutes the circadian clock in *Drosophila* (model described in section 1.3). Some TIM-independent properties of PER have already been discovered and these are discussed in chapter 4. Along with this, there are numerous papers that question the long-standing belief that the cycling of the RNA and protein products of clock genes is necessary for the manifestation of rhythms. For example, it has been shown that a lack of cycling of PDP1ε does not abolish molecular oscillations of other clock gene products (Benito *et al.*, 2007). It is already known that even though *Clk* transcript cycles, CLOCK protein is constitutively expressed in circadian neurons (Glossop *et al.*, 1999; Houl *et al.*, 2006). Conversely, PER protein can cycle in the peripheral clock of the eyes even in the lack of *per* mRNA cycles (Cheng and Hardin, 1998). It has also been demonstrated that abolishing the cycling of PDF in small cell termini of sLNvs does not obliterate behavioural rhythms (Kula *et al.*,

2006). All these papers indicate that there is still a lot to learn about the properties of 'well-characterised' clock gene products.

Dissecting the circadian clock provides one of the most exciting stories in gene regulation of the last 25 years. To do this in the primary model organism, *Drosophila*, has required simplifying the conditions under which behavioural genetic studies are performed. This is understandable and necessary. However the time is now ripe for a more comprehensive examination of these neurogenetic manipulations, under complex environments. Also, future work must take into consideration the natural temperature cycles that are usually ignored in the laboratory. The results in this thesis are quite novel, and show that the wild-type-like behaviour of canonical clock mutants in natural conditions may require some revision of the current model of how the circadian clock works in *Drosophila*.

Appendix 1

The components of 5X PCR buffer are listed in the table below.

Reagent	**Amount**
Reaction Buffer	500µl
$MgCl_2$	300µl
dATP	10µl
dCTP	10µl
dGTP	10µl
dUTP	10µl
Water	160µl

The following table contains a list of primers used in this project and their annealing temperatures.

Primer Name	**Primer Sequence**	**Annealing Temperature**
pGEX 5’	GGG CTG GCA AGC CAC GTT TGG TG	60°C
pGEX 3’	CCT CTG ACA CAT GCA GCT CCC GG	60°C
tim01 5’	GCT CAT CGC TTT TCA TAT GTT	57°C
tim01 3’	AGG ATG TGA TTG GTA ACC AC	57°C
per01 5’	TAC CAC CAC GAG GAC CTC TC	57°C
per01 3’	GAT GGT GTC CGA CGA CAA AT	57°C

Appendix 2

The position of Evening-onset - Lights-on (figure 2-1) and Evening-peak - Lights-on (figure 2-2) is reported here for the following genotypes.

(A) per^{01}

(B) tim^{01}

(C) $Clock^{jerk}$

(D) $per^{01}tim^{01}$

(E) per^{S}

(F) per^{L}

(G) cry^{0}

(H) Pdf^{01}

(I) $norpA^{p41}$

(J) *ywuashiduasreaper;pdfgal4/+/+*

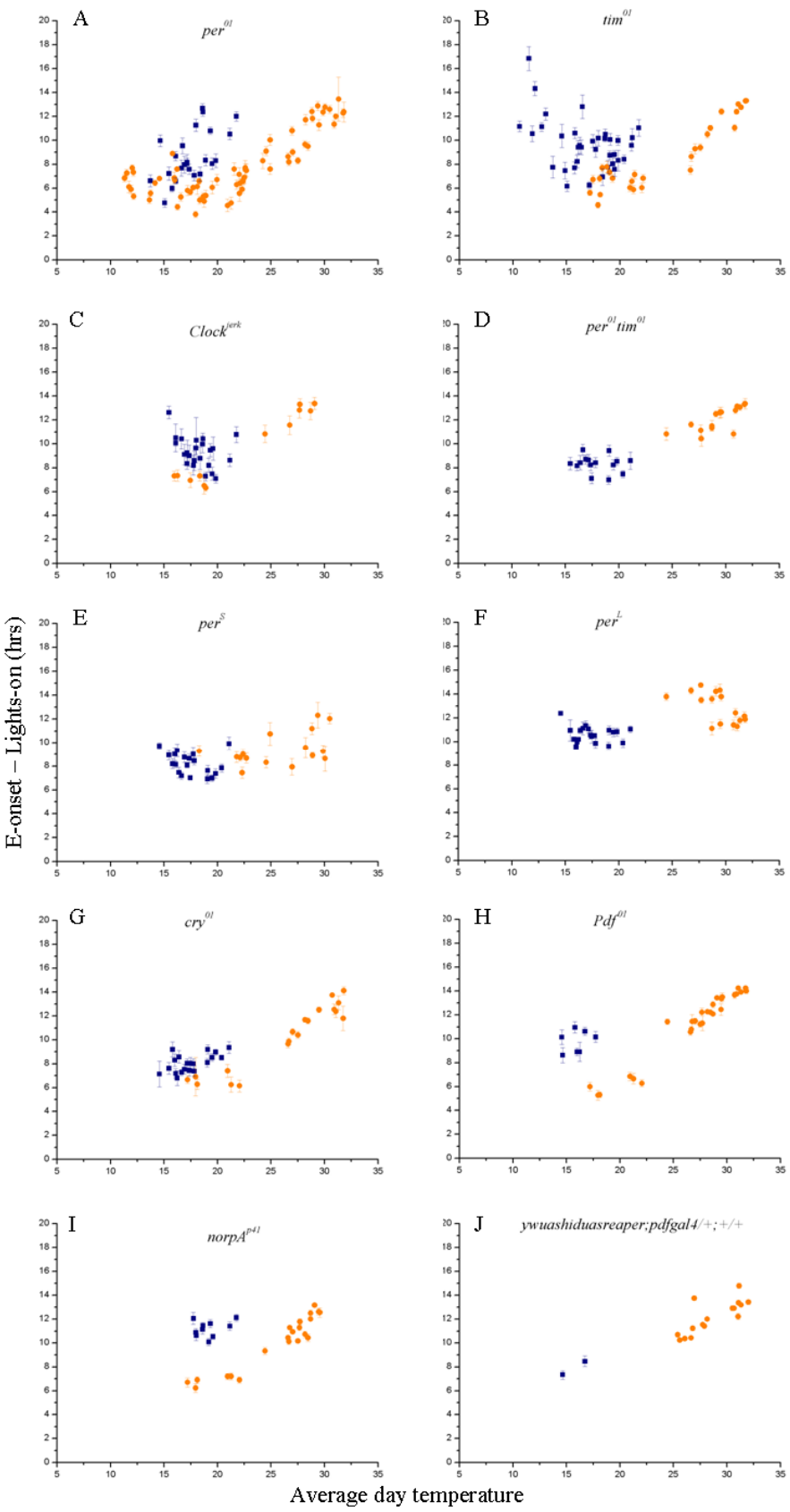


Figure 2-1: Evening-onset - Lights on (hrs ± SEM)

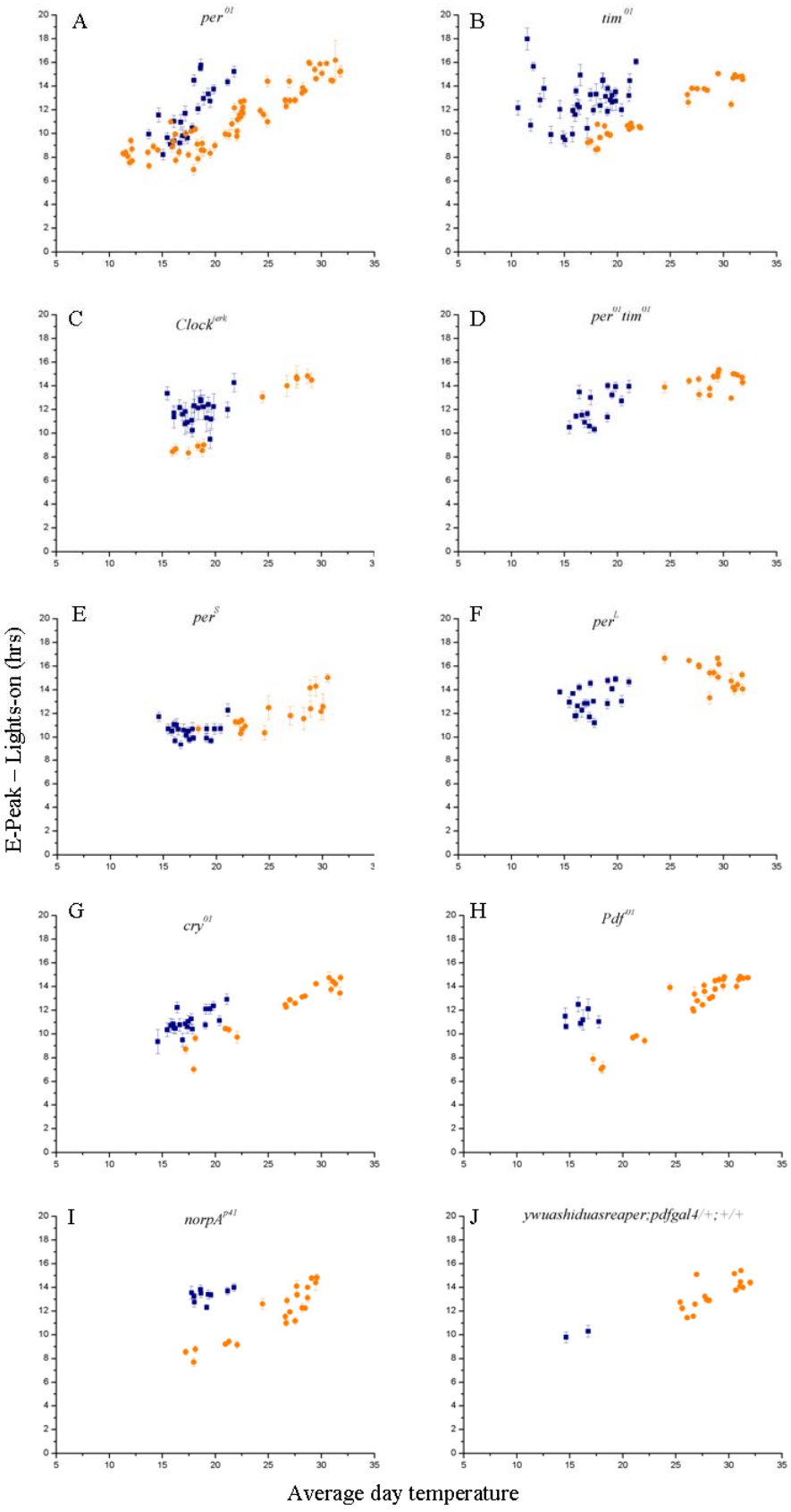


Figure 2-2: Evening-peak - Lights-on (hrs ± SEM)

Appendix 3

The position of evening onset (figure 3-1) and peak (figure 3-2) compared to time of temperature peak (hrs) has been plotted against average day temperature (°C). The data obtained for wild-type WTALA (black) has been compared to HU and each mutant (red) to illustrate the differences between them. This data has been analysed using an Analysis of Covariance (ANCOVA) with temperature as a covariate and the results are summarised in table 5.4 (section 5.3.3).

(A) WTALA compared to HU

(B) WTALA compared to per^{01}

(C) WTALA compared to tim^{01}

(D) WTALA compared to $Clock^{jerk}$

(E) WTALA compared to $per^{01}tim^{01}$

(F) WTALA compared to per^{S}

(G) WTALA compared to per^{L}

(H) WTALA compared to $norpA^{p41}$

(I) WTALA compared to cry^{0}

(J) WTALA compared to Pdf^{01}

(K) WTALA compared to *ywuashiduasreaper;pdfgal4/+;+/+*

(L) WTALA compared to *yw;uascycΔ103/pdfgal4;+/+*

(M) WTALA compared to *yw;uascycΔ103/timgal4;+/+*

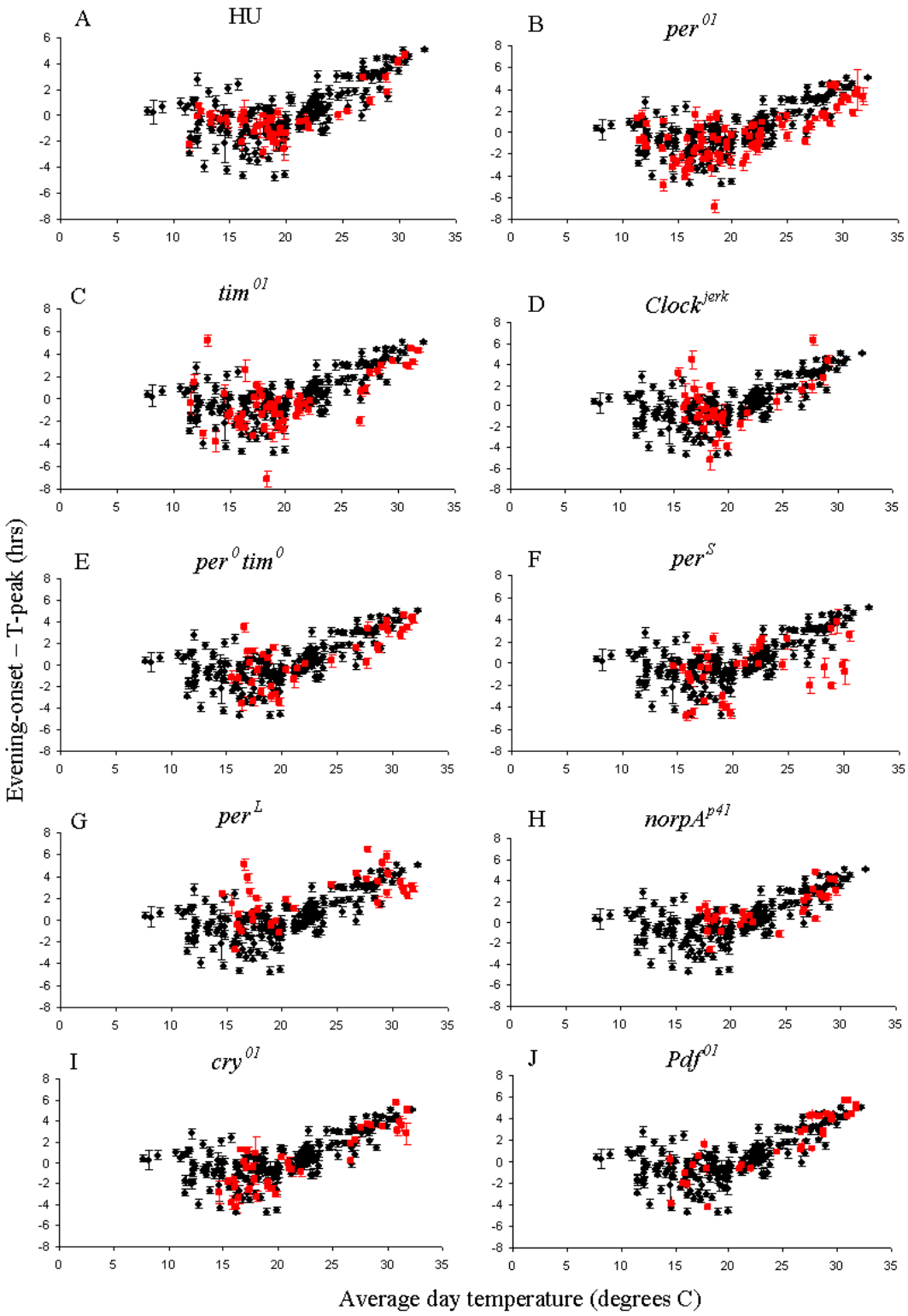


Figure 3-1: Evening-onset - T-peak (hrs ± SEM). WTALA (black) V/S other strains (red).

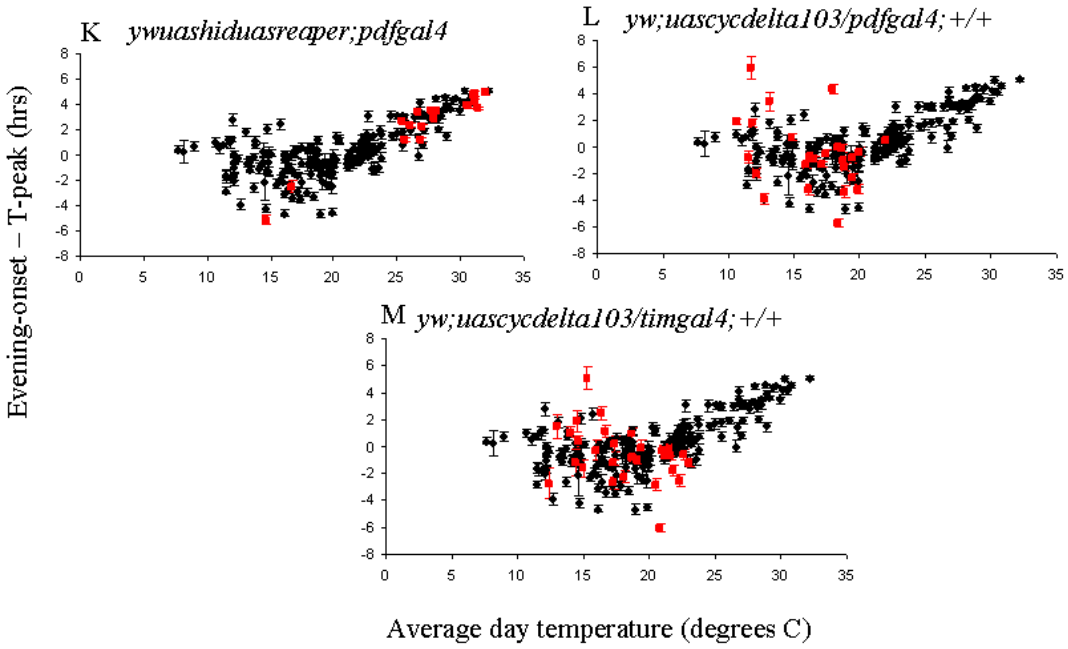


Figure 3-1 (continued)

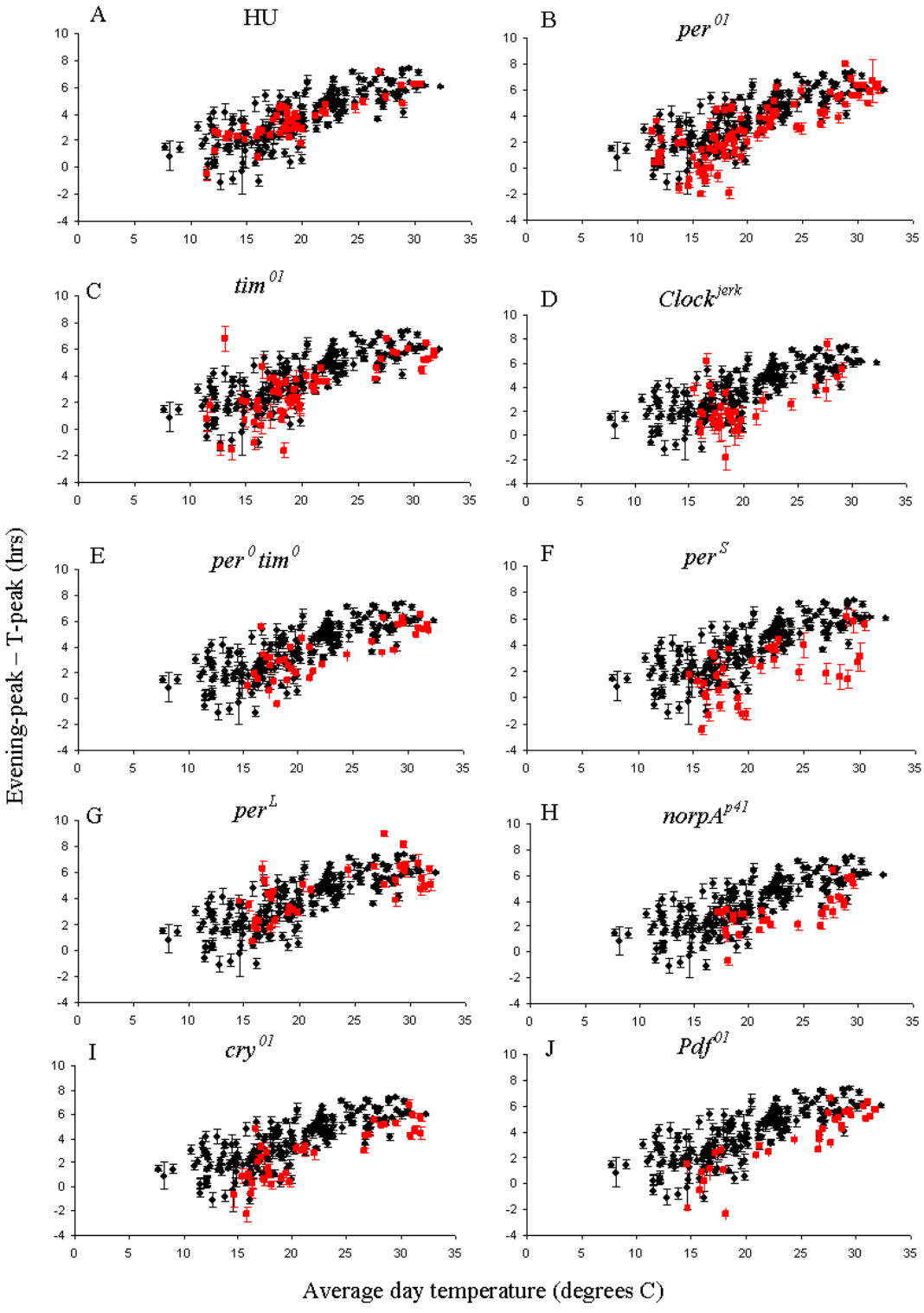


Figure 3-2: Evening-peak - T-peak (hrs ± SEM). WTALA (black) V/S other strains (red).

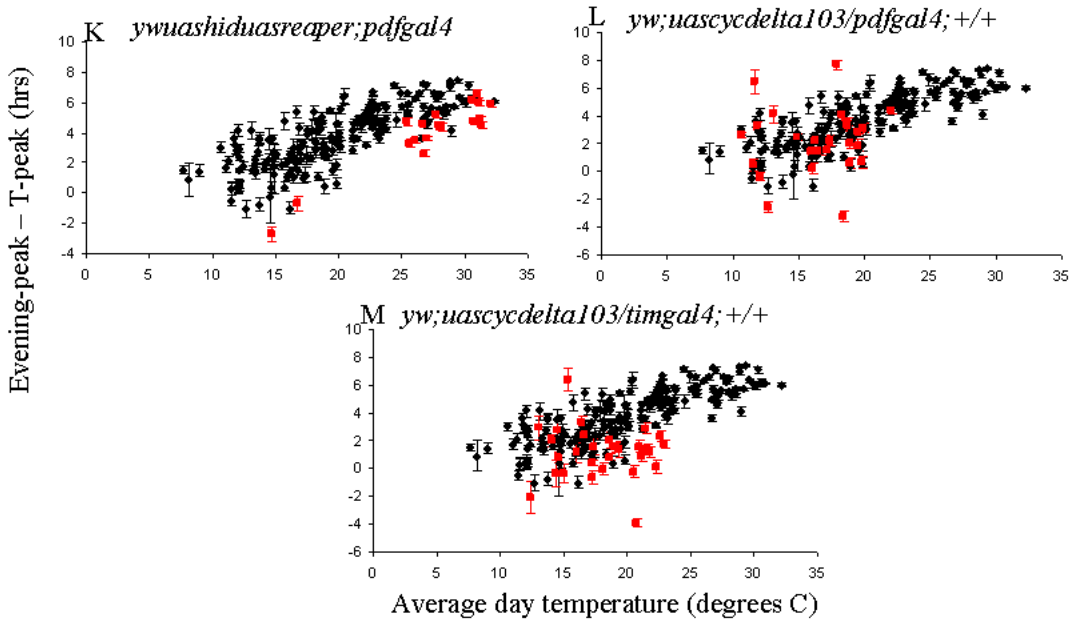
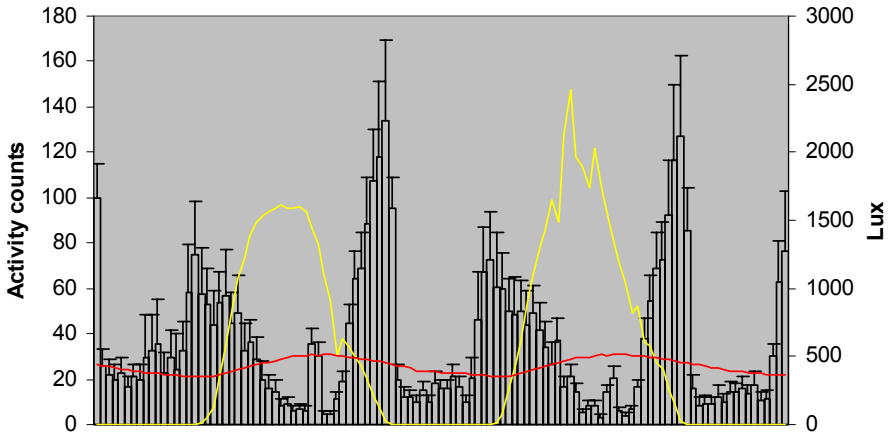


Figure 3-2 (continued)

Appendix 4

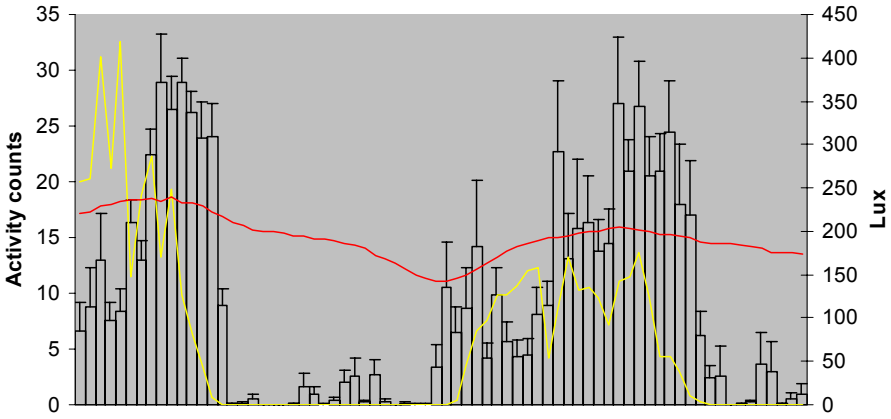
1. Locomotor activity profile of WTALA on 20-21st May 2009 in Padova

The locomotor activity profile of WTALA (n=25) is represented in bars, the temperature in red and the light intensity in yellow. The photoperiod during these days of collection was 15:9 and the temperature range was 31:21°C. The activity is bimodal with clear morning and evening peaks.



2. Locomotor activity profile of WTALA on 11-12th September 2008 in Leicester

The locomotor activity profile of WTALA (n=27) is represented in bars, the temperature in red and the light intensity in yellow. The photoperiod during these days of collection was 15:9 and the temperature range was 18:11°C. The activity is bimodal but with a reduced morning component and enhanced evening peak.



Appendix 5

Two inserts were used to over-express GST-fusion protein in order to generate an antibody against CRY. FL-CRY and NT-CRY inserts were cloned into pGEX-6p-1 vector at the *EcoRI* and *XhoI* restriction sites. The two maps are shown in figure 5-1A and B respectively. The NT-CRY insert was sequenced and aligned to the published sequence of *cry* in order to confirm its integrity. This sequence alignment can be seen in figure 5-2.

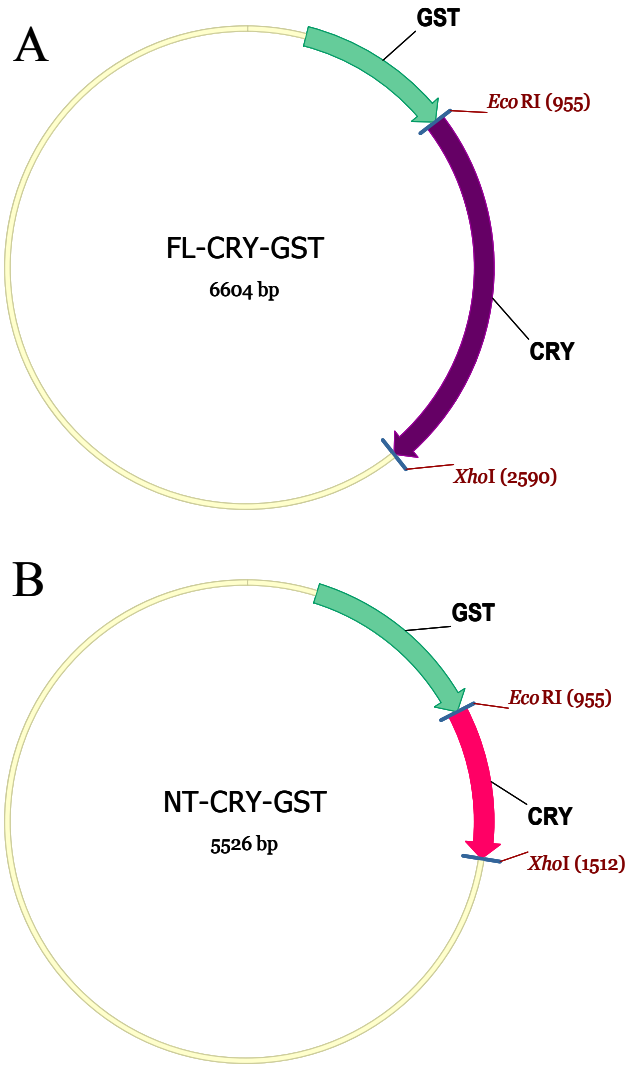


Figure 5-1: Plasmid maps of pGEX-6p-1 containing (A) FL-CRY and (B) NT-CRY.

```
cry             ATGGCCACGCGAGGGGCGAATGTGATTTGGTTTCGCCATGGATTGCGCCTCCATGA 56
NT-CRY          ATGGCCACGCGAGGGGCGAATGTGATTTGGTTTCGCCATGGATTGCGCCTCCATGA 123
                ********************************************************

cry          TAATCCCGCTCTATTGGCCGCCCTCGCCGATAAGGATCAGGGTATAGCCCTAATTCCCGT 116
NT-CRY       TAATCCCGCTCTATTGGCCGCCCTCGCCGATAAGGATCAGGGTATAGCCCTAATTCCCGT 183
             ************************************************************

cry          TTTCATATTCGATGGAGAGAGTGCAGGTACCAAGAATGTGGGTTACAATCGGATGCGTTT 176
NT-CRY       TTTCATATTCGATGGAGAGAGTGCAGGTACCAAGAATGTGGGTTACAATCGGATGCGTTT 243
             ************************************************************

cry          CCTCCTGGACTCGTTGCAGGACATCGATGATCAGCTACAGGCGGCAACTGATGGACGTGG 236
NT-CRY       CCTCCTGGACTCGTTGCAGGACATCGATGATCAGCTACAGGCGGCAACTGATGGACGTGG 303
             ************************************************************

cry          ACGCCTCCTGGTCTTCGAGGGCGAACCGGCTTATATCTTCCGCCGGCTACATGAGCAAGT 296
NT-CRY       ACGCCTCCTGGTCTTCGAGGGCGAACCGGCTTATATCTTCCGCCGGCTACATGAGCAAGT 363
             ************************************************************

cry          GCGTCTGCACAGGATTTGCATAGAGCAGGACTGCGAGCCAATTTGGAATGAGCGCGATGA 356
NT-CRY       GCGTCTGCACAGGATTTGCATAGAGCAGGACTGCGAGCCAATTTGGAATGAGCGCGATGA 423
             ************************************************************

cry          AAGCATCCGTTCTCTATGTCGGGAGCTGAATATCGACTTTGTCGAGAAGGTATCACACAC 416
NT-CRY       AAGCATCCGTTCTCTATGTCGGGAGCTGAATATCGACTTTGTCGAGAAGGTATCACACAC 483
             ************************************************************

cry          GCTTTGGGATCCGCAATTGGTGATTGAGACCAATGGTGGCATTCCACCGCTGACCTACCA 476
NT-CRY       GCTTTGGGATCCGCAATTGGTGATTGAGACCAATGGTGGCATTCCACCGCTGACCTACCA 543
             ************************************************************

cry          AATGTTCCTGCACACGGTGCAAATTATTGGGCTTCCACCGCGTCCCACCGCCGATGCTCG 536
NT-CRY       AATGTTCCTGCACACGGTGCAAATTATTGGGCTTCCACCGCGTCCCACCGCCGATGCTCG 603
             ************************************************************

cry          ACTAGAAGACGCCAC
NT-CRY       ACTAGAAGACGCCAC
             ***************
```

Figure 5-2: Alignment of sequence of NT-CRY insert with *cry*.

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