CIRCADIAN CLOCK GENES AND SEASONAL BEHAVIOUR

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Abstract

Circadian and photoperiodic phenomena serve to organize the temporal pattern of various biological processes. While the former generates endogenous daily rhythms, the latter is related to seasonality. In *Drosophila melanogaster*, the gene *timeless* (*tim*) encodes a cardinal component of the circadian clock and also contributes to photoperiodism, which is observed as an adult reproductive diapause. In this work, natural *tim* variants were examined for diapause across different temperatures and photoperiods. The newly derived allele, *ls-tim*, exhibited consistently higher diapause levels than the ancestral one, *s-tim*, implicating a putative adaptive advantage in the seasonal European environment and providing a perfect substrate for the recently proposed scenario of directional selection.

To investigate further genetic links between circadian and photoperiodic mechanisms, classical clock mutations and transgenes were placed on a natural congenic background and assayed for locomotor activity behaviour and diapause response. Surprisingly, the results not only highlighted the importance of *tim*, and its natural alleles, but also revealed the participation of other clock components in diapause, suggesting that both daily and seasonal timers might have molecularly co-evolved.

The phenotypic effects promoted by *ls-tim* arise from the protein isoform L-TIM, which expresses an additional N-terminal fragment. To study the adaptive significance of the N-terminal residues, including putative phosphorylation sites, a number of mutagenized TIM constructs were generated and functionally analysed. At the molecular level, it was demonstrated that both the N-terminus length and the order of its residues are important variables modulating the interaction dynamics between TIM and CRYPTOCHROME (CRY). At the behaviour level, the overall amino acid composition, rather than a particular order, appeared to be more critical for the phase-shift responses. Interestingly, despite the functional importance of the N-terminus, a deletion mapping analysis revealed that CRY directly binds to a protein sequence located at TIM C-terminus.

To my ‘inspirational’ wife Renata

One life is not enough time to love you

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Chapter 1: Introduction

1.1. The Circadian Clock

1.1.1. General properties

The rotation of the planet around its own axis drives a 24 hour oscillation in many environmental factors (i.e. light, temperature, humidity, etc), which exert a strong selection pressure on life. The ability to organize biological resources to anticipate daily oscillations in those factors would certainly be of an enormous adaptive significance. It is not surprising, then, to find that the behaviour and/or metabolism of most, if not all, living species evolved to follow a 24 hour schedule.

When isolated from environmental time cues (e.g. constant darkness, DD), organisms are still able to maintain biological rhythms with a period of approximately 24 hours, indicating the existence of an endogenous oscillator pacemaker, generally referred to as the circadian clock (Latin: *circa* = about, *dies* = day) (Moore-Ede *et al.*, 1982). Without the presence of environmental cycles, the circadian clock is put in *free-running* conditions to develop rhythms with inherent period values, which depend on a number of variables, including species specificity, illumination (i.e. light intensity), ambient temperature and developmental factors. For most of the species, the *free-running* period in DD ranges from 23 to 25 hours (Dunlap *et al*., 2004).

Interestingly, very little variation in the period is observed when an organism is submitted to different temperatures, indicating the presence of a compensatory regulation in the pacemaker mechanism (Pittendrigh, 1954). This fundamental aspect allows the circadian clock to adapt to daily and seasonal variations of temperature.

Despite its self-sustainable nature, the oscillator needs to be synchronized to environmental cycles on a daily basis to maintain an exact 24 hour rhythm. These environmental cycles embrace important time cues, or *zeitgebers* (German: "time giver"), which act as input factors to the pacemaker mechanism, regulating the turn-over dynamics of some of its components, in a process called entrainment. For many species, the strongest and most important *zeitgeber* is the daily light-dark (LD) cycle, although other non-photic cues (e.g. temperature cycles, humidity cycles, social interactions, food availability) have also been attributed to play an important role (Sharma and Chandrashekaran, 2005).

Finally, the circadian clock generates rhythmic outputs, expressed in many levels of the organism's behaviour, physiology and metabolism. The experimental analysis of the outputs allows the characterization of properties of the clock, such as its period and phase relationship to environmental cycles. In the fruit-fly *Drosophila melanogaster*, a number of rhythmic outputs have been identified and studied to date, including locomotor activity, eclosion (Konopka and Benzer, 1971), egg-laying (Allemand and David, 1984) and olfaction (Krishnan *et al.*, 1999). Due to its relative simplicity, automation, efficiency, reliability and robustness, locomotor activity is, by far, the most studied circadian phenotype in *D. melanogaster* (Rosato and Kyriacou, 2006). A general scheme of the main levels of complexity involved in circadian systems is illustrated in Figure 1.1.

When placed under 25^{o}C, on a generic day of 12 h of light followed by 12 h of darkness (LD 12:12), wild-type flies exhibit two distinct bouts of locomotor activity: one is centred at dawn (morning) and the other at dusk (evening), similar to many crepuscular insect species. The morning and evening activity peaks begin some hours before the light-dark transitions, indicating that flies anticipate these events through a

clock dependent mechanism. Hence, the behaviour readout of locomotor activity, either at entraining or free-running conditions, can be very informative about the oscillator's state.

The molecular analysis of circadian clock genes provides one of the major success stories of recent years in the study of gene regulation as applied to a complex behavioural/physiological character. Despite the enormous amount of knowledge accumulated to date, the intricate pathway used by the clock to generate behavioural rhythms remains unclear and subject to many current studies.

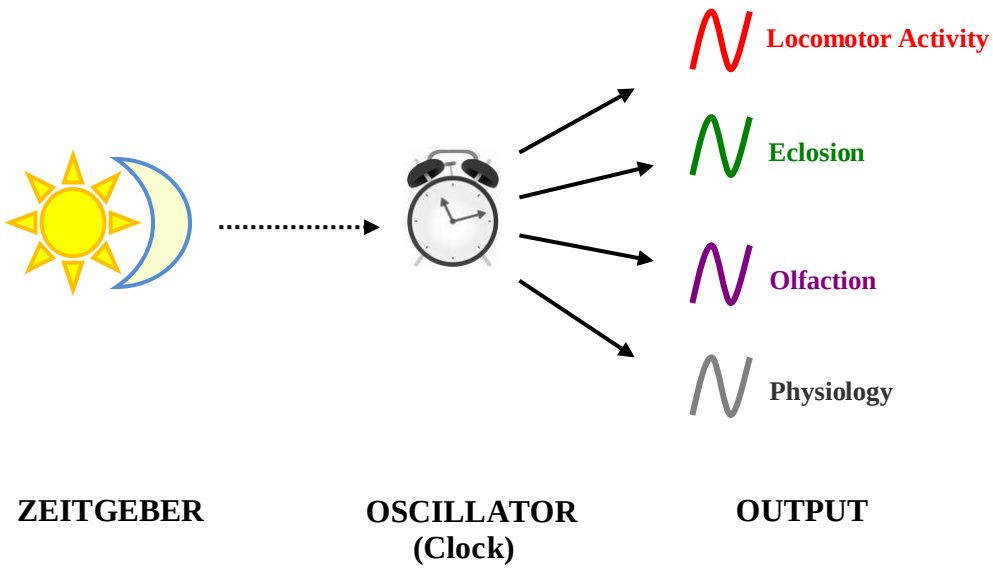


Figure 1.1. The various levels of a circadian system. A self-sustaining circadian clock lies at the centre, generating a wide range of rhythmic outputs. To maintain an exact 24 hour periodicity, the oscillator is reset on a daily basis by environmental cycles of light and temperature, often referred to as *zeitgebers* or entraining agents.

1.1.2. The pacemaker

Molecular genetic studies of the circadian clock of various organisms revealed that this mechanism consists of interlocked feedback loops that act on the transcriptional and post-transcriptional regulation of genes (Dunlap, 1999). In *D. melanogaster*, the pacemaker components have been extensively studied and characterized over the past four decades (Rosato *et al.*, 2006).

The first clock gene to be identified and cloned was *period* (*per*) (Konopka and Benzer, 1971; Bargiello *et al.*, 1984; Reddy *et al.*, 1984). Many years later, another gene emerged, *timeless* (*tim*) (Sehgal *et al.*, 1994; Myers *et al.*, 1995). The abundance of transcript and protein encoded by both genes were also found to cycle with an approximate 24 hour periodicity. The peak levels of the proteins, however, occur a few hours later than those of the mRNAs, suggesting the existence of a negative feedback loop of PER and TIM products on their own expression (Hardin *et al.*, 1990).

The feedback mechanism was confirmed with the identification of two other clock genes: *Clock* (*Clk*) and *cycle* (*cyc*), which belong to the bHLH/PAS family of transcription factors (Allada *et al.*, 1998; Darlington *et al.*, 1998; Rutila *et al.*, 1998). In the morning, the proteins CLK and CYC form a heterodimer and bind E-box elements (i.e. *CACGTG)* on the promoter sequences of *per* and *tim*, activating their expression (Kyriacou and Rosato, 2000). The transcript levels of both genes gradually increase to culminate in a peak early at night, but it is only in the middle of the night that the respective protein products PER and TIM reach maximum levels, promoting their interaction and translocation to the nucleus. Once in the nucleus, PER/TIM binds to CLK/CYC to inhibit their transcriptional activation role on *per* and *tim* expression,

closing the main feedback loop that dictates circadian rhythms in flies (Darlington *et al.*, 1998; Lee *et al.*, 1999; Chang & Reppert, 2003).

The levels of *Clk* transcripts also cycle, indicating that the expression of this factor is rhythmically regulated (Glossop *et al*., 1999). The molecular basis of this regulation involves the basic leucine zipper (bZIP) transcription factor *vrille* (*vri*), which inhibits the transcription of *Clk*, and the *PAR domain protein 1* (*Pdp1ε*), which activates it (Blau and Young, 1999; Lin *et al*., 1997; Cyran *et al.*, 2003; Glossop *et al*., 2003). VRI and PDP1ε proteins compete for the same regulatory elements (V/P boxes) in *Clk* promoter, but since the former accumulates earlier in the cell, the repression and activation events are temporally separated, thus creating rhythmic expression. Interestingly, both factors are also activated by the CLK/CYC complex (Blau and Young, 1999; Cyran *et al*., 2003), providing a second feedback loop at the oscillator's core.

Recently, the level of complexity of the molecular clock has gained an extra dimension. Through microarray (Lim *et al.*, 2007; Kadener *et al*., 2007) and RNA interference screens (Matsumoto *et al.*, 2007), the transcriptional regulator gene *clockwork orange* (*cwo*) was identified. Under CLK/CYC regulation, *cwo* mRNA oscillates with an amplitude and phase comparable with other direct CLK targets such as *vri*, *Pdp1*, *per*, and *tim*. Late at night, CWO feeds back and synergizes with PER to repress CLK/CYC activity (Kadener *et al.*, 2007). This additional negative transcriptional feedback loop helps to maintain circadian oscillations with high amplitudes, which assists the generation of robust behavioral rhythms (Matsumoto *et al.*, 2007; Lim *et al*., 2007; Kadener *et al.*, 2007; Richier *et al.*, 2008).

Based on these findings, a molecular model for the circadian pacemaker in *D. melanogaster* could be hypothesised to include three feedback loops on gene

expression, interconnected by the CLK/CYC activator complex. However, recent evidence has suggested that, although molecularly linked, the genes comprising these feedback loops might contribute to the maintenance of the oscillator's function and rhythmic behaviour in unique ways. PDP1ε, for instance, was initially thought to be a core component of the clock (Cyran *et al.*, 2003), but changes in its expression levels (by overexpression or RNAi-mediated silencing) does not impair *Clk* mRNA rhythms or disrupt other aspects of the oscillator's function (i.e. PER cycling in cell compartments), ruling out this possibility. Nevertheless, the same genetic manipulations resulted in behavioural arrhythmicity, suggesting that PDP1ε rather mediates output signalling pathways (Benito *et al.*, 2007).

The dynamics of PER/TIM complex formation and translocation to the nucleus is a key aspect of the determination of the oscillator's pace (Hardin, 2005). Thus, it is not surprising to find a number of kinases and phosphatases regulating the turnover of core clock components by post-translational modifications (Schoning and Staiger, 2006). During the day, for instance, the accumulation of PER in the cytoplasm is prevented by the activity of the kinase DOUBLETIME (DBT), which might explain why the protein levels peak some hours later than the corresponding mRNA (Price *et al.*, 1998). DBT phosphorylates PER and marks it for degradation in the ubiquitin/proteasome pathway, with the participation of the F-Box/WD40 protein Slimb (SLIMB) (Grima *et al.*, 2002; Ko *et al.*, 2002). The formation of the PER/TIM complex and the action of the protein phosphatase 2A (PP2A), on the other hand, stabilizes PER (Gekakis *et al.*, 1995; Sathyanarayanan *et al.*, 2004; Yang *et al.*, 2004). Finally, the translocation of PER and TIM to the nucleus depends on phosphorylation events by SHAGGY (SGG) (Martinek *et al.*, 2001) and casein kinase 2 (CK2) (Lin *et al.*, 2002; Akten *et al.*, 2003). Overall, the above mentioned regulatory proteins add

another level of complexity to the circadian system, promoting a fine-tuned delay on some core clock components which is crucial for the maintenance of the molecular rhythms. A simplified version of the fly's molecular clock, including some of its transcriptional and post-transcriptional regulation, is illustrated in Figure 1.2.

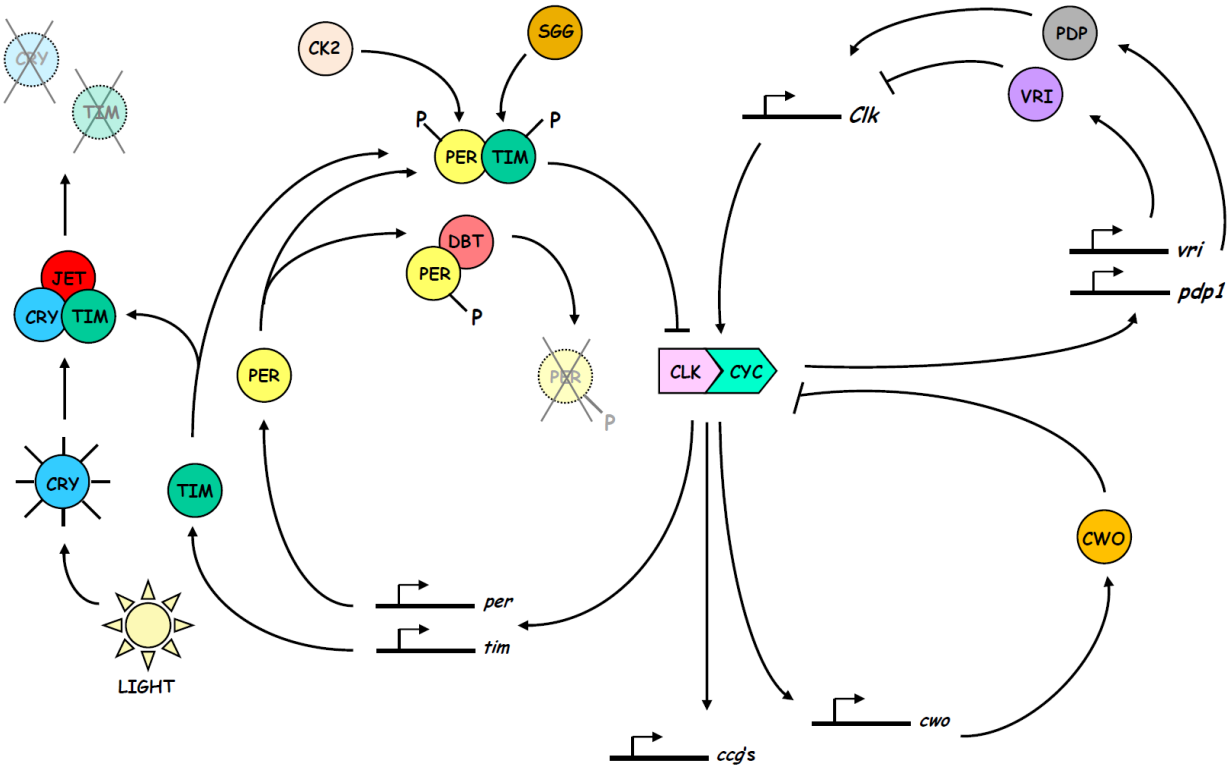


Figure 1.2. Molecular model of the circadian clock in *D. melanogaster*. The CLK/CYC transcriptional activation complex interconnects three self-sustaining feedback loops on gene expression and mediates downstream output processes by promoting the expression of clock-controlled genes (*ccg's*). The synchronization with the environmental cycles occurs primarily by light-mediated stimulation of CRY and subsequently TIM degradation.

1.1.3. Outputs

The output of the molecular clock was demystified through a series of microarray experiments using *Drosophila* tissues harvested at different time points along the day. Those studies identified a number of cycling transcripts, which seem to be under direct or indirect control of *Clk*, reinforcing its role on the generation of molecular and behavioural rhythmicity (McDonald and Rosbash, 2001; Claridge-Chang *et al.*, 2001; Ceriani *et al.*, 2002). Moreover, these clock-controlled genes (*ccg's*) are expressed in a tissue-specific manner, modulating various physiological processes ranging from protein stability and degradation, signal transduction, heme metabolism, detoxification, and immunity (Ceriani *et al.*, 2002).

The various pathways stemming from the central oscillator might govern different rhythmic outputs. Corroborating this idea, the gene *lark*, which encodes an RNA-binding protein, is important for controlling the eclosion gate timing, but does not seem to play any role in locomotor activity rhythms (Newby and Jackson, 1993; McNeil *et al.*, 1998). Likewise, one of *lark*'s molecular targets, the gene *E74*, exhibits comparable phenotypes (Huang *et al.*, 2007). The downstream clock gene *ebony*, on the other hand, affects locomotor activity, but not eclosion rhythms (Newby and Jackson, 1991). *ebony* expression is restricted to glial cells, some of which are found in close proximity of dopaminergic neurons. Moreover, *ebony* interacts genetically with the dopamine transporter gene *dDAT*. Taken together, these findings suggest that *ebony*-expressing glia might modulate dopamine signalling to circadian clock neurons and subsequently affect behaviour (Suh and Jackson, 2007).

Another clock controlled gene is *takeout* and its function comprises a wide range of behaviours, including feeding, locomotor activity and courtship (Sarov-Blat

et al., 2000; Dauwalder *et al.*, 2002; Meunier *et al.*, 2007). Recent evidence revealed that *takeout* expression exhibits a circadian profile similar to that of *Clk* and *cry* and is regulated by *Pdp1ε* (So *et al.*, 2000; Benito *et al.*, 2010).

The most extensively studied output gene in *D. melanogaster* is *Pigment-dispersing factor* (*Pdf*) (Renn *et al.*, 1999). *Pdf* ^{01} null mutants exhibit altered locomotor activity rhythms (i.e. most flies are arrhythmic in DD), but quasi-normal cycling of the core clock gene *period* in the pacemaker neurons (described in section 1.1.5. Neurobiology). In addition, *Pdf* expression and signalling is also crucial for the maintenance of eclosion rhythms (Myers *et al.*, 2003).

1.1.4. Entrainment

The synchronization (or entrainment) of the pacemaker to environmental cycles occurs, primarily, by light-dependent degradation of TIM, when the CLK/CYC complex becomes free of the inhibitory effects of PER/TIM to start a new molecular cycle. The key factor in this resetting mechanism is the flavoprotein CRYPTOCHROME (CRY), which represents the dedicated blue-light circadian photoreceptor (Stanewsky *et al.*, 1998). Upon light stimulation, CRY physically interacts with TIM (Ceriani *et al.*, 1999) and, possibly, with PER (Rosato *et al.*, 2001). This event induces a post-translational modification on TIM, which makes it a substrate for the ubiquitin ligase JETLAG (JET) and, ultimately, leads to its degradation by the proteasome (Koh *et al.*, 2006; Peschel *et al.*, 2006, 2009). JET also assumes a role in CRY degradation, which is ironically, inhibited by TIM (Peschel *et al.*, 2009).

Although CRY function in brain pacemaker neurons is a central aspect of light mediated entrainment, this phenomenon also includes the compound eyes, the ocelli and a pair of extraretinal Hofbauer–Buchner (H–B) eyelets (Helfrich-Förster, 2005). It seems that light information transduced by these photoreceptor organs reaches the clock neurons *via* synaptic connections. In fact, it has been reported that axonal projections stemming from the H-B eyelets make direct contact with the s-LNvs (described in section 1.1.5. Neurobiology) (Helfrich-Förster *et al.*, 2002; Mapel *et al.*, 2002). A similar functional connection may also exist between the compound eye photoreceptors (i.e. R1-R6 and R7/R8) and the fiber network derived from the l-LNvs (Helfrich-Förster *et al.*, 2002; Helfrich-Förster, 2005).

Both the retinal and extra-retinal photoreceptors express the phospholipase C (PLC) encoding gene *norpA* (Pearn *et al.*, 1996; Malpel *et al.*, 2002), which is an essential component of the classical phototransduction cascade. The behavioural analyses of flies carrying *norpA* or *cry* mutations (or even both together) has revealed that, notwithstanding the difficulties, these flies can still adjust their clock to light-dark (LD) cycles (Stanewsky *et al.*, 1998). The complete impairment of light entraining pathways to the pacemaker neurons has been reported in $glass^{60J}$ cry^{b} flies, which have no external or internal eye structures and lack CRY function (Helfrich-Förster *et al.*, 2001). However, recent evidence suggests that even these double mutant flies seem to carry an extra, non-identified, circadian photoreceptor (Breda C, 2010).

The circadian clock can also be entrained by environmental cycles of temperature, even when the difference between the warm and the cold phase of the day is only 3^{o}C (Wheeler *et al.*, 1993). Interestingly, the behavioural rhythms and the molecular clockwork can synchronize to temperature cycles not only in DD but also

under constant light (LL) conditions, which normally leads to arrhythmicity (Yoshii *et al.*, 2002; Glaser and Stanewsky, 2005). The molecular correlate for this phenomenon has been dissected with the identification of two mutant lines which could still entrain to LD cycles, but failed to synchronize to temperature cycles (Glaser and Stanewsky, 2005). One of the mutations was *norpA*, which immediately raised the possibility that light and temperature pathways might indeed overlap for some aspects. The second mutation, exhibiting a more severe phenotype than *norpA*, was called *nocte* (*no circadian temperature entrainment*). Surprisingly, it was recently reported that reducing the function of *nocte* in chordotonal organs dramatically interferes with temperature entrainment, at the molecular and behaviour level (Sehadova *et al.*, 2009). The same study also showed that temperature synchronization, resembling that by light, is a tissue autonomous process, with one important exception: the brain. When isolated in culture, the 'brain clock' seems to react to temperature changes but it is unable to synchronize to the right time of the day, unlike other peripheral organs. To sum up, the current evidence suggests that temperature entrainment occurs through two distinctive ways: a) the *norpA* mediated signal-transduction cascade (It is not known whether the canonical photoreceptors structures or alternative cell/tissues expressing *norpA*, and maybe other PLCs, would be involved in this phenomenon); b) the signalling pathways coming from sensory peripheral organs and involving the gene *nocte*.

The effects of light and temperature on the circadian clock are very important parameters to consider if one decides to study its seasonal adaptations. It has been shown that at low temperature and short photoperiods, mimicking winter conditions, the splicing of the 3' untranslated region (UTR) of *per* 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 ecologically advantageous because the bulk of the activity would occur during the middle of the day, when the temperatures are comparatively warmer. The opposite happens at the high temperatures and long photoperiods of summer, when splicing is inhibited and the peaks of activity are shifted towards dawn and dusk, leading to a siesta during the day. In *norpA* mutants, however, *per* splicing levels are high even at hot temperatures, advancing the molecular cycles and the evening peak of activity (Collins *et al.*, 2004; Majercak *et al.*, 2004). These results implicate that *norpA* not only participates in the daily entrainment of the clock, but also mediates its adjustments to seasonal changes in temperature and photoperiod.

1.1.5. Neurobiology

The investigations of the expression of clock factors at the cellular level revealed the presence of oscillators spread in diverse tissues of the fly body. Using the luciferase reporter fused with *per* promoter, a study showed cycling gene expression in the head, proboscis, antennae, legs, wings, eyes, malphigian tubules and testes of the fly (Plautz *et al.*, 1997). Interestingly, most of these tissues can be maintained in culture without losing its rhythmic output, indicating a certain level of self-sustainability. Despite the presence of multiple peripheral clocks, the centre responsible for the generation of behavioural rhythms is located in the brain and is referred to as "master" clock or central oscillator (Glossop and Hardin, 2002).

Of all the neurons in the fly brain, about 150 are believed to be involved in the circadian clock. Based on their size and anatomical location, these cells were classified in two main groups: the DNs (dorsal neurons) and the LNs (lateral neurons).

These were further subdivided into six classes: LN_ds (dorsal lateral neurons), s-LN_vs (small ventral lateral neurons), l-LN_vs (large ventral lateral neurons), DN_1s (dorsal neurons group 1), DN_2s (dorsal neurons group 2) and DN_3s (dorsal neurons group 3) (Kaneko and Hall, 2000; Helfrich-Förster, 2007) (Figure 1.3). Further studies also suggested incrementing this list by adding another cellular cluster, namely the lateral posterior neurons (LPNs) (Shafer *et al.*, 2006; Miyasako *et al.*, 2007). The role of each of these cellular groups and the way they interact to form the circadian neuronal network, controlling the fly's behaviour, are not yet completely understood. However, some insights have been obtained by ablation and clock gene manipulation of these cells (Grima *et al.*, 2004; Stoleru *et al.*, 2004, 2007; Helfrich-Förster *et al.*, 2007; Murad *et al.*, 2007).

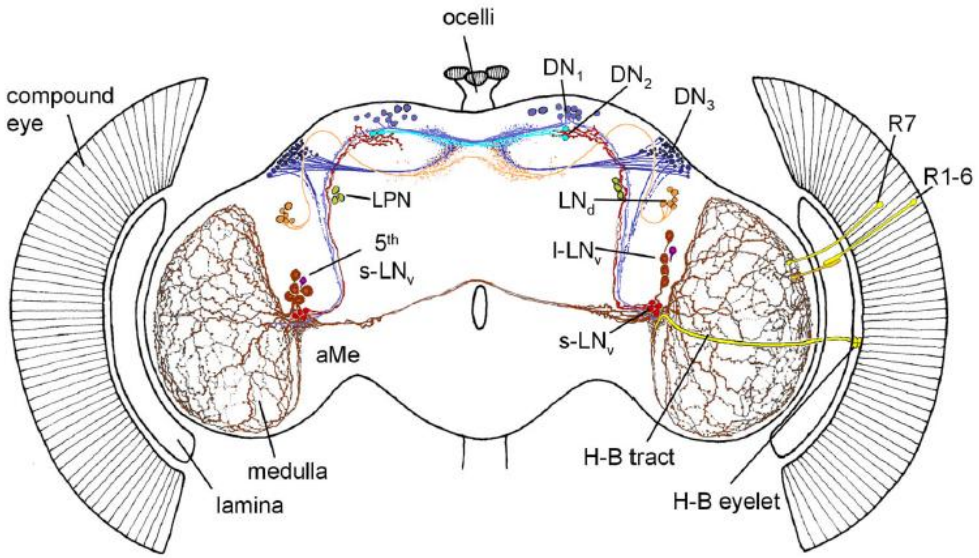


Figure 1.3. Illustration of the *Drosophila* brain showing the relevant clock neuron clusters and their arborization patterns. The photoreceptor structures, such as the compound eyes, ocelli and H-B eyelets are also depicted (Extracted from Helfrich-Förster *et al.*, 2007).

It has been demonstrated that the s-LN_vs are involved in controlling the anticipation of the morning locomotor activity, while the LN_ds and DN_1s control the anticipation of the evening (Stoleru *et al.*, 2004; Grima *et al.*, 2004). These cellular groups, known as "M" (morning) cells and "E" (evening) cells, seem to operate under a hierarchy to control the behavioural output (Stoleru *et al.*, 2005). Recently, a model has been proposed to explain how the oscillators of both "M" and "E" cells operate to adapt to seasonal variations in day length (Stoleru *et al.*, 2007). According to this model, in the winter, when the photoperiods are short, the "M" cells are the master clock, controlling the pace of the "E" cells slave clock. In the summer, the opposite situation applies: the "E" cells are the master clock and the "M" cells are the slave.

Besides establishing the location of the "master" clock in the *Drosophila* brain, it is important to understand the mechanisms by which the clock neurons communicate. A neuropeptide called Pigment Dispersing Factor (PDF), a homologue of the crustacean Pigment Dispersing Hormone (PDH), seems to have a key role in synchronization among the pacemaker cells, being especially important for those neurons unable to sustain circadian rhythmicity independently (Peng *et al.*, 2003). Supporting this finding, it was reported that the clock neurons cycle out phase in the absence of PDF (i.e. in Pdf^{01} mutant flies), which could explain the high degree of behavioural arrhythmicity observed in DD (Renn *et al.*, 1999).

Using antibody staining, a numbers of expression studies revealed, in detail, the distribution pattern for PDF in the adult nervous system, which includes all the l-LNvs, most of the s-LNvs (i.e. four out of five; the 5^{th} s-LNv is PER+ and PDF-) and a group of neurons located in the abdominal ganglion (Helfrich-Förster, 1995, 1997; Shafer and Taghert, 2009). Because PDF is also present in axonal and dendritic terminals of the LNvs, whereas other clock proteins like PER and TIM are only found

in their cell bodies, these expression studies have revealed an array of neuronal processes, thus providing good insights on the way the clock network is organized. While the s-LNvs send projections to the dorsal region of the brain, where some of the DN1s and DN2s are located, the l-LNvs arborize densely in the medulla (i.e. the second neuropil of the optic lobe) and project via the posterior optic tract (POT) to the other hemisphere (Helfrich-Förster, 1997).

Interestingly, comprehensive analyses of the PDF neuronal circuit have revealed additional levels of circadian regulation. First, PDF intensity appears to cycle in the dorsal termini of the s-LN_vs (Park *et al.*, 2000). This rhythmic release is under clock control and could represent a mechanism by which PDF transmits time information to different pacemaker clusters. The importance of this regulation, however, seems to be controversial, since genetic manipulations that collapsed PDF oscillations did not result in behavioural abnormalities (Kula *et al.*, 2006). A second level of circadian regulation was discovered by carefully observing the same dorsal projections. Surprisingly, the arborisation pattern of these termini seems to change in a circadian manner, raising the possibility that structural plasticity underlies the generation of rhythmic behaviour (Fernandez *et al*., 2008). s-LN_vs and other pacemaker neurons might use neuronal remodelling to establish synapses between themselves and effector output centres, in a spatial and temporal fashion. Supporting this idea, a number of cell adhesion genes were found to cycle in transcriptome studies (Mcdonald and Rosbash, 2001; Ceriani *et al.*, 2002).

Given the significance of PDF signalling to numerous aspects of behavioural and molecular rhythms, determining precisely where the PDF receptor (PDFR) is expressed and how it mediates the flow of information among pacemaker neurons has been a central question. Some years ago, several studies successfully identified the

PDFR as a class II G-Protein Coupled Receptor, but its spatial distribution in the brain was somewhat controversial (Hyun *et al.*, 2005; Lear *et al.*, 2005; Mertens *et al.*, 2005). A thorough analysis, carried out recently, showed that PDFR is found in the entire clock cellular network, in the visual system, and in non-clock brain cells (Im and Taghert, 2010). In fact, the most intensely stained neurons for PDFR were subsets of LNds, DN1s and the 5th s-LNv, all of which project to the same dorsal brain region where PDF is secreted. Curiously, PDFR was also found in the PDF expressing LNvs, indicating a certain degree of autocrine regulation, which, in turn, could be important for the maintenance of the oscillator's amplitude and phase in these cells (Lear *et al.*, 2005).

Although PDF seems to exert a crucial function in the clock network, it is reasonable to expect that other neurotransmitters are also involved (Taghert *et al.*, 2001). The neuropeptide IPNamide (IPNa), for instance, appears to be expressed in anterior DN1s, but its functional significance remains unknown (Shafer *et al*., 2006). Another example is the neuropeptide F (NPF), which was found to be present in some LN_ds of males, but not females, suggesting that it might contribute to the sexually dimorphic profiles of locomotor activity (Lee *et al.*, 2006). In addition to the neuropeptides, other small molecules such as acetylcholine, histamine, serotonin, glutamate and γ-amino-butyric acid (GABA) can also mediate synaptic input to the clock circuitry (Wegener *et al.*, 2004; Yuan *et al.*, 2005; Hamasaka *et al.*, 2005, 2007; Hamasaka and Nassel, 2006). Most of the current data focus on the spatial distribution of neurotransmitters and its receptors, as well as the ability of the pacemaker neurons to respond to their stimuli, but lack functional analyses. Notwithstanding, recent evidence with glutamate and serotonin have revealed that, while the first influences

rhythmicity under LD and DD conditions, the latter seems to modulate signalling pathways leading to photoentrainment (Yuan *et al.*, 2005; Hamasaka *et al.*, 2007).

Finally, behavioural and cellular studies on the circadian rhythms in *D. melanogaster* have successfully located pacemaker neurons and revealed a complex circuitry underlying their interaction pathways. Although a coupled dual-oscillator model, with "M" and "E" cells, has been initially created to explain the bimodal pattern of locomotor activity, recent evidence suggests it might be too simple. Depending on the genetic and environmental conditions chosen, the pacemaker neurons could assume a primary or secondary role in the control of behaviour rhythms, suggesting that the system is rather plastic and composed of multiple oscillators (Nitabash and Taghert, 2008; Sheeba *et al.*, 2008; Helfrich-Förster, 2009). The connection between pacemaker and output centres is also unclear and subject to current research efforts. New technologies applied to this field, such as electrophysiology (Cao and Nitabach, 2008; Sheeba *et al.*, 2008b) and FRET-based cAMP sensors (Shafer *et al.*, 2008), will certainly contribute to unravelling the clock neuronal network.

1.2. The Photoperiodic Clock

1.2.1. Overview

The Earth's annual revolution around the sun and its daily rotation on its axis determine the light-dark patterns to which most living organisms are exposed. As its axis of rotation is tilted with respect to its plane of revolution, the light incidence at a certain locality oscillates along the year, creating seasons. These are characterized not

only by light-dark regimes (i.e. photoperiod) but also by associated environmental factors, like temperature, humidity, etc. Thus, it is not surprising to find out that a wide range of organisms, especially plants and animals living in higher latitude zones, express annual or seasonal cycles in their behaviour and metabolic functions (Moore-Ede *et al.*, 1982).

This phenomenon, called photoperiodism, allows organisms to distinguish the long days (or short nights) of summer from the short days (or long nights) of autumn and winter, and thereby obtain calendar information from the environment (Saunders *et al.*, 2002). Like the circadian clock, photoperiodism uses "noise-free" light information as the preferred environmental signal to control various seasonally appropriate switches in metabolism, most of which have a clear functional significance in nature (Tauber and Kyriacou, 2001; Saunders *et al.*, 2002).

The first report about the existence of photoperiodism was by Garner and Allard (1920). It was demonstrated that many plants, including tobacco, soybean, radish, carrot and lettuce, could only flower and fruit when day length fell between certain limits, with some plants responding to long days and others to short. In insects, photoperiodism is often expressed as a dormant state known as diapause, which allows them to overwinter or to withstand the dry season (Saunders *et al.*, 2002).

Unlike quiescence, which is another type of dormancy, diapause is an "actively induced" state, involving the cessation or alteration of neuroendocrine activity, usually at species-specific points in the insect's life cycle. Moreover, this response is triggered by environmental cues which are predicting forthcoming unfavourable conditions, but are not unfavourable themselves (Tauber and Kyriacou, 2001; Saunders *et al.*, 2002). Usually, the diapause state is associated with reduced morphogenesis, increased resistance to environmental stresses and altered or reduced

behavioural activity (Tauber *et al.*, 1986). Depending on its duration and how easy it is terminated, diapause may be regarded as either "intense" or "shallow" (Saunders *et al.*, 2002).

The photoperiodic system can be schematically divided into three components: a) a receptor, to detect light and other environmental cues; b) a clock, to measure seasonal time (i.e. the length of the day or the night) and integrate this information through a counter mechanism; and c) an effector system, to trigger the appropriate metabolic switch to induce the diapause state. In overwintering organisms, the induction occurs when the day length is perceived to be lower than a critical value (i.e. critical day length), which is often well defined and varies between and within species (Saunders *et al.*, 2004).

In insects, diapause is usually classified according to the stage of life cycle in which the arrest occurs (e.g. embryonic, larval, pupal or adult). In *D. melanogaster*, for instance, it has been demonstrated that short photoperiods (i.e. day length) and low temperatures induce a reproductive diapause in the adults, expressed as a block to vitellogenesis that can be visualized by the lack of yolk inside the ovarian oocytes (Figure 1.4) (Saunders *et al.*, 1989; Saunders and Gilbert, 1990; Schmidt *et al.*, 2005a). However, *D. melanogaster* presents a shallow response, being quickly terminated when the flies are transferred to higher temperatures or longer photoperiods (Saunders *et al.*, 1989). That is to be expected since the evolutionary history of photoperiodism is quite recent in this species, whose ancestral home is the "non-photoperiodic" sub-Saharan African environment.

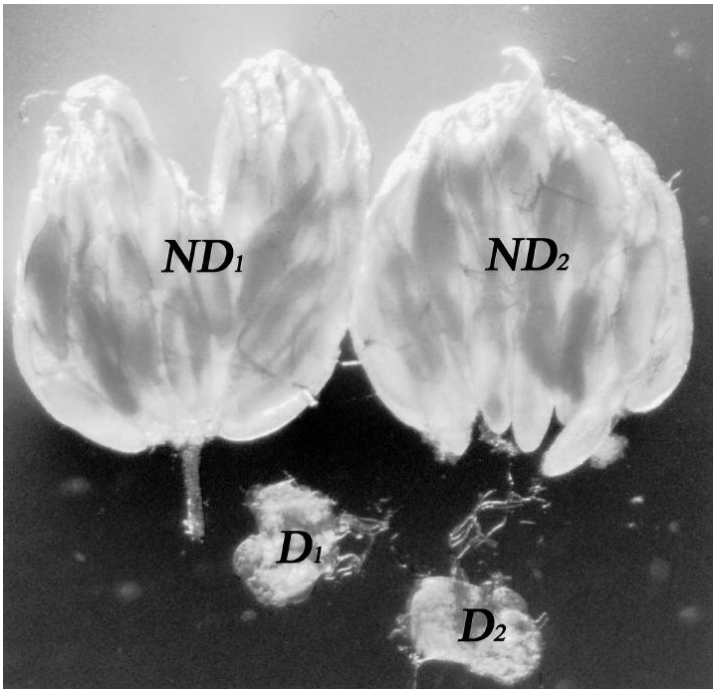


Figure 1.4. The vitellogenesis arrest in *D. melanogaster* ovaries. ND, non-diapausing ovaries; D, diapausing ovaries (from Schmidt *et al.*, 2005a).

Unlike the circadian system, which has been very well molecularly dissected, most of the studies regarding insect photoperiodism have been carried out using a "black box" strategy in which the environmental input to the system (the light–dark cycle) was systematically changed and the output (diapause or development) scored, with very little understanding of the factors involved in time measurement (the so-called "photoperiodic clock") itself. Many of these formal approaches were used to address the question of whether the seasonal time measurement involved the circadian clock or was based on a non-oscillatory hourglass-like mechanism (Saunders *et al.*, 2002). The importance of this distinction is significant because if the photoperiodic clock is found to be a derivative of the circadian system, then both daily and seasonal timing mechanisms might also share a common array of genes and cellular pathways.

As a result of those black box experiments, a number of different theoretical models have been proposed to explain how the photoperiodic clock works (Nunes and Saunders, 1999). The models assuming the involvement of the circadian pacemaker are classified into two main groups: external- and internal-coincidence models. In the first, originally proposed by Bunning (1936) and further developed by Pittendrigh and Minis (1964), light had two roles: the entrainment of the circadian system and photoperiodic induction. As for the latter, light had only one role: the entrainment of multiple oscillators (Pittendrigh, 1972), which would be associated with either dawn or dusk. In this case, photoperiodic induction would occur when day length changes the internal phase relationships between these oscillators. As an alternative model, Lees (1973) and others suggested that the photoperiodic clock was a dark-phase hourglass. In other words, night length was "measured" by a non-oscillatory (i.e. non-circadian) timer set in motion at dusk and used to distinguish short from long nights.

At present, depending on the species and the type of "black box" protocol adopted, most of the theoretical models can be used or slightly modified to explain the functioning of the photoperiodic clock. However, the increasing amount of experimental data, especially at the molecular level, will soon be putting these models to test in a more complete and complex manner.

1.2.2. Genetics

Drosophilidae have been extensively used to uncover the genetics underlying diapause. Most of the data accumulated to date were obtained by classical segregation and linkage analysis and a very superficial mapping of the locus or loci involved in this complex physiological response (Saunders *et al.*, 2002). Moreover,

its genetic bases appeared to change from one species to another, suggesting that it may have arisen independently many times during evolution (Hoy, 1978).

In one of the early studies, Lumme *et al.* (1975) found that the photoperiodic response of *D. littoralis* was mapped to a single autosomal Mendelian unit. Some years later, this unit, called *Cdl* (*Critical daylength for photoperiodic diapause*), was mapped to the fused chromosome 3-4 (Lumme, 1981). In *D. lummei*, evidence suggested an X-linked character (Lumme and Keranen, 1978), while in *D. melanogater*, the response was associated with an autosomal recessive trait (Williams and Sokolowski, 1993).

Currently, new evidence from *D. melanogaster* and *D. triauraria*, as well as many other insect species, like the pitcher-plant mosquito *Wyeomyia smithii*, indicates that diapause is probably a polygenic trait, involving both sex-linked and autosomal loci (Kimura and Yoshida, 1995; Williams *et al.*, 2006; Schmidt *et al.*, 2008; Mathias *et al.*, 2007). These genes are probably under strong selection pressure in the natural environment, thus making the analyses of natural allelic variants a must for understanding the molecular bases and the evolution of photoperiodism.

Because latitude usually correlates with the climate conditions (i.e. temperature, photoperiod, etc) is not unreasonable to speculate that species, or even populations, of insects living in the more northern localities should express a stronger diapause, which allows them to bypass the harsh winter conditions they are exposed to. In fact, it was documented that the incidence of diapause in North American populations of *D. melanogaster* follows a latitudinal cline (Schmidt *et al.*, 2005a). Associated with this photoperiodic response were a number of fitness-related traits, including: life span, age-specific mortality, fecundity, cold resistance, starvation stress, lipid content, developmental time and embryonic viability (Schmidt *et al.*,

2005a, 2005b, 2006). Motivated by these findings, a growing number of recent studies have implicated genes regulating these traits as part of the effector mechanism underlying diapause (Williams *et al.*, 2006; Schmidt *et al.*, 2008; Emerson *et al.*, 2009).

By using a method of chromosomal deletion mapping, it has been demonstrated that the natural variation of diapause incidence in *D. melanogaster* was associated with the insulin-regulated phosphatidylinositol 3-kinase (PI3-kinase) gene, *Dp110* (Williams *et al.*, 2006). Flies overexpressing *Dp110* in the entire nervous system exhibited lower diapause levels, the same not being reproduced when the overexpression was directed to the eye photoreceptor cells. Thus, it appears that this gene is acting in particular cell groups, located in the brain, to negatively regulate diapause. Interestingly, the *Dp110* orthologue in the worm *Caenorhabditis elegans*, *age-1*, plays a role in *dauer* formation, which, like diapause, is an arrest in response to environmental stress (Morris *et al.*, 1996).

The role of PI3K in diapause raises the possibility that other genes acting in the insulin signalling pathway might also be involved. In insects, insulin-like peptides, secreted from the dorsal neurosecretory cells in the brain, promote yolk uptake and ecdysteroid production by the ovaries (Brown *et al.*, 2008), lipid mobilization, increased fecundity and shorter lifespan (Broughton *et al.*, 2005). Under stressful conditions (e.g. caloric restriction, oxidative stress, etc) the insulin signalling pathway is downregulated, resulting in a number of characteristic phenotypes (e.g. cessation of development, increased life-span, etc) that resemble those observed in diapausing flies, suggesting that the insulin signalling pathway might be acting as a whole to modulate the hormonal regulation of diapause (Emerson *et al.*, 2009).

The observed latitudinal cline in diapause propensity of North American populations of fruit-flies was also attributed to a polymorphism in the gene *couch potato* (*cpo*) (Schmidt *et al.*, 2008). The same study revealed that this gene is expressed in the ring gland and has a series of upstream ecdysone response elements, suggesting that its effect on diapause is mediated by ecdysteroids. In the face of these results, it has been postulated that *cpo* might provide the link between the insulin signalling pathway and the downstream hormones involved directly in the regulation of vitellogenesis (Emerson *et al.*, 2009).

The involvement of circadian clock genes in photoperiodism is a polemic subject in the field of chronobiology. It was first tested by Saunders *et al.* (1989), using *D. melanogaster* as study model. Well-known mutations in the *period* gene that would shorten, lengthen or even abolish circadian rhythms were tested for their effect on the photoperiodic response. Although some effect was reported for the arrhythmic mutant per^{01}, which showed a critical day length a few hours shorter than the wild-type, all the genotypes were photoperiodic, still being able to distinguish short from long days. These results were taken as evidence that *period* played no crucial role on diapause, corroborating the hypothesis that the circadian clock is not fundamental for photoperiodism.

However, this scenario has changed with some recent studies in another drosophilid, *Chymomyza costata*, which provided strong evidence for the participation of *timeless* on its photoperiodic response (Pavelka *et al.*, 2003; Stehlik *et al.*, 2008). The mature wild-type larvae of this fly enter a robust diapause when exposed to short days or low temperature. By contrast, in a specific strain isolated from a Sapporo (Japan) population, called *npd*, the photoperiodic induction of this response is abolished (Riihimaa and Kimura, 1988). The *npd* mutants are arrhythmic for adult

eclosion and the larvae lack circadian oscillations on *per* and *tim* transcription (Kostal and Shimada, 2001; Pavelka *et al.*, 2003). Interestingly, when wild-type embryos were injected with double-stranded interference RNA (dsRNAi) to disrupt *tim* expression, a good proportion of the larvae was found to be photoperiodically neutral and, resembling the *npd* mutants, failed to enter diapause (Pavelka *et al.*, 2003). Ultimately, genomic mapping studies finally associated the *npd* mutation with the *tim* locus (Stehlik *et al.*, 2008). Nevertheless, it is still not clear whether the influence of *tim* on diapause is exerted through the circadian clock or a putative pleiotropic effect.

In *D. melanogaster*, *timeless* has also been reported to have an effect on photoperiodism (Tauber *et al.*, 2007). Natural variants isolated from different European populations, carrying either the *ls-tim* or *s-tim* polymorphism, were assessed for diapause under different photoperiods. The results indicated that *ls-tim* flies, which can produce both long and short isoforms of TIM, enter diapause earlier in the year than *s-tim* flies, which produce only the short isoform. The photoperiodic response of the null mutant tim^{01} appeared to be abolished, since diapause incidence was constitutively high irrespective of the day length. Although *tim* is certainly influencing the levels of diapause in this species, it has been suggested that its effect would be restricted to processing the light information at the input level of the photoperiodic clock (Tauber *et al.*, 2007; Emerson *et al.*, 2009).

1.2.3. Photoperiodic clock location

The anatomical location of the photoperiodic clock has been studied in various species of insects (Saunders *et al.*, 2002). Since the elimination of either the photoreceptors or the clock would result in the same phenotype (i.e. the insect's

inability to distinguish between long and short days), most of the studies opted to treat them together. At present, however, the evidence suggests that the photoperiodic clock is probably brain-centred and the photoreceptors might be located either in the compound eyes or in the brain itself.

Recent studies with the black blow fly *Protophormia terraenovae* demonstrated that the removal of certain neurosecretory neurons in the *pars intercerebralis* (PI) ceased vitellogenesis in both diapause (short-days) and non-diapause (long-days) conditions. The opposite situation occurred with the ablation of the *pars lateralis* (PL), when ovarian development was stimulated in both rearing conditions (Shiga and Numata, 2000). Conversely, a similar study in the bean bug *Riptortus pedestris* revealed that the removal of the PI did not have any effect on diapause incidence, but once the PL were ablated the diapause levels decreased substantially (Shimokawa *et al.*, 2008).

Based on those and other evidence, a model has been proposed where the *pars lateralis* (PL) neurons work on the effector mechanism regulating photoperiodism in insects (Shiga and Numata, 2007). Through its axonal projections, the PL innervates the corpus cardiacum (CC) and corpus allatum (CA), which are neuroendocrine brain centres involved in the production and secretion of hormones necessary for gonadal development and reproduction (Saunders *et al.*, 2002). During the photoperiodic induction of diapause, those neurons would have an inhibitory effect on the CC and CA centres, resulting in an ovarian developmental arrest. Interestingly, PDF-immunoreactive fibers form synaptic connections with the PL neurons in *P. terraenovae*, revealing an intriguing link between circadian clock cells and a putative photoperiodic effector centre (Hamanaka *et al.*, 2005).

Corroborating this idea, a recent study involving surgical ablation of PER and PDF immunoreactive neurons in *P. terraenovae* uncovered interesting new information about the location of the photoperiodic clock in this species (Shiga and Numata, 2009). Immunocytochemical analysis on the brain, using antibodies against PDF and PER, revealed a pattern of expression very similar to that observed in *D. melanogaster*, with both factors co-localizing in two cellular groups: the large ventral lateral neurons (l-LN_v) and the small ventral lateral neurons (s-LN_v). Interestingly, bilateral ablation of the anterior base of the optic lobe medulla, where the PDF+ neurons are located, affected not only the circadian rhythms but also the photoperiodism (Shiga and Numata, 2009). Diapause incidence levels were about 50% for both long and short photoperiods, suggesting that the photoperiodic clock rather than the effector's mechanism was impaired. Put simply, the diapause phenotype was still being processed, but with no coupled timing mechanism to modulate the response accordingly. Therefore, it appears that, at least for some aspects, circadian behavioural rhythms and photoperiodism share common elements in their underlying neural networks.

Since it was considered for a long time as a commensal non-diapausing species, it is not surprising to find that there are no specific studies on the anatomical location of the photoperiodic clock in *D. melanogaster*. The genetic tools available for this species, however, are unique among the insects and can be certainly explored in an attempt to map the location of the photoperiodic clock. Ablation experiments, for instance, could be performed by using pro-apoptotic genes driven by a series of different enhancers with the UAS/GAL4 system (Duffy *et al.*, 2002). As a result, the knowledge obtained with fruit-flies could be used in comparative studies with other species, shedding light on the way the photoperiodic clocks evolved in insects.

1.3. Natural *timeless* variants

Previous studies on the genomic sequences of *timeless* (*tim*) identified a polymorphism in this gene that involves the insertion/deletion of a single nucleotide situated between two alternative translation starts (Rosato *et al.*, 1997). The implications of this mutational event are that the *ls-tim* allele, with the insertion, could potentially produce both long (L-TIM_{1421}) and short (S-TIM_{1398}) isoforms of the protein, while the *s-tim* allele, with the deletion, produces only the shorter isoform, since translation from the first *ATG* codon is truncated by a premature stop codon (Tauber *et al.*, 2007) (Figure 1.5).

The analysis of natural populations from different regions of Europe revealed that this polymorphism follows a strong latitudinal cline, ranging from southern Italy to Sweden, which suggests an adaptive function (Tauber *et al.*, 2007). It was also shown that the *ls-tim* allele appears to be spreading throughout Europe by directional selection, from an epicentre located in southern Italy, close to the city of Novoli. Phylogenetic analysis suggested that this polymorphism is very recent, arising a few thousand years ago (Tauber *et al.*, 2007; Valeria Zonato, personal communication), long after the time *D. melanogaster* colonised Europe (i.e. about 10,000 years ago). Corroborating with this hypothesis, the frequency of *ls-tim* is zero in sub-Saharan Africa, the ancestral home of this species (Tauber *et al.*, 2007).

In order to find out whether the two alleles had any interesting differences in circadian related phenotypes, a number of ecologically relevant characteristics have been examined (Sandrelli *et al.*, 2007; Tauber *et al.*, 2007). To avoid complications with genetic background or linkage disequilibria, the analysis with the natural variants

were extended to transformant lines carrying engineered *tim* transgenes that could generate only L-TIM or S-TIM isoforms.

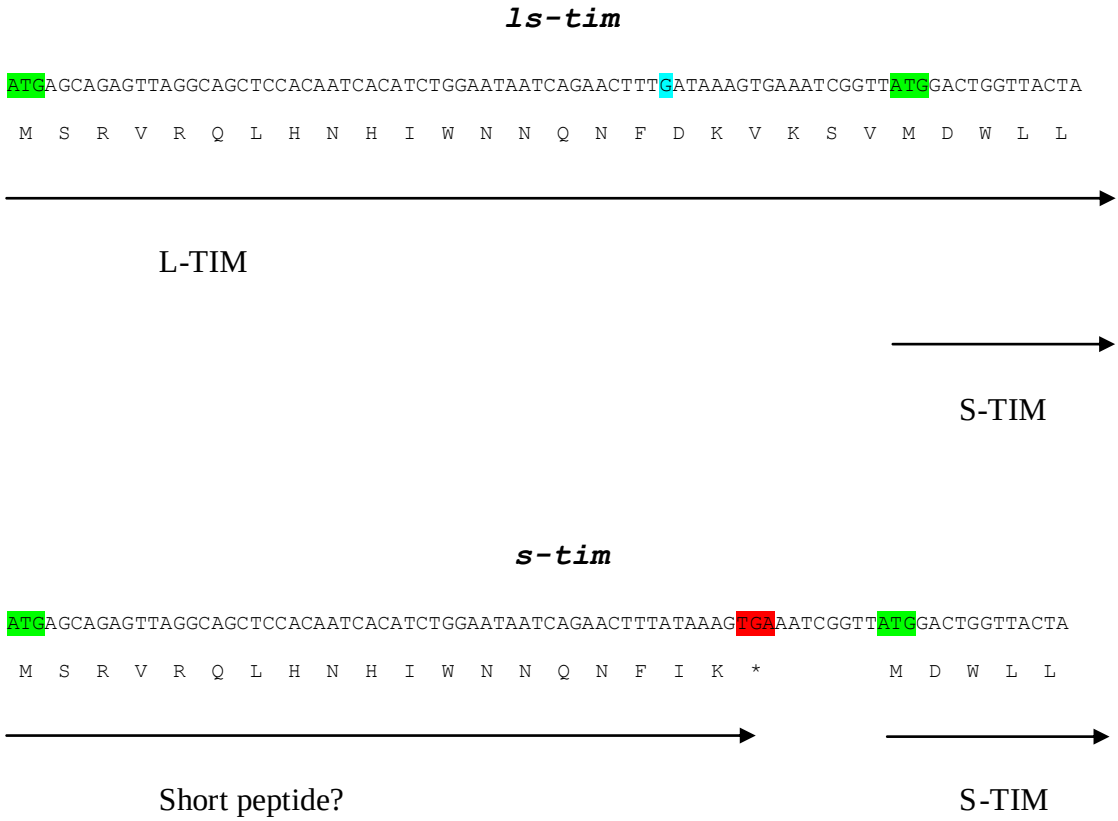


Figure 1.5. The genomic DNA sequences of *timeless* (*tim*) natural alleles, followed by their respective amino acid translations. The *ls-tim* polymorphism (upper panel) is characterized by the insertion of a guanine (G, highlighted in blue), which results in the translation of both L-TIM and S-TIM isoforms (Tauber *et al*., 2007). The deletion of the guanine in the *s-tim* polymorphism (lower panel) causes a premature stop codon (highlighted in red), which results in the translation of S-TIM and a putative 19 residue N-terminal peptide. Highlighted in green, translation starts.

At the physiological level, it was observed that females carrying *ls-tim* or the *P[L-tim]* transgene showed a longer critical day length for diapause induction than those carrying *s-tim* or *P[S-tim]*. In other words, *ls-tim* females would go into diapause earlier in the year, when day length was still long, thereby anticipating winter earlier than *s-tim* females (Tauber *et al.*, 2007).

At the behavioural level, it was shown that the circadian clock is generally less light responsive in *ls-tim* individuals, the same occurring with transgenic correlates (Sandrelli *et al.*, 2007). This reduced photoresponsiveness provides a possible explanation for the longer critical day length of *ls-tim* flies. In other words, these flies could be "seeing" less light than they actually were exposed to, thus going into diapause earlier. Supporting this view, it has been proposed that in temperate zones, which have dramatic seasonal changes in photoperiod, the circadian clock adapts with either increased amplitude of oscillation ("buffering" against light perturbations) or filtering light input, which would prevent it from becoming de-synchronised (Pittendrigh *et al.*, 1991). Since the amplitude of TIM oscillation in "free-running" conditions was similar for both alleles, it appears that *ls-tim* is actually filtering the light input signal.

The observed phenotypic differences between natural variants and transformants were also extrapolated to the molecular level. Using the yeast two-hybrid system, it was observed that the strength of the L-TIM/CRY physical dimerization is dramatically reduced when compared to S-TIM/CRY (Sandrelli *et al.*, 2007; Peschel *et al.*, 2009). Further analyses showed that, in the presence of light, TIM is more stable in *ls-tim* and *P[L-tim]* transformants, highlighting the significance of the yeast results *in vivo* (Sandrelli *et al.*, 2007; Peschel *et al.*, 2006). These findings provide the molecular correlate for the behavioural phenotypes, explaining in an elegant way the reduced light sensitivity of *ls-tim* flies.

Therefore, a recent mutation seems to be spreading under natural selection due to the adaptive phenotypes it confers in the seasonal European environment. However, it is still not completely understood what special feature in the L-TIM sequence is determining its peculiar function. Further investigations on the extra 23 N-terminal residues, including the structure/function analysis on any relevant post-translational motif, are necessary to improve our knowledge on this beautiful example of clock gene evolution.

1.4. Aims

This work intends to provide a better understanding of the molecular bases underling circadian and seasonal adaptations of the fruit-fly *D. melanogaster* in the natural environment. I am particularly interested in providing genetic links between the circadian and photoperiodic clocks, which would suggest a co-evolution between these mechanisms. Previous studies with *tim* natural variants have revealed a role for this locus in the photoperiodic response (diapause). At 13^{o}C and different day length regimes, the diapause levels of *ls-tim* and *s-tim* flies were significantly and consistently different in several populations analysed (Tauber *et al*., 2007). However, it appeared that day length changes affected both genotypes the same way (i.e. no interaction), suggesting that *tim* is not part of the photoperiodic clock and rather acts as a photoreceptor, providing light information to the mechanism. I decided to further investigate the photoperiodic response (diapause) of *tim* natural variants under different temperature conditions to infer how robust is the difference between genotypes and whether there is an yet obscure interaction between them (Chapter 3). The findings with *tim* urge a complete re-evaluation of the participation of circadian

clock genes in photoperiodism. Yet, given the polygenic nature of this phenomenon, any genetic analysis needs to be performed on a homogeneous genetic background. For that reason, I generated a collection of congenic flies carrying canonical clock mutations (Chapter 4), which are well-studied for its effect on the oscillator's core, input and output pathways. These flies were analysed for their circadian behaviour and photoperiodic response.

I also turned my attention to the *ls-tim* polymorphism and its molecular, behaviour and physiological implications. As previously mentioned, flies carrying this polymorphism generate an additional protein isoform, L-TIM, which accounts for its adaptive phenotypes. The effects promoted by L-TIM derive from its extra 23 N-terminal residues, which somehow modulate protein function. I investigated the importance of these residues and putative post-translational modifications by performing structure/function analyses with genetically modified TIM constructs (Chapter 5). These engineered proteins were tested for their ability to interact with CRY (i.e. in Yeast Two-Hybrid assays) and to respond to light (i.e. in behavioural phase-shift experiments). To provide an interesting background for the functional analyses, I also performed a deletion mapping assay to describe which particular region of TIM sequence physically interacts with CRY (Chapter 6).

Chapter 2: Material and Methods

2.1. Fly stocks and husbandry

2.1.1. Fly Stocks

Natural lines

LS-Hu: This strain was originated from an isofemale line obtained from a natural population of Houten (Holland), which lies at a latitude of 52^{o} N. Using PCR genotyping combined with classical genetic crossing methods, this line was made homozygous for the polymorphism *ls-tim* (Tauber *et al.*, 2007; Sandrelli *et al.*, 2007)

S-Hu: This strain was derived from the same isofemale line as LS-Hu (see the description above). However, using the same genotyping and crossing techniques as for the latter, care was taken to create a line homozygous for another natural polymorphism, called *s-tim* (Tauber *et al.*, 2007; Sandrelli *et al.*, 2007).

"Houtenized" flies

The strains cited below refer to well described mutations, which were put in the natural genetic background of Houten (Holland) by repetitive backcrossing (8 generations) to LS-Hu flies (See Chapter 4 for more details). Balancer chromosomes and some transgenes (i.e. UAS, GAL4 and GAL80 constructs) were also placed in this wild background, but for the latter, the original lines were crossed to $w^{1118}{}_{HU}$ flies.

This strategy allowed the tracking of the segregation of transgenic elements in every crossing step, simply by selecting the "red" eyed flies. All the lines, although preserving the original mutations or transgenic elements, were labelled with an additional "HU" in their names, meaning that they are now "Houtenized".

$w^{1118}{}_{HU}$: Loss-of-function *white* mutant commonly used as a genetic background in transgenic manipulations. The flies have *white* eyes phenotype.

ln(2LR)O,Cy_{HU}: Balancer for the second chromosome. Contains the dominant wing mutation *Curly* (*Cy*). Maintained over the dominant marker *Sco*.

$TM6b_{HU}$: Balancer for the third chromosome. Carries a dominant allele of *Antennapedia* (*Hu*) and *Tubby* (*Tb*). Maintained over another balancer, *MKRS*.

$per^{01}{}_{HU}$: The classic "clock" mutant, isolated by EMS-induced mutagenesis (Konopka and Benzer, 1971). It is molecularly characterized by a nucleotide substitution which results in a STOP codon (Q464STOP) (Yu *et al.*, 1987).

$tim^{01}{}_{HU}$: Obtained by *P-element* mutagenesis (Sehgal *et al.*, 1994), these flies carry a deletion of 64 bp causing a frame shift in the coding sequence (Myers *et al.*, 1995).

$Clk^{jrk}{}_{HU}$: This mutant was isolated by chemical mutagenesis (EMS) (Allada *et al.*, 1998). At the DNA level, there is a nucleotide substitution leading to a STOP codon (Q776STOP).

***cry*$^{b}_{HU}$**: EMS-induced mutant. The molecular nature of the lesion is an amino acid replacement (D410N) (Stanewsky *et al.*, 1998).

***cyc*$^{01}_{HU}$**: These flies carry a nonsense mutation (K159STOP), induced by EMS (Rutila *et al.*, 1998) .

***Pdf*$^{01}_{HU}$**: Isolated by EMS screening, this mutant carries a nucleotide substitution causing a premature STOP codon (Y21STOP) (Renn *et al.*, 1999).

***norpA*$^{p24}_{HU}$**: This EMS-induced mutant carries a 28 bp deletion in its DNA, which results in a frameshift and a premature stop codon (Pearn *et al.*, 1996).

***gmr-GAL4*$_{HU}$**: Transgenic flies expressing GAL4 in the eyes (Freeman *et al.*, 1996). The *P-element* insertion used in the backcrossing scheme was the one named *P{GAL4-ninaE.GMR}12* (Bloomington Stock N^{o} 1104).

***tim-GAL4*$_{HU}$**: Transgenic flies expressing GAL4 under the control of *tim* promoter (Emery *et al.*, 1998). The *P-element* insertion used in this work is known as *A3*, which was kindly provided by Ben Collins (New York University).

***elav-GAL4*$_{HU}$**: These transgenic flies express GAL4 in all neurons of the nervous system. The *P-element* insertion used at the beginning of the backcrossing steps is named *C155*, which was isolated during an enhancer trap screening using the construct *P{GawB}* (Lin and Goodman, 1994).

Pdf-GAL4$_{HU}$: Transgenic flies expressing GAL4 under the control of *Pdf* promoter (Renn *et al.*, 1999). For the backcrossing to Houten genetic background, I chose the *P-element* insertion *P{Pdf-GAL4.P2.4}2* (Bloomington Stock N^0 6900) given its broad use in the circadian field.

cry-GAL4$_{HU}$: Transgenic flies expressing GAL4 under the control of the *cry* promoter (Emery *et al*., 2000). The *P-element* insertion *P{cry-GAL4.E}13* was the one chosen to be put in the LS-HU natural background.

Pdf-GAL80$_{HU}$: Transgenic flies expressing GAL80 under the control of *Pdf* promoter (Stoleru *et al.*, 2004). Due to its ability to suppress GAL4-mediated transcription activation, the line *Pdf-GAL80$_{96A}$* was chosen for the backcrossing scheme.

cry-GAL80$_{HU}$: Transgenic flies expressing GAL80 under the control of *cry* promoter (Stoleru *et al.*, 2004). Considering the efficiency to suppress GAL4-mediated transcription activation, I chose the line *cry-GAL80$_{2e3m}$*.

uas-sgg$_{HU}$: Transgenic flies carrying an EP element (Rorth *et al.*, 1996) in the *sgg* locus, which can be used for misexpression/overexpression studies in the binary system UAS/GAL4. This insertion is referred to as *P{EP}sgg^{EP1576}* (Bloomington Stock N^0 11008).

uas-rpr, ***uas-hid$_{HU}$***: Transgenic flies carrying two different *UAS* constructs in the first chromosome (X), kindly provided by François Royer (University of Paris). The *UAS*

sequence was fused either with the cDNA of *reaper* (*rpr*) or *head involution defective* (*hid*), both of them being proapoptotic genes (Zhou *et al.*, 1997)

uas-tim$_{HU}$: Transgenic flies carrying an *UAS* element to misexpress (or overexpress) *timeless* (*tim*) (Yang and Sehgal., 2001). The insertion line used here was the one termed *P{UAS-tim.Y}3-1*, which was kindly provided by Amita Sehgal (University of Pennsylvania Medical School).

2.1.2. Fly husbandry

All fly stocks were maintained in plastic vials containing "sugar meal" (4.6 % w/v Sucrose, 1 % w/v Agar, 0.8 % w/v dried yeast, 0.04 % w/v Nipagen) at either 25^{o}C or 18^{o}C temperature-controlled rooms, which can accelerate or delay the developmental time, respectively. The flies were subjected to a 24 h light-dark (LD) schedule, with lights turning on at 08:00 h and turning off at 20:00 h.

2.2. Genotyping mutant flies

2.2.1. Preparation of Genomic DNA

The genomic DNA of *D. melanogaster* was extracted according to Gloor *et al.* (1993). The method was developed for rapid and reproducible isolation of DNA from single flies, reducing the possibility of contamination by cloned or amplified DNA. Initially, each fly was placed inside a 1.5 ml tube and mashed for 5 to 10 s in 50 µl of Squishing Buffer (Table 2.1). The tubes were, then, incubated at 37^{o}C (or room

temperature) for 1 h, and heated to 95°C for 2 min, to inactivate the Proteinase K. For genotyping the flies being backcrossed to Houten genetic background, the wings, instead of the whole-fly, were used as a source of genomic DNA. In this case, in order to maximize the yield and concentration of the DNA, the wings were incubated overnight at 37°C in only 20 μl of Squishing Buffer. The rest of the protocol proceeds without any alteration.

Table 2.1. Squishing Buffer

Squishing Buffer
10 mM Tris-HCl pH 8.2
1 mM EDTA
25 mM NaCl
200 μg / ml Proteinase K *

* The Proteinase K was added to the Squishing Buffer, just prior to use, from a frozen stock.

2.2.2. Polymerase Chain Reaction (PCR)

The PCR was the technique I chose in order to identify and follow mutant alleles over every generation in the backcrossing scheme. For each of the candidate genes, specific primers were designed and tested in preliminary assays to determine optimal conditions. To sum up, different concentrations of Mg^{2+} ($MgCl_2$ raging from 1.0 to 2.0 mM) and annealing temperatures (55 to 70°C) were tested, both known to be essential parameters for the efficiency of the amplification reaction (McPherson and Moller, 2000). The volumes and concentrations of the reagents used in all the reactions, irrespective of the primers, are listed in Table 2.2.

Table 2.2. PCR reaction mix.

Reagents	Volumes
Ultrapure H_2O	4.7 µl
*[1] PCR buffer (5X)	2.0 µl
Forward (5') Primer (10 µM)	0.5 µl
Reverse (3') Primer (10 µM)	0.5 µl
Taq polymerase (5 units/ µl)	0.3 µl
*[2] DNA	2.0 µl
	Total = 10 µl

*[1] The PCR buffer is supplemented with $MgCl_2$ (7.5 mM) and dNTPs (1.0 mM each).

*[2] The DNA should be added to the reaction together with the fly wings, whenever it is possible. This was shown to maximize the yield of the PCR product.

Specific primers for each gene were designed using the Primer Premier Software version 5.0 (Premier Biosoft). The parameters were set to satisfy some important criteria: length (18 to 25 bp), Tm (55 to 70°C), GC content (40 to 60 %), 3' end stability (ΔG higher than - 9 kcal/mol) and weak or no formation of primers dimmers and hairpins. Also, product lengths ranging from 150 to 400 bp were preferred so as to generate high yields.

For genotyping the flies carrying mutant alleles, two strategies based on PCR were taken. The first consisted of amplifying the DNA flaking the mutation and, then, digesting it with a restriction endonuclease capable of cutting only the mutant DNA. As a result, flies carrying the mutant DNA would show more than one band in the gel, in a pattern that perfectly reflects their genotypes. The

second, and alternative strategy for the first, was based on primer sequences in which the last base (3' end) anneals only with the mutant allele. This technique was very useful in cases where there was no suitable enzyme or when the latter was not working efficiently. It is important to mention that the second technique is not applied to determine genotypes, given that individuals homozygous or heterozygous for the mutation would both show one band in the gel. However, since the aim of the technique was to be able to identify and follow mutant alleles over a number of generations, it was a perfect solution.

The genotyping information (e.g. primer sequence, PCR conditions, etc), for each of the mutations backcrossed to the Houten strain, is described below. For those cases where a restriction analysis would follow the PCR reaction, the details are found in section 2.2.3 (Digestion of PCR fragments):

Clk^{jrk}

Forward primer (*5DmjrkA*): 5' GATGGGATTCGCACCTGG 3'

Reverse primer (*3DmjrkA*): 5' TGCCTCCATTGTAGCTTTGATT 3'

Temperature cycles: 92^{o}C / 2 min, 35 x (92^{o}C / 30 s, 60^{o}C / 30 s, 72^{o}C / 30 s), 72^{o}C / 6 min, 10^{o}C / 2 min or more.

Product length: 530 bp

Restriction analysis: YES (*BfaI*)

cry^{b}

Forward primer (*5Dmcry*b*B*): 5' GCATGGAGGGCAATGACAT 3'

Reverse primer (*3Dmcrybmut1*): 5' GACCGACCAATCCGCATT 3'

Temperature cycles: 92^{o}C / 2 min, 35 x (92^{o}C / 30 s, 66^{o}C / 30 s, 72^{o}C / 30 s), 72^{o}C / 6 min, 10^{o}C / 2 min or more.

Product length: 257 bp

Restriction analysis: NO

cyc^{01}

Forward primer (*5Dmcyc01B*): 5' AGCGGCAGCTTACATCCA 3'

Reverse primer (*3Dmcyc01mut1*): 5' CCTTAACCTTGCCTATGTCCTA 3'

Temperature cycles: 92^{o}C / 2 min, 35 x (92^{o}C / 30 s, 66^{o}C / 30 s, 72^{o}C / 30 s), 72^{o}C / 6 min, 10^{o}C / 2 min or more.

Product length: 241 bp

Restriction analysis: NO

norpA^{p24}

Forward primer (*5Dmnorp24A*): 5' GCAACTCAACCAGGGCAAGT 3'

Reverse primer (*3Dmnorp24A)*: 5' CCGAGGGCAATCCAAACA 3'

Temperature cycles: 92^{o}C / 2 min, 35 x (92^{o}C / 30 s, 64^{o}C / 30 s, 72^{o}C / 30 s), 72^{o}C / 6 min, 10^{o}C / 2 min or more.

Product length: 193 bp (mutant fragment) and 221 bp (wild-type fragment)

Restriction analysis: NO

Pdf ^{01}

Forward primer (*5Dmpdf01mut1*): 5' TGCTGCCAGTGGGGATAA 3'

Reverse primer (*3Dmpdf01A*): 5' CTTACTTGCCCGCATCGT 3'

Temperature cycles: 92°C / 2 min, 35 x (92°C / 30 s, 66°C / 30 s, 72°C / 30 s), 72°C / 6 min, 10°C / 2 min or more.

Product length: 265 bp

Restriction analysis: NO

per ^{01}

Forward primer (*5Dmper01B*): 5' ACGGTAATGAAGAAGGGTCAGA 3'

Reverse primer (*3Dmper01B*): 5' GGGTCCTGGAAGGTGAAATG 3'

Temperature cycles: 92°C / 2 min, 35 x (92°C / 30 s, 60°C / 30 s, 72°C / 30 s), 72°C / 6 min, 10°C / 2 min or more.

Product length: 236 bp

Restriction analysis: YES (*BfaI* or *XbaI*)

tim ^{01}

Forward primer (*5Dmtim01A*): 5' GCTCATCGCTTTTCATATGTT 3'

Reverse primer (*3Dmtim01A*): 5' AGGATGTGATTGGTAACCAC 3'

Temperature cycles: 92°C / 2 min, 35 x (92°C / 30 s, 57°C / 1 min, 72°C / 45 s), 72°C / 6 min, 10°C / 2 min or more.

Product length: 538 bp (mutant fragment) and 602 bp (wild-type fragment)

Restriction analysis: NO

To genotype the flies according to *ls/s-tim* polymorphism, the PCR conditions and primers used in the reaction were the same as previously described (Tauber *et al.*, 2007). For each tested fly, two different PCR reactions were done, using either a primer specific for *ls-tim* or for *s-tim* polymorphism. Both reactions employed the same reverse primer and also a pair of two other primers, used to amplify another region of the same locus, thus representing a control for the efficiency of the reaction.

ls/s-tim haplotypes

Forward *ls-tim* primer: 5' TGGAATAATCAGAACTTTAT 3'

Forward *s-tim* primer: 5' TGGAATAATCAGAACTTTGA 3'

Reverse primer: 5' AGATTCCACAAGATCGTGTT 3'

Control forward primer: 5' CATTCATTCCAAGCAGTATC 3'

Control reverse primer: 5' TATTCATGAACTTGTGAATC 3'

I also developed some primers to check the authenticity of the transgenic fly stocks before starting the backcrossing process. These primers were optimized to amplify *GAL4*, *GAL80* or *UAS* sequences and can be extremely useful for a variety of purposes. Initially, the idea was to use them to follow the transgenes over the generations of backcrossing to Houten flies. However, after the creation of the strain $w^{1118}{}_{HU}$, the procedure would no longer need a PCR approach and this use was discarded. It is important to mention that the primers to detect UAS sequences were designed based on the *pUAST* transformation vector (Brand and Perrimon, 1993) and do not work with *EP* lines (Rorth, 1996).

GAL4

Forward primer (*5Gal4A*): 5' CATTGGGCTTGAATAGGGA 3'

Reverse primer (*3Gal4A)*: 5' TTAGAGCGGTGGTGGAAAT 3'

Temperature cycles: 92°C / 2 min, 35 x (92°C / 30 s, 64°C / 30 s, 72°C / 30 s), 72°C / 6 min, 10°C / 2 min or more.

Product length: 393 bp

Restriction analysis: NO

GAL80

Forward primer (*5Gal80A*): 5' TTCCCACTTTAGAGTCATTTGC 3'

Reverse primer (*3Gal80A)*: 5' CCGTTCCCGATTTCATAGA 3'

Temperature cycles: 92°C / 2 min, 35 x (92°C / 30 s, 64°C / 30 s, 72°C / 30 s), 72°C / 6 min, 10°C / 2 min or more.

Product length: 385 bp

Restriction analysis: NO

UAS

Forward primer (*5uasA*): 5' TGTCCTCCGAGCGGAGACTCTAG 3'

Reverse primer (*3uasA)*: 5' TTCTTGGCAGATTTCAGTAGTTGCAG 3'

Temperature cycles: 92°C / 2 min, 35 x (92°C / 30 s, 64°C / 30 s, 72°C / 30 s), 72°C / 6 min, 10°C / 2 min or more.

Product length: 238 bp

Restriction analysis: NO

2.2.3. Digestion of PCR products

For genotyping the mutant flies per^{01} and Clk^{jrk}, the PCR amplification reactions were followed by a restriction analysis, carried out according to manufacturer's instructions (New England Biolabs). 5 μL of each PCR product was added to 12.2 μl of H_2O, 2.0 μl NEB buffer 4 and 0.8 μl of *BfaI* (5 units/μl). The solution was then incubated at 37°C for 4 h and the resulting DNA fragments were visualized in an agarose gel.

It is important to mention that the digestion was never complete, possibly because the buffer and the polymerase present in the PCR reaction were interfering with the activity of the restriction enzyme. Thus, the band corresponding to the intact amplified DNA could always be visualized in the gel, even if the sample was the DNA from a fly homozygous for the mutation.

For Clk^{jrk} flies, the PCR generated a product of 530 bp, which was cut into two DNA fragments of 325 bp and 205 bp. As for per^{01} flies, the PCR product of 236 bp was cut into two fragments of 170 bp and 66 bp, the latter being hardly seen on a standard agarose gel. As an alternative restriction enzyme to *BfaI*, one can use *XbaI* to cut per^{01} mutant DNA.

2.2.4. Agarose Gel Electrophoresis

The amplified DNA fragments, cut or not with a restriction enzyme, were analysed in agarose gels (0.5 – 2%) supplemented with ethidium bromide (0.5 μg/ml). Typically, 10 μl of the PCR product were mixed with 2 μl of 6X Loading dye (0.25% Bromophenol blue, 0.25% Xylene cyanol, 40% Sucrose) and applied in individual gel

lanes. The electrophoresis was performed under constant voltage (100V) in a tank containing 1X TBE. The usual DNA markers, used along the samples to verify the size of the DNA fragments, were ΦX-174 HaeIII Digest (New England Biolabs), λ HindIII Digest (New England Biolabs) and Hyperladder I (Bioline). After the electrophoresis, the gels were visualized under an UV transilluminator.

2.3. Recombinant *timeless* constructs

2.3.1. N-terminal mutant fragments

To generate a number of N-terminal mutagenized *tim* fragments, the plasmid construct Tim4 (Ousley *et al.*, 1998) was used as the DNA template in site-directed mutagenesis assays based on PCR. Through the use of mutated oligonucleotides (primers), designed above the target site, specific changes were introduced in the resulting DNA. Initially, two independent amplification reactions were performed: A) a standard forward primer (*5DmtimMUTA*) with a mutated reverse primer, using the following temperature cycles: 98°C / 30 s, 35 x (98°C / 10 s, 61°C / 20 s, 72°C / 10 s), 72°C / 2 min; and B) a mutated forward primer with a standard reverse primer (*3DmtimMUTB*), using the following cycling conditions: 98°C / 30 s, 35 x (98°C / 10 s, 70°C / 20 s, 72°C / 20 s), 72°C / 6 min. Because the mutated primers, forward and reverse, were complementary in most, if not all, of their sequences, a small overlapping region between each PCR product was created. This was of fundamental importance for the next and final PCR round, which consisted in purifying and mixing both amplicons in a new reaction, utilizing the distally located standard primers (*5DmtimMUTA* and *3DmtimMUTB*) and the following temperature cycles: 98°C / 30

s, 35 x (98°C / 10 s, 65°C / 20 s, 72°C / 25 s), 72°C / 6 min. A schematic representation of this strategy can be found in Figure 5.4. The appropriate volumes and concentrations of the reagents used in the PCR reaction mix are described in Table 2.3. It is important to mention that all amplifications were done with the proof-reading enzyme "Phusion® Hot Start High-Fidelity DNA Polymerase" (Finnzymes). Nevertheless, as an extra precaution for PCR-induced mutations, I confirmed the DNA sequence of the amplified DNA, discarding any clones carrying a non-expected nucleotide mismatch.

Table 2.3. PCR reagent mix for the *tim* fragment cloning.

Reagents	Volumes
Ultrapure H_2O	12.64 μl
*[1] Phusion HF buffer (5X)	4.0 μl
Forward (5') Primer (10 μM)	1.0 μl
Reverse (3') Primer (10 μM)	1.0 μl
dNTPs (25 mM each)	0.16 μl
Phusion® polymerase (2 units/ μl)	0.2 μl
*[2] DNA	1.0 μl
	Total = 20 μl

*[1] The Phusion HF buffer is supplemented with $MgCl_2$ (7.5 mM), so that the final concentration in the reaction is 1.5 mM .

*[2] The DNA concentration varied from 1 to 30 ng/μl.

The nucleotide sequences of the standard and mutated primers described above can be found in Tables 2.4 and 2.5, respectively. The information regarding the combination of primers used for generating each type of mutation is documented in

Table 2.6. For obvious reasons, the generation of the L-TIM control fragment didn't use any mutated primers. The amplification of such wild-type DNA was performed just by challenging the Tim4 template construct with a combination of the forward (*5DmtimMUTA*) and reverse (*3DmtimMUTB*) standard primers, in a one-step only PCR.

Table 2.4. Standard primers.

Name	Sequence (5' > 3')
5DmtimMUTA	TGCAATTCGGCTAAAACTAAAACAGTAA
3DmtimMUTB	CCCAATTTGACCGCAGATACGT

Table 2.5. Mutated primers.

Name	Sequence (5' > 3')
5DmtimMUTB	AAGTGAAAGCGGTTATGGACTGGTTAC
3DmtimMUTA	TAACCAGTCCATAACCGCTTTCACTTT
5DmtimMUTC	AAGTGAAAGACGTTATGGACTGGTTAC
3DmtimMUTC	TAACCAGTCCATAACGTCTTTCACTTT
5DmtimMUTD	AAGTGAAAGCGGTTGGGGACTGGTTAC
3DmtimMUTD	TAACCAGTCCCCAACCGCTTTCACTTT
5DmtimMUTE	AAGTGAAAGACGTTGGGGACTGGTTAC
3DmtimMUTE	TAACCAGTCCCCAACGTCTTTCACTTT
5DmtimMUTF	ATCAGAACTTTATAAAGTGAAATCGGTTATG
3DmtimMUTF	ACCGATTTCACTTTATAAAGTTCTGATTATTC
5DmtimMUTI	AAGTGAAATCGGTTGGGGACTGGTTAC
3DmtimMUTI	TAACCAGTCCCCAACCGATTTCACTTT
5DmtimMUTJ	GCAGTCGACAGGGAGCAGAGTTAG

3DmtimMUTJ	CCTAACTCTGCTCCCTGTCGACTG
5DmtimMUTK	GCAGTCGACAATGGCCAGAGTTAG
3DmtimMUTK	CCTAACTCTGGCCATTGTCGACTG
5DmtimMUTL	GCAGTCGACAATGGACAGAGTTAGG
3DmtimMUTL	CCTAACTCTGTCCATTGTCGACTGC
3DmtimMUTMIX	CATTGTCGACTGCGAACATTGAG
5DmtimL(01/12)fly	CTCAATGTTCGCAGTCGACAATGAGCAGAGTTAGGCAGCTCCAC
5DmtimL(05/15)fly	CTCAATGTTCGCAGTCGACAATGAGGCAGCTCCACAATCACAT
5DmtimL(12/23)fly	CTCAATGTTCGCAGTCGACAATGTGGAATAATCAGAACTTTGATAAAGTGA
5DmtimL(FLIP)fly	CTCAATGTTCGCAGTCGACAATGAATAATCAGAACTTTGATAAAGTGAAATC
5DmtimL(Random)fly	CTCAATGTTCGCAGTCGACAATGCTCTGGAAAGATAGCTTTAATCAGA

Table 2.6. Primers used to generate each N-terminal *tim* mutant fragment.

Construct	**1st PCR round** **(Standard primers vs. Mutated primers)**	**2nd PCR round** **(Standard primers only)**
SS-TIM	a) *5DmtimMUTA* x *3DmtimMUTF* b) *5DmtimMUTF* x *3DmtimMUTB*	*5DmtimMUTA x 3DmtimMUTB*
S-TIM	a) *5DmtimMUTA* x *3DmtimMUTJ* b) *5DmtimMUTJ* x *3DmtimMUTB*	*5DmtimMUTA x 3DmtimMUTB*
L-TIM	————————	*5DmtimMUTA x 3DmtimMUTB*
L(S2,A22)	a) *5DmtimMUTA* x *3DmtimMUTA* b) *5DmtimMUTB* x *3DmtimMUTB*	*5DmtimMUTA x 3DmtimMUTB*
L(A2,S22)	a) *5DmtimMUTA* x *3DmtimMUTK* b) *5DmtimMUTK* x *3DmtimMUTB*	*5DmtimMUTA x 3DmtimMUTB*
L(A2,A22)	a) *5DmtimMUTA* x *3DmtimMUTK* b) *5DmtimMUTK* x *3DmtimMUTB*	*5DmtimMUTA x 3DmtimMUTB*
L(S2,D22)	a) *5DmtimMUTA* x *3DmtimMUTC* b) *5DmtimMUTC* x *3DmtimMUTB*	*5DmtimMUTA x 3DmtimMUTB*

L(D2,S22)	a) *5DmtimMUTA* x *3DmtimMUTL* b) *5DmtimMUTL* x *3DmtimMUTB*	*5DmtimMUTA x 3DmtimMUTB*
L(D2,D22)	a) *5DmtimMUTA* x *3DmtimMUTL* b) *5DmtimMUTL* x *3DmtimMUTB*	*5DmtimMUTA x 3DmtimMUTB*
L- TIM (G24)	a) *5DmtimMUTA* x *3DmtimMUTI* b) *5DmtimMUTI* x *3DmtimMUTB*	*5DmtimMUTA x 3DmtimMUTB*
L(S2,A22,G24)	a) *5DmtimMUTA* x *3DmtimMUTD* b) *5DmtimMUTD* x *3DmtimMUTB*	*5DmtimMUTA x 3DmtimMUTB*
L(S2,D22,G24)	a) *5DmtimMUTA* x *3DmtimMUTE* b) *5DmtimMUTE* x *3DmtimMUTB*	*5DmtimMUTA x 3DmtimMUTB*
L(01-12)	a) *5DmtimMUTA* x *3DmtimMUTMIX* b) *5DmtimL(01/12)fly* x *3DmtimMUTB*	*5DmtimMUTA x 3DmtimMUTB*
L(05-15)	a) *5DmtimMUTA* x *3DmtimMUTMIX* b) *5DmtimL(05/15)fly* x *3DmtimMUTB*	*5DmtimMUTA x 3DmtimMUTB*
L(12-23)	a) *5DmtimMUTA* x *3DmtimMUTMIX* b) *5DmtimL(12/23)fly* x *3DmtimMUTB*	*5DmtimMUTA x 3DmtimMUTB*
L(Flip)	a) *5DmtimMUTA* x *3DmtimMUTMIX* b) *5DmtimL(FLIP)fly* x *3DmtimMUTB*	*5DmtimMUTA x 3DmtimMUTB*
L(Random)	a) *5DmtimMUTA* x *3DmtimMUTMIX* b) *5DmtimL(Random)fly* x *3DmtimMUTB*	*5DmtimMUTA x 3DmtimMUTB*

The amplified DNA fragment was then ligated into the vector pSC-B, following the protocol of the "Strataclone Blunt PCR cloning kit" (Stratagene) (section 2.3.5). Clones containing the appropriate insert size were selected (section 2.3.9) and their DNA sequences were confirmed (section 2.3.10). Finally, each of the resulting constructs was digested with SwaI and MluI, releasing a ~1.5 kb N-terminal fragment to be cloned into the vector *pattB-timcold* described in the next section.

2.3.2. Fly constructs

A ~9,5 kb SpeI(blunt)-KpnI fragment of Tim4 construct (Ousley *et al.*, 1998), containing the promoter and the cDNA of *timeless*, was cloned at the HpaI and KpnI sites of the *pattB* vector (http://www.frontiers-in-genetics.org/flyc31/), to create the vector *pattB-Tim4*.

A ~4.2 kb SacI-KpnI fragment of Tim4, containing ~80% of the coding region and the entire 3'UTR sequence, including termination signals (e.g. poly-adenylation), was cloned in pBluescript® II KS (+) plasmid (Stratagene). The resulting construct was then digested and ligated with a ~2.8 kb MluI-ClaI fragment from the vector BC_Tim3 (kindly provided by Federica Sandrelli; carries a portion of *tim* cDNA). The DNA exchange with BC_Tim3 ensured that our construct would not carry a 237 bp intron and the missense mutation S1312F (position numbering according to Genbank accession AAN10371), both present in the original Tim4 sequence. The next step consisted of replacing a 324 bp BsmBI-ClaI fragment of our plasmid for an analogous BsmBI-ClaI fragment including the tim^{cold} intron (last intron before the stop codon), obtained by amplification of the genomic DNA from flies collected in Houten (Holland) and carrying the *ls-tim* polymorphism. I opted to include this intron in our final construct given a strong evidence that it is retained in most transcripts of flies collected at cold temperatures (Boothroyd *et al.*, 2007; Rodolfo Costa, personal communication), thus being relevant for our functional studies (i.e. diapause). The resulting construct, which contains most of the *tim* cDNA plus the intron, in a pBluescript backbone, was referred to as *pBS-timcold*.

Subsequently, a ~3.6 kb MluI-KpnI fragment of *pattB-Tim4* was substituted for a ~4.2 kb MluI-KpnI fragment from *pBS-timcold* to make the vector *pattB-*

timcold. Finally, a ~1.5 kp SwaI-MluI fragment of the latter was replaced by a series of equivalent fragments originated by site-directed mutagenesis (see section 2.3.1), thus creating the various N-terminal *tim* mutant constructs. These were sent to a company (Bestgene Inc.) for the generation of transgenic flies, using a new cutting-edge method based on phiC31 site-specific integration (Bischof *et al.*, 2007).

2.3.3. Yeast constructs

N-terminal mutants

The N-terminal *tim* fragments generated by site-directed mutagenesis (section 2.3.1) were used as DNA templates for new PCR reactions. Forward primers were designed in order to include an EcoRI restriction site at the 5' end (Table 2.7), which would guarantee a correct reading frame for the *tim* coding sequences in the yeast Two-Hybrid vectors. The names and nucleotide sequences of forward primers used in each of the yeast constructs are represented in Tables 2.7 and 2.8, respectively.

Table 2.7. Primers for the creation of N-terminal *tim* fragments in the Two-Hybrid System.

Construct	Forward Primer
S-TIM	*5DmtimS-Y2H*
L-TIM	*5DmtimL-Y2H*
L(S2,A22)	*5DmtimL-Y2H*
L(A2,S22)	*5DmtimL(A2)-Y2H*
L(A2,A22)	*5DmtimL(A2)-Y2H*
L(S2,D22)	*5DmtimL-Y2H*

L(D2,S22)	*5DmtimL(D2)-Y2H*
L(D2,D22)	*5DmtimL(D2)-Y2H*
L- TIM (G24)	*5DmtimL-Y2H*
L(S2,A22,G24)	*5DmtimL-Y2H*
L(S2,D22,G24)	*5DmtimL-Y2H*
L(01-12)	*5DmtimL(01/12)Y2H*
L(05-15)	*5DmtimL(12/23)Y2H*
L(12-23)	*5DmtimL(05/15)Y2H*
L(Flip)	*5DmtimL(Flip)Y2H*
L(Random)	*5DmtimL(Random)Y2H*

Table 2.8. Yeast N-terminal primer sequences

Name	* Sequence (5' > 3')
5DmtimL-Y2H	GAATTCATGAGCAGAGTTAGGCAGCTCCAC
5DmtimS-Y2H	GAATTCATGGACTGGTTACTAGCAACTCCG
5DmtimL(A2)-Y2H	GAATTCATGGCCAGAGTTAGGCAGCTCCAC
5DmtimL(D2)-Y2H	GAATTCATGGACAGAGTTAGGCAGCTCCAC
5DmtimL(01/12)Y2H	GAATTCATGAGCAGAGTTAGGCAGCTCCACAATCACATCTGG GTTATGGACTGGTTACTAGCAACTCCG
5DmtimL(05/15)Y2H	GAATTCATGAGGCAGCTCCACAATCACATCTGGAATAATCAG GTTATGGACTGGTTACTAGCAACTCCG
5DmtimL(12/23)Y2H	GAATTCATGTGGAATAATCAGAACTTTGATAAAGTGAAAT
5DmtimL(Flip)Y2H	GAATTCATGAATAATCAGAACTTTGATAAAGTGAAATCGAGC AGAGTTAGGCAGCTCCACAATCACATCTGGGTTATGGACTGGT TACTAGCAACTCCG
5DmtimL(Random)Y2H	GAATTCATGCTCTGGAAAGATAGCTTTAATCAGAGAAATCAC AAACAGAATGTTATCGTGTCGCACAACAGGGTTATGGACTGG TTACTAGCAACTCCG

* The nucleotide sequences contain a 5' end EcoRI tail (highlighted in yellow).

All the forward primers were used in combination with the standard reverse primer *3DmtimMUTB* (for sequence information, see Table 2.4). Due to its high processivity and proof-reading activity, my standard choice for thermophilic enzyme was the "Phusion® Hot Start High-Fidelity DNA Polymerase" (Finnzymes). The temperature cycling conditions were as follows: 98°C / 30 s, 35 x (98°C / 10 s, 67°C / 20 s, 72°C / 20 s), 72°C / 6 min. The volumes and concentrations for each reagent in the PCR reaction mix were exactly as described in Table 2.3.

Following the PCR reaction, the amplified fragments were first analysed and, then, extracted from agarose gels to be ligated into pSC-B vector using the "Strataclone Blunt PCR cloning kit" (Stratagene) (see section 2.3.5). After screening for clones containing the insert, their nucleotide sequence was confirmed to avoid PCR-induced mutations.

The resulting plasmids described above were cut with EcoRI and MluI to release a ~1.4 kb DNA fragment, which corresponds to the N-terminal portion of *tim*. Meanwhile, a ~2.9 kb MluI-XhoI fragment containing the C-terminal region of the gene was isolated from the plasmid BC_Tim3. Finally, the yeast two-hybrid vectors pJG4-5 and pEG202 were cut with EcoRI and XhoI to serve as receptor molecules for both EcoRI-MluI and MluI-XhoI *tim* fragments in a three-way ligation reaction.

TIMΔ fragments

To generate a series of *tim* fragments, the construct S-TIM (see item above, "N-terminal mutants") was used as a template in standard PCR reactions. Forward and reverse primers were designed at strategically located sites to amplify different sequence regions, such as those coding for the putative ARM domains (Vodovar *et*

al., 2002). Importantly, an XhoI 5' tail was carefully added to the primers to allow a subsequent cloning in pJG4-5, as well as a correct reading frame when the recombinant proteins were expressed. Further information about the primers used for the generation of each TIMΔ construct can be found in Tables 2.9 and 2.10.

Table 2.9. TIMΔ constructs

* Construct	Primer pair
(1-466)	*5DmtimDELTA1* X *3DmtimDELTA1*
(458-1078)	*5DmtimDELTA2* X *3DmtimDELTA2*
(1056-1398)	*5DmtimDELTA3* X *3DmtimDELTA3*
(1-1078)	*5DmtimDELTA1* X *3DmtimDELTA2*
(458-1398)	*5DmtimDELTA2* X *3DmtimDELTA3*
(1-1398)	*5DmtimDELTA1* X *3DmtimDELTA3*

* The numbers refer to the range of residues included in each construct, according to the full-length S-TIM coding sequence (NCBI Accession number NP_722914).

Table 2.10. TIMΔ primer sequences

Name	* Sequence (5' > 3')
5DmtimDELTA1	CTCGAGATGGACTGGTTACTAGCAACTCCGCA
3DmtimDELTA1	CTCGAGTTAGCCCAATTTGACCGCAGATACGT
5DmtimDELTA2	CTCGAGTCCCACGTATCTGCGGTCAAATTG
3DmtimDELTA2	CTCGAGTTAGCCCAGCTTGTGGAGCAGGAGAAC
5DmtimDELTA3	CTCGAGCAGTGGAACAACGAGCAATCCACTAC
3DmtimDELTA3	CTCGAGGTCGACGGTATCGATTGG

* The nucleotide sequences contain a 5' end XhoI tail (highlighted in yellow).

To amplify the fragments (1-466), (458-1078) and (1056-1398), the following temperature cycles were used: 98°C / 30 s, 35 x (98°C / 10 s, 65°C / 20 s, 72°C / 35 s), 72°C / 6 min. For slightly longer fragments, such as (1-1078) and (458-1398), the cycling conditions were as follows: 98°C / 30 s, 35 x (98°C / 10 s, 67°C / 20 s, 72°C / 1 min 20 s), 72°C / 6 min. And, finally, for the full-length *tim* fragment (1-1398), the temperature cycles were: 98°C / 30 s, 35 x (98°C / 10 s, 67°C / 20 s, 72°C / 1 min 50 s), 72°C / 6 min.

As described for the "N-terminal mutants", the amplified *tim* fragments were first ligated into pSC-B and their nucleotide sequences were verified by sequencing. In case no PCR-induced mutations were detected, these plasmids were digested with XhoI and the *tim* fragments were finally transferred the two-hybrid vector pJG4-5.

2.3.4. Purification of DNA fragments

The PCR products and restriction digest fragments were purified from the agarose gel with the kits "MinElute Gel Extraction" and "QIAquick Gel Extraction" (QIAGEN), according to manufacturer's instructions. After an approximate determination of the fragment length, scalpel blades were used cut the DNA band out of the gel and put it inside microcentrifuge tubes. The weight of the agarose was verified and, for every milligram (mg) of it, I added 3 μl of buffer QG. Then, the tube was incubated at 50°C for 10 min until all the agarose has melted. Subsequently, I added the equivalent of 1 μl of isopropanol for every mg of the initial agarose and mixed the solution. Next, this was applied to the appropriate silica column (the MinElute spin column purifies DNA ranging from 70 bp to 4 Kb, and the QIAquick spin column is suitable for fragments ranging from 100 bp to 10 Kb) and centrifuged

at 16,000 x *g* for 1 min, discarding the flow-through in the end. After that, I followed 2 steps of washing / centrifugation (1 min at 16,000 x *g*) by adding to the column 500 μl of buffer QG (to remove traces of agarose) and, later, 750 μl of buffer PE. An additional one minute centrifugation was necessary to remove residual ethanol (present in the buffer PE). Finally, the DNA was eluted either in 10 μl (for the MinElute collumn) or 30 μl (for the QIAquick column) of the buffer EB (10 mM Tris-Cl, pH = 8.5).

2.3.5. Ligation

The purified PCR fragments were ligated into pSC-B plasmid vector according to the instructions of the "StrataClone Blunt PCR Cloning kit" (Stratagene). The reaction mixture was prepared by adding 2 μl of the purified PCR product (variable concentration), 3 μl of "StrataClone Blunt Cloning Buffer" and 1 μl of "StrataClone Blunt Vector Mix". The solution was then incubated for 5 min at room temperature and placed on ice until the transformation step (section 2.3.6).

For the cloning of fragments generated by digestion of the DNA by restriction endonucleases, the ligation reaction was performed as follows. First, both the restriction fragments from the donor plasmid (insert) and the receptor plasmid (vector) had their sizes (in number of base pairs) and concentration (in ng/μl) measured. Then, the equivalent volume for 50 ng of the vector, a standard amount in the ligation reaction, was estimated. The volume of the corresponding insert solution was calculated by using an insert:vector molar ratio of 3:1. Subsequently, 1 μl of T4 DNA Ligase (New England Biolabs), 2,5 μl of T4 Ligase Buffer (New England Biolabs) and both insert and vector solutions were added to a 1,5 ml microcentrifuge tube to a

final volume of 25 μl, which was adjusted with ultrapure distilled water. The reaction mixture was incubated overnight at 16 °C to follow a transformation protocol.

For long-term storage, the ligation solution was kept in the freezer (-20 °C).

2.3.6. Transformation

The transformation protocol was carried out according to Sambrook and Russel (2001). At first, 2 μL of the ligation solution was added to a tube containing 50 μl of chemically competent cells (*Eschericha coli* DH5α). Then, it was incubated for 20 min on ice, heat-hocked for 45 s at 42^{o}C, and placed on ice again for 2 min. Next, the transformed cells were allowed to recover in 1 ml of liquid LB medium for 1 h at 37^{o}C, and spread in plates containing solid culture medium LB-Agar supplemented with ampicilin (100 μg/ml), X-gal (80 μg/ml) and IPTG (0.5 mM). Finally, the plates were incubated for 16 h (overnight) at 37^{o}C to allow the growth and blue/white selection of bacterial colonies.

For the ligation solutions obtained with the "StrataClone Blunt PCR Cloning kit" (Stratagene), the transformation protocol described above was carried out in "SoloPack" competent cells, which are part of the kit. These cells express Cre recombinase, which is necessary for the circularization of the linear DNA molecules produced by topoisomerase I-mediated ligation.

2.3.7. Inoculation

White colonies were selected from culture plates and allowed to grow overnight at 37^{o}C in 5 ml of LB medium supplemented with ampicillin (100 μg/ml).

Then, the bacterial cultures were centrifuged repeatedly for 2 min at maximum speed (13,000 x *g*) in 1.5 ml tubes. After each centrifugation step, the supernatant was discarded. The resulting cellular pellet was kept at - 20^{o}C (freezer) until it was used for the plasmid DNA extraction (Miniprep).

2.3.8. Miniprep

The extraction of plasmid DNA (Miniprep) was done with the "QIAprep Spin Miniprep Kit" (QIAGEN), according to manufacturer's instructions. This protocol is based on alkaline lysis of bacterial cells, followed by adsorption of DNA onto silica in the presence of high salt concentrations. Initially, the cell pellet was resuspended in 250 μl of buffer P1 until no clumps were visible. Then, 250 μl of buffer P2 were added and the solution was mixed by inverting the tube several times. Following that, 350 μl of the buffer N3 was added and, again, the solution was mixed by inversion. After a centrifugation step (10 min at 13,000 x *g*), the supernatant was collected and applied to the silica column provided. The latter was centrifuged for 1 min at 13,000 x *g*, discarding the flow-through in the end. Then, repeating this procedure, two washing/centrifugation steps took place: the first with 500 μl of buffer PB and the second with 750 μl of buffer PE. Residual ethanol present in the buffer PE was removed by an additional spin of 1 min at 13,000 x *g*. Finally, the plasmid DNA was eluted in 50 μl of buffer EB (10 mM Tris-Cl, pH = 8.5).

2.3.9. Plasmid digestion

The digestion of plasmid DNA was carried out with different restriction endonucleases, supplied by different companies (e.g. New England Biolabs, Fermentas). As a result, the reagents used (e.g. buffer, BSA) and the reaction conditions (e.g. incubation temperature and time) varied enormously depending on the enzyme or the DNA to be digested. Therefore, I simply followed the instructions of the manufacturer, which proved to work in most of the cases. The amount of enzyme (units) and the incubation time were sometimes increased to achieve complete digestion.

However, in some cases, a quite standard digestion protocol was used for the diagnosis of the plasmids containing the DNA fragment of interest. Following the ligation of PCR products to the vector pSC-B (supplied with the "StrataClone Blunt PCR Cloning kit", see section 2.3.5), the plasmids extracted from several bacterial clones were digested with EcoRI. This enzyme cuts this plasmid twice at strategically located sites, which flank the PCR product and thus allow us to easily verify its presence and size. A reaction mixture containing 12.7 µl of water, 2 µl of NEB buffer for EcoRI (New England Biolabs), 5 µl of plasmid and 0.3 µl of EcoRI (20 units/µl) was incubated at 37 °C for 1 to 16 h (overnight).

The digested DNA fragments were analysed by electrophoresis in agarose gels (0.5 - 2%) as described before (section 2.2.4).

2.3.10. DNA Sequencing

The plasmids containing an insert of expected size were aliquoted in 1.5 ml tubes and sent to a company for automated DNA sequencing (Macrogen Inc.). According to the company, the sequencing reaction was done using BigDye TM terminator (Applied Biosystems) and analysed by capillary electrophoresis with the DNA sequencer 3730x (Applied Biosystems).

2.3.11. Sequence Analysis

For the edition and analysis of the DNA sequences, I used different bioinformatics tools including BLASTX (Altchul *et al.*, 1997), ClustalX version 1.83 (Thompson *et al.*, 1997), CHROMAS 2 (Technelysium Pty Ltd), GCG version 11.0 (Accelrys Inc.) and Vector NTI Advance version 10 (Invitrogen).

2.4. Yeast Two-Hybrid System

2.4.1. Strain and plasmids

The yeast strain and plasmids used in this study are part of the interaction trap developed by Golemis and Brent (1997), which is a LexA-based version of the two-hybrid assay, originally developed by Fields and Song (1989). The *E.coli* LexA system resembles the GAL4 system, but comprises additional features that make it more sensitive and less deleterious to the yeast (Gyuris *et al.*, 1993).

Fusions to the DNA-biding domain encoded by the LexA protein were obtained from a *HIS3*-containing plasmid named pEG202. The strong alcohol dehydrogenase (*ADH1*) promoter in this vector was directing the expression of the recombinant LexA-fusion protein.

Another plasmid, pJG4-5, was used for the generation of protein fusions to the activation domain encoded by the acidic peptide B52. This vector carries the selection marker *TRP1* and an expression cassette under the control of GAL1 promoter, in order that the fusion proteins are expressed in the presence of galactose but not glucose.

The reporter vector, pSH18-34, carries the *URA3* marker and a very sensitive expression cassette comprising 8 LexA operators directing the transcription of the *lacZ* gene.

The yeast strain EGY48 (*MAT alpha, his3, trp1, ura3, 6LexAop-LEU2*) was used as our standard (and only) choice for transforming the plasmids described above and performing the TIM-CRY interaction assays.

2.4.2. Yeast media and solutions

Yeast cells can grow in minimal media containing only glucose, as a carbon source, and some salts which supply nitrogen, phosphorus and metals. However, they grow much faster in the presence of proteins and yeast cell extract hydrolysates, providing amino acids, nucleotide precursors, vitamins and other metabolites which the cells would normally have to synthesize. Like *E. coli*, yeast can grow in liquid media or on the surface of solid agar plates.

The *S. cerevisiae* strain EGY48 is an auxotroph. Its genetic deficiencies render it unable to grow in defined media lacking tryptophan, histidine, uracil and leucine. For that reason, this strain was initially maintained in a rich and complex medium, also called YPD (**y**east extract, **p**eptone, **d**extrose). Afterwards, however, the auxotrophic requirements were used as a tool for obtaining genetic transformants. By growing the cells in minimal media leaving out one of those nutrient requirements, I could select for clones able to grow in its absence, containing a plasmid which rescues the deficient gene. For the selection of the "prey" plasmid pJG4-5, the minimal media was formulated without tryptophan. As for the "bait" plasmid pEG202 and the reporter pSH18-34, the media lacked Histidine or Uracil, respectively. The recipes for the rich and the selection media, adapted from Gietz and Schiestl (2007), are described in Table 2.11.

Table 2.11. Yeast media recipes.

YPD (rich medium)	Synthetic Complete drop-out medium (selection medium)
1 % (w/v) Yeast Extract	0.67 % (w/v) Yeast Nitrogen Base without amino acids
2 % (w/v) Peptone	*[2] 0.14 % (w/v) Synthetic Complete Drop-out Supplement (Y2001)
2 % (w/v) Glucose	Amino acid supplements (see Table 2.12)
*[1] 1.8 % (w/v) Agar	2 % (w/v) Glucose
	*[1] 1.8 % (w/v) Agar

The ingredients were all mixed in water and the pH was adjusted to 5.6 with NaOH, before the addition of agar (if applicable). The solutions were then autoclaved at 121°C for 15 min.

*[1] Agar was added for the solid media only.

*[2] The Synthetic Complete Drop-out Supplement Y2001 (Sigma) lacks Histidine, Leucine, Tryptophan and Uracil. Its addition to the media promotes the yeast growth and the transformation efficiency.

Table 2.12. Amino acid supplements.

Amino acid	Stock concentration	Final concentration
Tryptophan	10 mg/ml	0.05 mg/ml
Histidine	20 mg/ml	0.02 mg/ml
Leucine	20 mg/ml	0.02 mg/ml
Uracil	5 mg/ml	0.10 mg/ml

The amino acid stock solutions were formulated according to Saghbini *et al*. (2001) to complement the selection medium described in Table 2.11. Tryptophan, Histidine and Uracil were not added (i.e. drop-out) if one wanted to select for pJG4-5, pEG202 or pSH18-34, respectively.

2.4.3. Transformation

The transformation of the plasmids into yeast cells was carried out according to Gietz and Shiestl (2007), with some slight modifications. At first, 5 ml of YPD (or appropriate selective media) was inoculated with a single yeast colony and placed overnight in an incubator shaker at 30°C. On the next day, about 100 μl was taken from the overnight culture and added to 5 ml of fresh medium (OD_{600} ~0.2). Subsequently, the cells were incubated at 30°C for another 4 h, when they could grow to mid or late-log phase (OD_{600} = 0.5 – 1.0). Then, using a tabletop centrifuge, the cells were centrifuged for 5 min at 3,000 rpm and the supernatant was discarded. The pellet was resuspended in 1 ml of sterile distilled water, transferred to a 1.5 ml microcentrifuge tube and, then, centrifuged for 1 min at 13,000 x *g*. After removing the supernatant, the cell pellet was washed in sterile distilled water twice again. Next, the cells were resuspended in 1 ml of 0.1 M LiAc and the solution was divided into

two new tubes (one would be the control). These were centrifuged at 13000 x *g* for 1 min and the supernatant was discarded in the end. Then, I added to each tube the following reagents: 240 μl of 50% (w/v) PEG 3350, 36 μl of 1M LiAc and 50 μl of 2 mg/ml single-stranded carrier DNA (boiled to 95^{o}C for 5 minutes before use). An extra 34 μl of a solution comprising the plasmid DNA (200 ng – 1 μg) plus water, or just water (for the control), was added to both tubes and the pellets were resuspended carefully. Subsequently, the cells were heat-shocked at 42°C for 20 min and centrifuged at 13,000 x *g* for 30 sec. After removing the supernatant, the pellets were resuspended in 1 ml of sterile distilled water and centrifuged at 13,000 x *g* for 1 min. This last washing step was repeated once more and, finally, the cells were spread in solid selective media and kept at 30°C for 3 to 5 days, until colonies started to form.

2.4.4. β-Galactosidase Plate assay

The colonies obtained from the transformation of yeast cells with the plasmids described above (i.e. pEG202-CRY, pJG4-5-TIM and pSH18-34) were streaked in X-Gal plates containing galactose. These were incubated at 30°C for 16 h under constant light, which promotes the interactions of between the target proteins, or constant darkness (the plates were covered with two layers of foil), which constituted our negative control. The reaction was monitored every hour with a digital camera. A visible blue staining (an indicator of interaction) started to show after 8 h and a nearly complete saturation was achieved after 15 h. The recipe used for making the X-Gal plates, adapted from Golemis and Brent (1997), is described in Table 2.13.

Table 2.13. X-Gal Plates recipe.

Reagents
0.67 % (w/v) Yeast Nitrogen Base without amino acids
*[1] 0.14 % (w/v) Synthetic Complete Drop-out Supplement (Y2001)
*[2] 0.02 mg/ml Leucine
2 % (w/v) Galactose
1 % (w/v) Raffinose
1.8 % (w/v) Agar
*[3, 4] 1 X BU salts
*[3] 80 μg/ml X-Gal

The reagents were mixed and autoclaved for 15 min at 121°C. The solution was cooled down to 50°C before pouring into Petri dishes.

*[1] The Synthetic Complete Drop-out Supplement Y2001 (Sigma) lacks Histidine, Leucine, Tryptophan and Uracil.

*[2] Added from the stock solution illustrated in Table 2.12.

*[3] Added after autoclaving when the solution temperature was down to 50°C.

*[4] Added from a 10X solution (3,8 M Na_2HPO_4, 4 M NaH_2PO_4, pH = 7.0).

2.5. Locomotor Activity Experiments

2.5.1. Experimental Set-up

For the locomotor activity experiments, individual male flies (1-4 days old) were placed in small glass tubes filled with sugar food (about 2 cm) and sealed with a black cap at one end and a cotton plug at the other. These tubes were placed individually in channels of monitors (TriKinetics Inc) used to record the locomotor

activity of single flies. Each monitor has 32 independent channels able to detect motion as the flies walk back and forth within the tube interrupting an infrared beam. The monitors were housed inside boxes equipped with LED bulbs, which emit lights intensities of 300 to 500 lux. Each box was individually programmed to turn lights on and off according to the experimental regime, described in each of the results chapters. Finally, the light boxes were positioned inside incubators (Scientific Laboratories Supplies Ltd) set to 25^{o}C until the end of the experiment.

2.5.2. Data Collection and Analysis

The locomotor activity monitors were connected to a PC using a Power Supply Interface Unit (TriKinetics Inc) and the data from them were collected using the DAMSystem2.1.3 software (TriKinetics Inc).

The period (τ) of individual flies was calculated by both autocorrelation and CLEAN spectral analyses (Rosato and Kyriacou, 2006). For the autocorrelation, the data (collected in 30 minute bins) are shifted point by point and compared to itself to create correlation coefficients (thus called autocorrelation), which are then plotted together. As for the rhythmic data identified by CLEAN, Monte Carlo simulations were used to generate the confidence limits of 95% and 99% by doing 100 randomisations of the data for each fly. The data obtained after CLEAN was assembled together and further processed by the BeFly (version v5.3) collection of macros written in Bambos' lab by Edward Green. Those individuals with significant autocorrelation and spectral analysis were considered rhythmic and used for the computation of the average period. Those genotypes with less than 20% of rhythmic individuals were classified as arrhythmic and the average period was not calculated.

The average activity for each genotype (actogram) was plotted using Microsoft Excel® 2007 and the flies that died during the experiment were excluded from the analysis.

2.5.3. Phase-shift

For the phase-shift experiments, flies were entrained for three days in LD 12:12, at 25°C, and a light pulse (10 min) was administrated during the last day at ZT15. Then, the flies were maintained in DD for five days. The same procedure was carried out for another group of flies (control) of the same genotype, but with no light pulse intervention. The analysis of the data was performed using a Befly macro (also written by Edward Green), which uses an automated cross-correlation function to measure the degree of phase-shift. Cross-correlation analysis examines the correlation between the individual 30 min activity bins of the experimental data series against the individual activity bins of the control data series. For example, bin 1 of the experimental series is correlated with bin 1 of the control series, bin 2 with bin 2, etc, until the end of the data sets. The control series is then moved by one bin and the two data sets are again cross-correlated. This will continue until the control data set has been moved down the entire experimental data set. The direction of the movement is dependent upon whether a phase advance or phase delay is expected. If a phase delay is expected, the control data set is moved down by one bin. A correlogram is then produced by plotting the cross-correlation against an axis of 30 min time lags. The phase difference between the control and experimental data sets can then be calculated by extrapolating the highest cross-correlation value onto the time lag

axis. The automated method was validated by calculating phase-shifts manually, which is extremely laborious and gives similar results.

2.6. Diapause

Diapause was studied by raising flies at 25°C in LD 12:12, then placing between 30-50 individuals (males and females), depending on availability, in a food vial at cold temperatures and different photoperiods. Importantly, the flies were collected within a few hours of emergence (<10 h) to allow a proper diapause induction (Saunders and Gilbert, 1990). After 15 days, the females were etherized and their ovaries were dissected in cold saline (1 X PBS). Diapause was scored according to the developmental stage of the oocytes (Saunders *et al.*, 1989). Ovaries containing oocytes comprising the stages 1-7 were regarded as previtellogenic (or "diapausing"), whereas those with oocytes at stage 8 and over were regarded as vitellogenic (or "non diapausing"). An individual was considered to be in diapause when all the oocytes, from both ovaries, were previtellogenic. If one (or more) oocyte(s) was found to be in more advanced developmental stage, the individual was classified as "non diapausing". For each vial, 20 females were dissected and the proportion of "diapausing" flies was an experimental replicate.

2.7. Statistics

Analysis of variance (ANOVA) was applied to the behavioural (i.e. free-running rhythms, phase-shifts) and physiological data (i.e. diapause) using the STATISTICA software package version 8.0 (StatSoft, Inc.). This software was also

useful for building the graphs illustrated in Chapter 3 (Figure 3.2) and Chapter 4 (Figures 4.10 and 4.11).

Chapter 3: Temperature effect on the photoperiodic response of natural variants of *timeless*

3.1. Introduction

The study of the photoperiodic response (diapause) in populations of European flies revealed a surprising role for *timeless* (*tim*) in this phenomenon (Tauber *et al.*, 2007). Individuals carrying the newly derived *ls-tim* allelic variant exhibited significantly higher diapause levels than those carrying the ancestral one, *s-tim*, in a very consistent pattern for all the populations analysed (Figure 3.1). A thorough observation of the same data also revealed that both genotypes show roughly "parallel" profiles, indicating that they are equally affected by day length changes. For that reason, it has been speculated that *tim* is not causally associated with the photoperiodic clock, instead it would simply modulate the threshold for diapause levels (Emerson *et al.*, 2009).

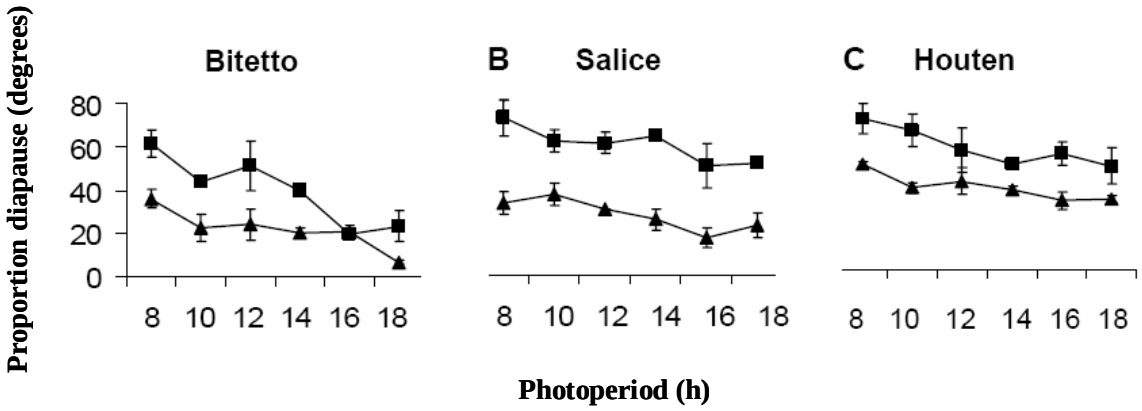


Figure 3.1. Photoperiodic response (diapause) of *tim* natural variants from three different European populations of *D. melanogaster*. *ls-tim*, squares; *s-tim*, triangles (from Tauber *et al.*, 2007).

It has also been reported that temperature has a defining role on this phenotype, affecting its levels and amplitude (Saunders and Gilbert, 1990). This raises the possibility that temperature could be interacting with the diapause profiles of wild population of flies such as those previously studied by Tauber *et al.* (2007). For this reason, I decided to further investigate the diapause of *ls-tim* and *s-tim* natural variants across a temperature range, re-evaluating how robust is the difference between these genotypes and analyzing any new interactions that might emerge among the relevant variables.

3.2. Methods

3.2.1. Flies

An isofemale line, derived from a population collected in Houten (Holland), was selected as our study model due to its accentuated diapause phenotype (Tauber *et al.*, 2007). Using PCR and standard crossing techniques, the *ls-tim* or *s-tim* alleles were made homozygous in separate fly stocks (LS-Hu and S-Hu, section 2.1.1), which were given to me as gift by Dr. Eran Tauber.

3.2.2. Diapause assays

Following the protocol described in section 2.6, diapause assays were performed under five temperatures (7°C, 9°C, 11°C, 13°C and 15°C), at two photoperiods (LD 10:14 and LD 16:8) and for the two different genotypes (homozygous *ls-tim* and *s-tim* flies).

3.3. Results

The results revealed that, at a constant temperature of 15°C, most if not all the flies do not show any sign of diapause, irrespective of the genotype analysed (Figure 3.2; Table 3.1). When the temperature was decreased to 13°C, however, the reproductive arrest characterizing diapause was triggered, especially in short-days, but the effect was restricted to the *ls-tim* genotype. At 11°C, this response was accentuated, affecting virtually all the *ls-tim* individuals. This temperature was also critical for inducing diapause in the *s-tim* genotype, but the effect was reduced to about half of the flies. At 9°C, the diapause levels of *ls-tim* flies were stabilized at their highest values, whereas for *s-tim* it was slightly more pronounced than at 11°C. When the experiment was performed at 7°C, the mortality rate was higher than 50% and very few surviving individuals were assessed for diapause, resulting in an erratic profile (data not shown).

Three-way ANOVA (Table 3.2) revealed highly significant effects for Genotype, Temperature and Photoperiod. An interaction was observed for Genotype x Temperature, but not for Genotype x Photoperiod or Temperature x Photoperiod. Surprisingly, a significant effect was also revealed for the Genotype x Temperature x Photoperiod term.

To further investigate the source of statistical interaction between the variables studied, the data were grouped by temperature and new two-way ANOVAs were performed (Table 3.3). Interestingly, Genotype X Photoperiod interactions were observed at 9°C and 13°C, but not at 11°C or 15°C.

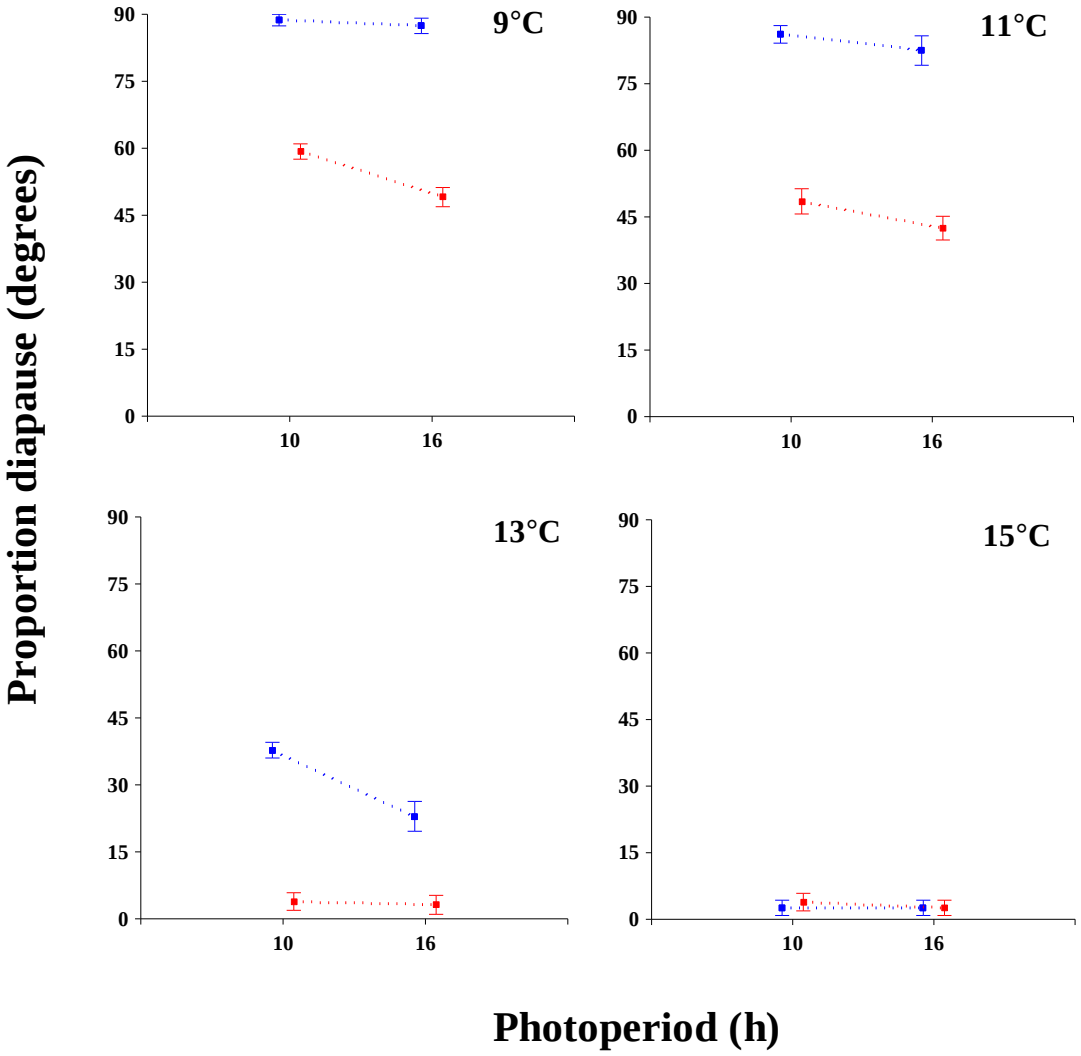


Figure 3.2. Photoperiodic response (diapause) of *ls-tim* and *s-tim* natural variants from Houten (Holland) under different temperature regimes. X axis, photoperiod (in hours of light). Y axis, diapause incidence in degrees (mean proportions, arcsin ± SEM). Represented in blue, *ls-tim*, and in red, *s-tim*.

Table 3.1. Diapause incidence for *tim* natural variants.

Temperature	Photoperiod	Genotype	N	Mean	SEM
15˚C	16:8	*ls-tim*	10	2.58	1.72
		s-tim	10	2.58	1.72
	10:14	*ls-tim*	10	2.58	1.72
		s-tim	10	3.88	1.97
13˚C	16:8	*ls-tim*	10	22.95	3.34
		s-tim	10	3.13	2.13
	10:14	*ls-tim*	11	37.78	1.75
		s-tim	10	3.88	1.97
11˚C	16:8	*ls-tim*	10	82.48	3.32
		s-tim	10	42.47	2.66
	10:14	*ls-tim*	10	86.12	1.97
		s-tim	10	48.50	2.85
9˚C	16:8	*ls-tim*	10	87.41	1.72
		s-tim	10	49.06	2.15
	10:14	*ls-tim*	10	88.70	1.29
		s-tim	10	59.27	1.72

The data were analysed as mean proportions in degrees (arcsin transformed). N represents the number of experimental replicates, consisting of 20 flies each (Total of flies dissected = 3,320).

Table 3.2. Three-way ANOVA.

	SS	d.f.	MS	*F*	*P*
Genotype	24606.8	1	24606.8	504.823	$< 10^{-6}$
Temperature	140099.2	3	46699.7	958.071	$< 10^{-6}$
Photoperiod	909.9	1	909.9	18.667	$< 10^{-4}$
Genotype X Temperature	9310.3	3	3103.4	63.668	$< 10^{-6}$
Genotype X Photoperiod	1.4	1	1.4	0.028	0.867
Temperature X Photoperiod	272.8	3	90.9	1.865	0.138
Genotype X Temperature X Photoperiod	723.1	3	241.0	4.945	0.003
Error	7067.8	145	48.7		

Significant values ($P < 0.05$) were highlighted in red.

Table 3.3. Two-way ANOVA (grouped by temperature).

9˚C	SS	d.f.	MS	*F*	*P*
Genotype	11489.3	1	11489.3	376.020	$< 10^{-6}$
Photoperiod	330.9	1	330.9	10.831	0.002
Genotype X Photoperiod	199.0	1	199.0	6.512	0.015
Error	1100.0	36	30.6		

11˚C	SS	d.f.	MS	*F*	*P*
Genotype	15066.9	1	15066.9	199.940	$< 10^{-6}$
Photoperiod	234.3	1	234.3	3.109	0.086
Genotype X Photoperiod	14.3	1	14.3	0.190	0.665
Error	2712.9	36	75.4		

13˚C	SS	d.f.	MS	*F*	*P*
Genotype	7383.7	1	7383.7	129.907	$< 10^{-6}$
Photoperiod	620.0	1	620.0	10.909	0.002
Genotype X Photoperiod	507.7	1	507.7	8.932	0.005
Error	2103.0	37	56.8		

15˚C	SS	d.f.	MS	*F*	*P*
Genotype	4.2	1	4.2	0.130	0.720
Photoperiod	4.2	1	4.2	0.130	0.720
Genotype X Photoperiod	4.2	1	4.2	0.130	0.720
Error	1152.0	36	32.0		

Significant values ($P < 0.05$) were highlighted in red.

3.4. Discussion

Previous work regarding the temperature dependency of diapause used the Canton-S strain as a study model, which has been domesticated in laboratory conditions for over 50 years (Saunders and Gilbert, 1990). Although this strain exhibited a clear photoperiodic response, it is quite likely this adaptive seasonal phenotype was no longer under selection in the laboratory. The data presented in this study were obtained from flies recently collected in Holland, where a strong and fine-tuned photoperiodic response is imperative for surviving the harsh winter conditions. Under such a strong selection in this northern European environment, these flies should represent an excellent model for studying seasonal related phenotypes and their underlying genetics.

My results corroborated previous findings (Saunders and Gilbert, 1990; Tauber *et al.*, 2007) and also provided new insights. The first (and most obvious) observation concerns the major effect of temperature in the regulation of diapause in *D. melanogaster*. This seasonal response appears to be only induced when the average daily temperature falls in the range from 9°C to 13°C. Within this range, a gentle alteration of about 2°C could lead to a prominent effect on the diapause profiles (Figure 3.2; compare 13°C to 11°C). When temperature was raised to 15°C, no response was observed. In contrast, when it was decreased to 7°C, most of the flies did not survive. Whether this happened because there was no lead in time to accustomise the flies to gradually colder temperatures, remains an important question to be answered. Together, these data indicate that temperature, rather than photoperiod, is probably the most important environmental factor modulating the induction of diapause in this species.

Another important observation is that diapause in the wild "Houten" strain is indeed a photoperiodic response (i.e. its incidence varies according to the photoperiod), although its amplitude is not very pronounced. Temperature appears to modulate the strength of the photoperiodic effect, but that depends on the genotype analysed, as revealed by the significant Genotype X Temperature X Photoperiod term in the ANOVA (Table 3.2). Hence, while the initial analysis showed no significant Genotype x Photoperiod interaction, suggesting that an increase or decrease in day length has the same effect on diapause in both *ls-tim* and *s-tim* lines, this conclusion was somewhat contradicted by the three-way interaction and when the data was grouped by temperature. In other words, the photoperiod influence on the genotype depends on the temperature (observe the slope of the lines connecting the means in Figure 3.2). The presence of significant interactions, however, must be considered very carefully since the photoperiodic response is induced in a small range of temperatures. In this respect, an interaction event might represent an experimental artefact, simply because one of the genotypes analysed reached its maximum or minimum levels. Indeed, the Genotype x Photoperiod interactions at 9°C and 13°C appear to be due to *ls-tim* reaching its maximum at the former, and *s-tim* its minimum in the latter temperatures.

As one could have expected, *tim* natural alleles played a very important part in the diapause output, with *ls-tim* showing consistently higher levels than *s-tim*, for both short and long photoperiods. This pronounced difference was very robust at all temperatures analysed except for 15°C. The induction of diapause in *ls-tim* flies occurs at warmer temperatures, while *s-tim* individuals are still reproductively active. This observation supports the suggestion that *ls-tim* is generally more prone to diapause than *s-tim* (Tauber *et al.*, 2007), triggering this response earlier in the year,

when the days are longer and also warmer. These functional differences between the alleles might favour their distributions (and formation of latitudinal clines) by natural selection in the seasonal European environment.

Based on the above observations, one could speculate that, in the wild, flies enter diapause at appropriate conditions, which comprises a restricted range of temperature and photoperiod. The genetic background (i.e. ls/s-tim polymorphism), in turn, would modulate when and how the photoperiodic response is induced and maintained. The adaptive advantage of *ls-tim* over *s-tim* might be even more pronounced in the natural environment, where the decreasing day length is normally associated with colder temperatures.

Evidence obtained from other insects are rather controversial on the role that *tim* plays on diapause. A quantitative trait loci (QTL) map for photoperiodism in the mosquito *Wyeomyia smithii* revealed that *tim* does not fall within any of the markers identified, although it might interact epistatically with some (Mathias *et al.*, 2007). In *Chymomyza costata*, a Japanese drosophilid, analysis of the genomic structure of *tim* revealed that *npd* mutants, which do not respond to photoperiod, carry a 1855 bp-long deletion in the 5'-UTR region, which might be causally associated with reduced transcription rates and the disruption of both circadian and photoperiodic clock functions (Stehlik *et al.*, 2008).

Because the previous studies involving natural populations and transformants, expressing either one or the other *tim* allele, revealed no genotype by day length interactions (Tauber *et al.*, 2007), it has been proposed that both factors have an additive and independent effect on the incidence of diapause in *D. melanogaster* (Emerson *et al.*, 2009). This implies that *tim* would not be crucial for the photoperiodic effect and its role would be limited to the regulation of diapause levels.

Given its light-sensitivity, TIM could be acting at the input level of the photoperiodic clock, feeding this mechanism with the environmental cues necessary for the induction of diapause. Alternatively, *tim* could be acting downstream of the clock, acting as a modulator of hormonal signals or even the ovarian developmental arrest characterizing diapause, although this hypothesis is unlikely.

My data indicates that despite of some positive evidence, the suggestion that *timeless* is not an integral part of the photoperiodic mechanism in *D. melanogaster* may be misleading (Emerson *et al.*, 2009). As already discussed above, the shape of the photoperiodic response in *D. melanogaster* seems to be largely influenced by small variations in the environmental temperature, which can possibly mask the effect of the photoperiod on the natural *tim* variants. Moreover, since both of these alleles are natural and fully functional, it is now crucial to add to the analyses the tim^{01} loss-of-function mutation, as this will shed light on the role of *tim* on photoperiodism.

Chapter 4: A "Circadian" Photoperiodic clock?

4.1. Introduction

The investigation of the molecular bases of complex behavioural and physiological phenomena can be performed either by forward or reverse genetics approaches. The former has been the major choice in circadian rhythms research, especially in the fruit-fly *D. melanogaster* (Wager-Smith and Kay, 2000; Allada *et al.*, 2001). A number of genetic screen designs have been used to uncover mutants that disturb the free-running oscillator (Konopka and Benzer, 1971; Sehgal *et al.*, 1994; Allada *et al.*, 1998; Rutila *et al.*, 1998) or its entrainment pathways (Stanewsky *et al.*, 1998; Glaser and Stanewsky, 2005). The great success of these studies could be attributed, at least in part, to the relative robustness of the circadian phenotype and automated screening methods (Rosato and Kyriacou, 2006).

Recently, forward genetics has also been successfully applied to photoperiodism. Several QTLs involved in the photoperiodic response (diapause) have been characterized in mosquitos (Mathias *et al.*, 2007) and also in fruit-flies (Schmidt *et al.*, 2008). In spite of the importance and the contribution of such studies, *D. melanogaster* is not a perfect model for a large scale screening of diapause genes. First of all, contrary to other insect species living in temperate environments, *D. melanogaster* manifests a rather shallow photoperiodic response (Saunders *et al.*, 1989), which affects a quite variable proportion of individuals, thus requiring the analysis of multiple experimental replicates. Second, because these experiments are performed with very young flies (less than 10 h old), each line (or genotype) to be tested needs to have its population expanded beforehand, which demands work and

time. And finally, the diapause phenotype scoring is not an automated process; instead it involves the manual dissection and classification of specimens.

Regardless of the limitations described above, *D. melanogaster* offers unmatched genetic resources, which could certainly be adapted to the study of photoperiodism. If screening designs are replaced by a "candidate gene" approach, following the premises of reverse genetics, then the workload would be lightened and *D. melanogaster* may turn out to be an excellent model.

This study focuses on a crucial debate in chronobiology: the role of the circadian clock in the photoperiodic response (i.e. diapause). While the circadian clock has been cellularly and genetically dissected, the photoperiodic response has been mainly treated as a "black box" in experiments trying to understand intrinsic properties of any clock underlying it (Saunders *et al*., 2004). As a result of the "black box" approach, a number of theoretical models emerged to explain how the seasonal clock functions, either based on an oscillator (probably involving the circadian system) or an hourglass-like mechanism (Nunes and Saunders, 1999). A few attempts have also been made in order to find a relationship between circadian and photoperiodic clocks at the molecular level, but the results of these studies have been quite controversial and not conclusive (Saunders *et al.*, 1989; Saunders, 1990; Pavelka *et al.*, 2003; Tauber *et al.*, 2007; Stehlik *et al.*, 2008). The gene *period*, for instance, is essential for the maintenance of the circadian but not the photoperiodic clock since its hypomorphic and amorphic mutants are still able to distinguish short from long days (Saunders *et al.*, 1989). On the other hand, another clock gene, *timeless*, the molecular partner of *period* in the circadian clock machinery, has been associated with natural variation of diapause in *D. melanogaster* (Tauber *et al.*, 2007) and seems to play a central role in the photoperiodic clock of another drosophilid, *C. costata* (Stehlik *et*

al., 2008). Such studies have stimulated new discussions regarding the interplay between circadian and photoperiodic clocks. Partially supported by the findings with *per*, sceptics have refused to accept any causal relationship between both timing phenomena and argued that any effect that might emerge, as for *tim*, are probably due to gene pleiotropy (Emerson *et al*., 2009). However, the alternative hypothesis that the photoperiodic clock is circadian-based, thus sharing a common array of genes, cannot be discarded. Therefore, the current scenario urges a re-evaluation of molecular links between the clocks, which will certainly contribute to a better understanding of seasonal timing and will shed light on this incessant debate.

In this chapter, I investigate the photoperiodic response of a series of previously characterized mutations which affect different aspects of the circadian clock of *D. melanogaster*. Because photoperiodicity has a polygenic nature (Williams *et al*., 2006; Tauber *et al*., 2007; Schmidt *et al.*, 2008), the analysis of each of the candidate mutations had to be performed in a common and uniform genetic background. To achieve that, I generated I collection of congenic flies carrying mutations and transgenes commonly used in circadian rhythms research. Each genotype was first analysed for the circadian behaviour, in order to confirm previously reported effects in entraining and free-running conditions, and later for diapause incidence under short and long-day rearing conditions.

4.2. Methods

4.2.1. Flies

Classical mutations and transgenic constructs, as well as balancer chromosomes, commonly used in the circadian biology research, were placed in the wild "Houten" (Holland) background with 8 generations of backcrossing to the LS-Hu strain (see section 2.1.1). After this procedure, the predicted residual variation between the lines is 0.5^8 (or 1/256 or ~0.4% or 0.5 Mb). The approach used for each candidate, including genotyping information (i.e. primer sequences, PCR cycling conditions, restriction enzymes and gel analysis) is described in section 2.2.

Following the standardization of the background, all the clock mutants (except for tim^{01}) and transgenic flies carried the *ls-tim* allele. To place the same mutations in the *s-tim* background, additional crosses were performed using balancer chromosomes to swap entire second chromosome with the S-Hu strain.

The clock mutant cyc^{01} was very weak after the standardization of the background and was not used for behavioural or physiological (i.e. diapause) analyses.

4.2.2. Behaviour assays

The locomotor activity of the flies (3-7 days old) was monitored for 3 days in LD (12:12) followed by 7 days in DD. The details regarding the experimental set-up, data collection and analysis are described in section 2.5. The activity plots for entrained and free-running conditions are exhibited in 30 min bins. The LD profiles

were generated from the data of the two last days of entrainment before flies were transferred to DD.

4.2.3. Diapause assays

Diapause was scored according to the protocol on section 2.6. In this chapter, flies were analysed at short (LD 8:16) or long photoperiods (LD 16:8), under the temperature of 12°C.

4.3. Results

4.3.1. "Houtenized" collection of flies

To investigate the influence of the circadian clock on the photoperiodic response (diapause), a number of classical mutations and transgenic constructs have been carefully selected (Figure 4.1). These mutations affect the function of genes involved in the core, input and output of the circadian clock whereas the transgenic constructs (i.e. UAS, GAL4 and GAL80 elements) direct gene expression to particular tissues, or even cells, implicated in the circadian behaviour. While the mutations can provide information about the molecular nature of seasonal clocks, transgenic manipulations can assess its anatomical location and determine how time cues are interpreted and transmitted to the effector system. However, all these mutants and transgenic lines have diverse genetic backgrounds and, therefore, could not be promptly used to investigate phenotypes of polygenic nature such as the photoperiodic response. Consequently, a standardization of the genetic background of

these strains was an important and necessary step to allow a reliable comparison between the genotypes.

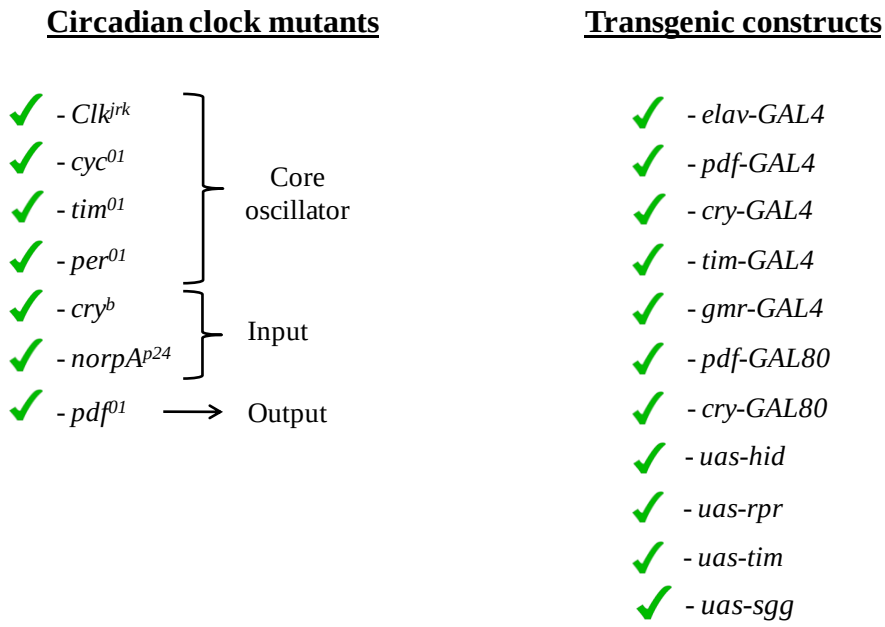


Figure 4.1. Mutants and transgenic lines commonly used in circadian rhythms research.

An ideal genetic background for this study should exhibit robust levels of diapause, preferentially high at short photoperiods (winter phenotype), but low at long photoperiods (summer phenotype). Standard Drosophila strains, like Canton-S, have been maintained in laboratorial conditions for many decades and, as a result, their genomes have been gradually "domesticated", possibly losing part of the genetic variability involved in seasonal adaptation. This may explain why sometimes they exhibit low and irregular diapause profiles (Saunders *et al.*, 1989; Saunders and Gilbert, 1990). In the past few years, a number of wild populations, coming from

northern and southern Europe, have been brought and maintained in laboratory conditions as separate isofemale lines. Some of these have been scored for diapause, revealing that this phenotype is enhanced at shorter day lengths, at northern latitudes and also by the newly derived *ls-tim* allele (Tauber *et al.*, 2007). These flies would, therefore, represent good substrates for the study of a number of life-history traits involved in seasonal adaptation, such as diapause. One particular isofemale line (LS-Hu) from Houten (Holland), which carries the allelic variant *ls-tim*, was chosen as the source of genomic background for this study (Tauber *et al.*, 2007; see also Chapter 3).

Accordingly, all the candidate mutations and transgenes have been transferred to the Houten background (LS-Hu) by repeated backcrossing. To ensure maximal DNA exchange, and since males lack meiotic recombination, females carrying the mutation/transgene were selected after each cross. Assuming that in every generation there is an uptake of about 50% of the Houten DNA, then after 8 rounds of backcrossing, approximately 99.6% of the genetic background will be derived from the natural strain (Figure 4.2). Thus, using this strategy, a collection of "Houtenized" mutants and transgenic lines was created for circadian and photoperiodic studies. It is important to mention, though, that these flies still carry some genetic variability since they derive from an isofemale, but not isogenic, line.

Special care was taken to avoid the mobilization of *P*-elements during the crosses, which could cause a diverse array of genetic abnormalities, including high rates of mutations, recombination in males, sterility resulting from aberrant gonadal development, and chromosomal rearrangements (Engels, 1983). These deleterious effects, collectively known as "hybrid dysgenesis", were only observed when males isolated from wild populations (P cytotype strain) were mated with females from laboratory populations (M cytotype strain) (Kidwell *et al.*, 1977). It is well-known

that the great majority of the wild strains are "infested" with *P*-elements, which do not transpose autonomously due to a repression mechanism. In a dysgenic cross, however, the repression is lost and *P*-element mobilization occurs (Rio, 1990). Therefore all the backcrossing schemes were initiated by mating a male from the lab strain (hosting a mutation or transgene) with a female from the wild. The resulting progeny, males and females, had a P cytotype, thus repressing *P*-element mobilization.

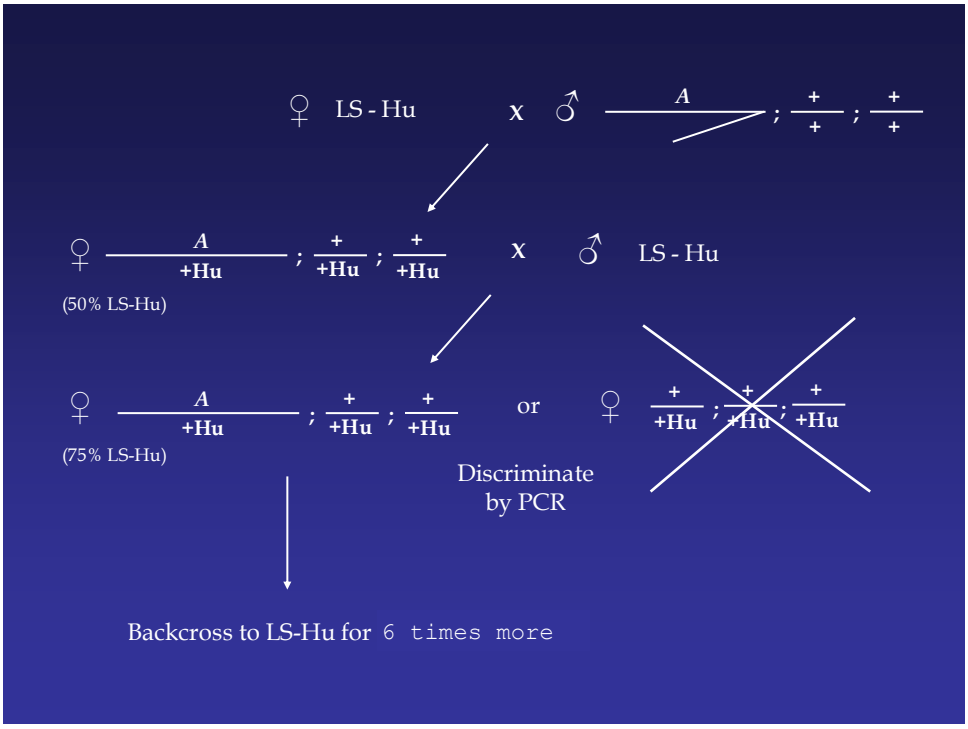


Figure 4.2. General backcrossing scheme. In the example, flies carrying the mutation "A", identified by PCR, are crossed repeatedly with the LS-Hu strain. After 8 generations of crosses, the mutation "A" will be inserted in a genomic background similar to LS-Hu (>99%).

In order to follow the "clock" mutations over multiple generations of backcrossing, a molecular genotyping methodology was developed. Even though some of the mutant alleles show semi-dominant effects, most of them are recessive,

thus complicating any behavioural approach. So, a PCR-based strategy was used, which proved to be a fast and reliable way for identifying positive individuals.

A standard amplification reaction, with primers flanking the mutant site, was used for screening the alleles tim^{01} (Figure 4.3A) and $norpA^{p24}$ (Figure 4.3B), which are characterized by deletions of 64 bp and 26 bp, respectively. In these two cases, the mutant DNA resulted in a significantly smaller product than the wild-type, being easily distinguished on an agarose gel.

For all the other clock mutations, the molecular lesion was encoded by a nucleotide substitution. Consequently, a standard PCR reaction using primers flanking the polymorphic site would result in DNA fragments with the same size for both mutant and wild-type flies, making it an inadequate genotyping method. To circumvent this obstacle, the amplification products were digested with restriction enzymes which would only cut the mutant DNA. This was successfully applied to per^{01} (Figure 4.4A) and Clk^{jrk} (Figure 4.4B) alleles, whose amplification reactions were immediately followed by restriction analyses. It is important to mention that when optimal conditions for the PCR and digestion assays were experimentally tested, restriction digests hardly ever reached completion (i.e. the amplified DNA was never totally cut), making it difficult to distinguish between flies homozygous or heterozygous for the mutation. This was not a problem since the sample would only include flies either wild-type or heterozygous for the mutation and, at the end of the backcrossing scheme, "Houtenized" balancer chromosomes were used to generate a true-breeding mutant stock. Taken as an example, the backcrossing scheme of the autosomal mutation Clk^{jrk} is illustrated in Figure 4.5.

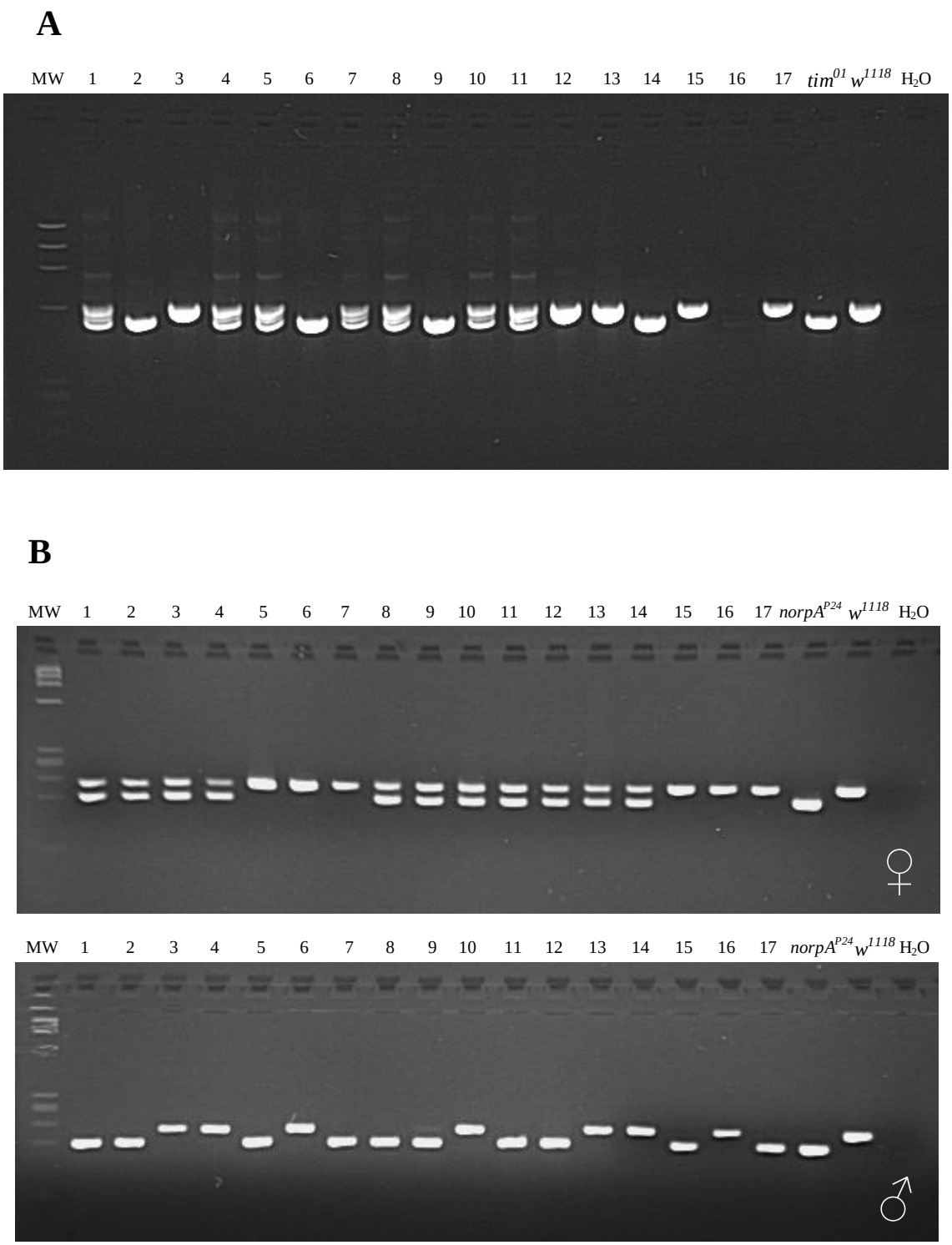
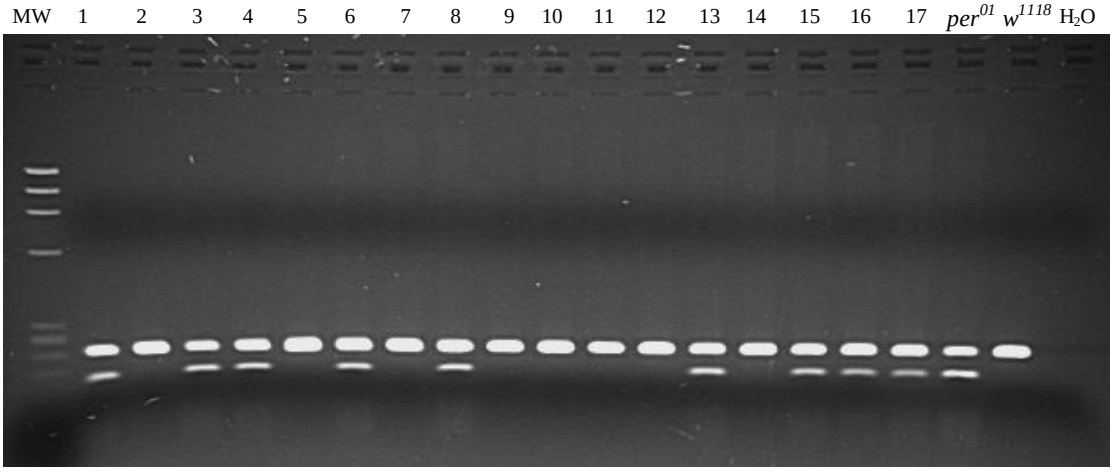


Figure 4.3. Molecular genotyping by PCR with primers flanking a deletion. A) *tim*^{01}. B) *norpA*^{P24}. The amplified fragments were resolved on a 2% (panel A) or 3.5% agarose gel (panel B). The numbers above each lane represent different individuals. *w*^{1118}, negative control. H_2O, water control (no DNA). MW, molecular weight marker ΦX Hae III.

A



B

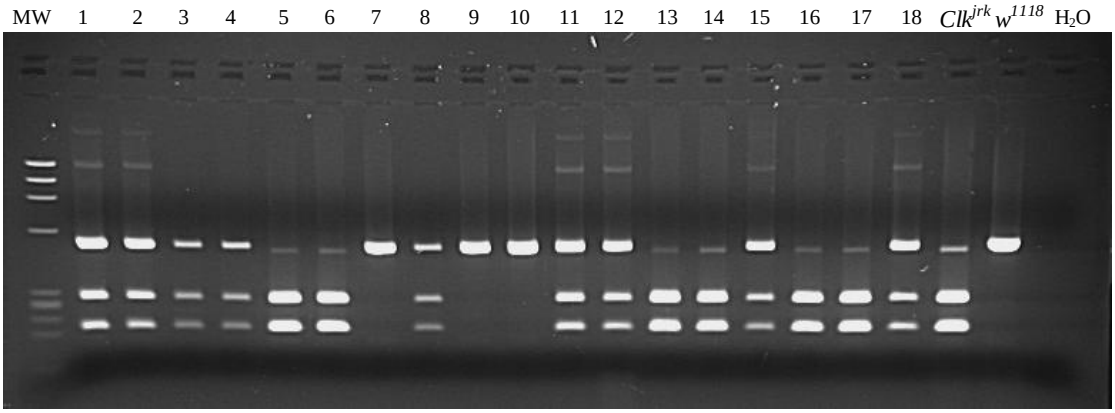


Figure 4.4. Molecular genotyping by PCR followed by restriction digest. A) *per*^{01}. B) *Clk*^{jrk} . The amplified DNA was cut with BfaI and resolved on a 2% agarose gel. The numbers above each lane represent different individuals. *w*^{1118}, negative control. H_20, water control (no DNA). MW, molecular weight marker ΦX Hae III.

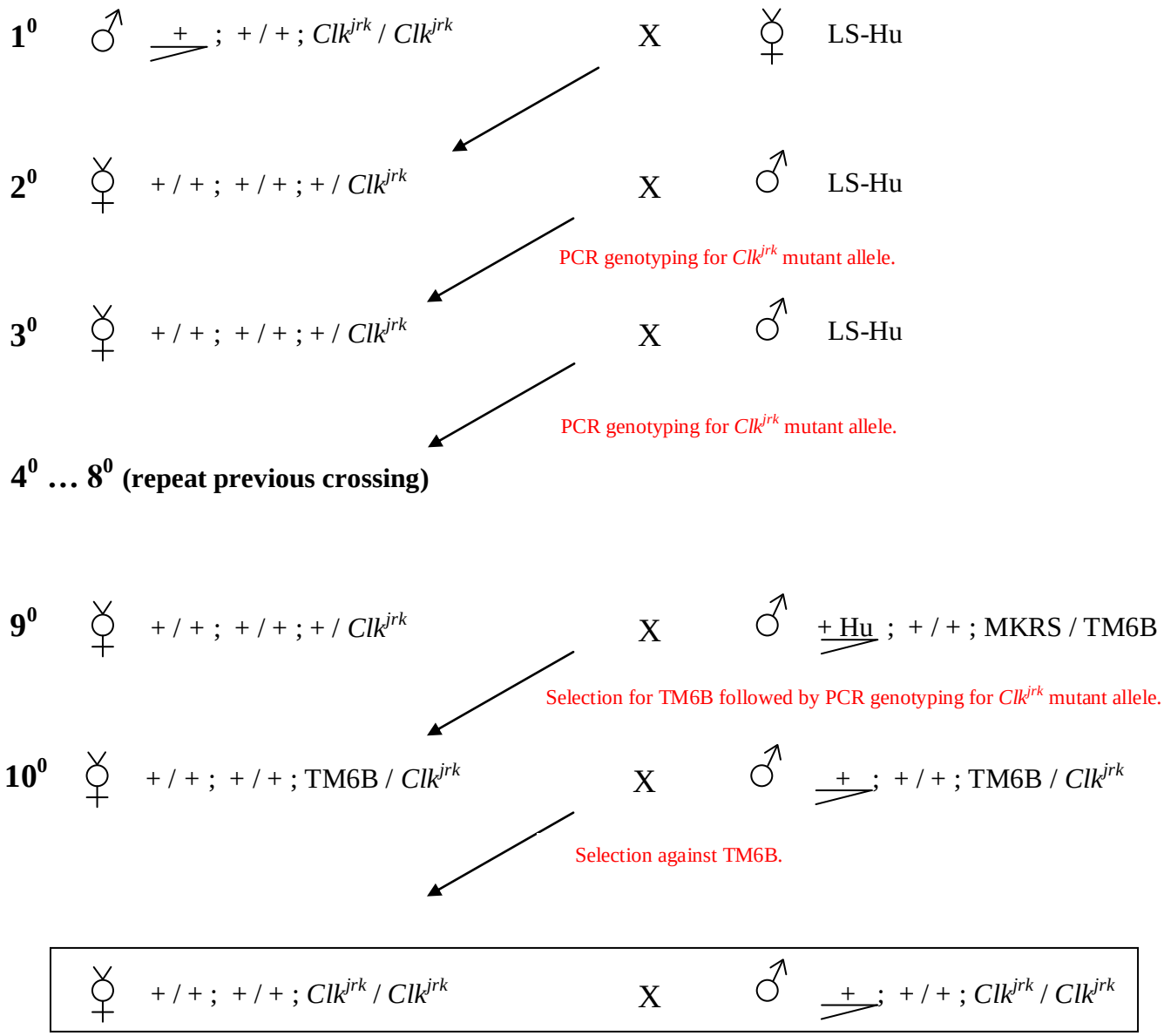


Figure 4.5. Backcrossing the *Clk*^{jrk} mutant allele to the LS-Hu genetic background.

In those cases where suitable restriction endonucleases could not be found, an alternative allele-specific PCR method was developed. This strategy involved the design of primer sequences including the polymorphic site on its 3' end, in an attempt to amplify only the mutant DNA. The success of this approach relies on a good optimization of the parameters affecting the reaction (e.g. annealing temperature), since there is a very delicate balance between a specific and an unspecific product (i.e.

amplification of the wild-type DNA). This method was used for genotyping flies carrying the mutations *cyc*^{01} (Figure 4.6A), *cry*^{b} (Figure 4.6B) and *Pdf* ^{01} (Figure 4.6C). Again, at the end of the backcrossing scheme, "Houtenized" balancer chromosomes were used to create a stock of mutant flies.

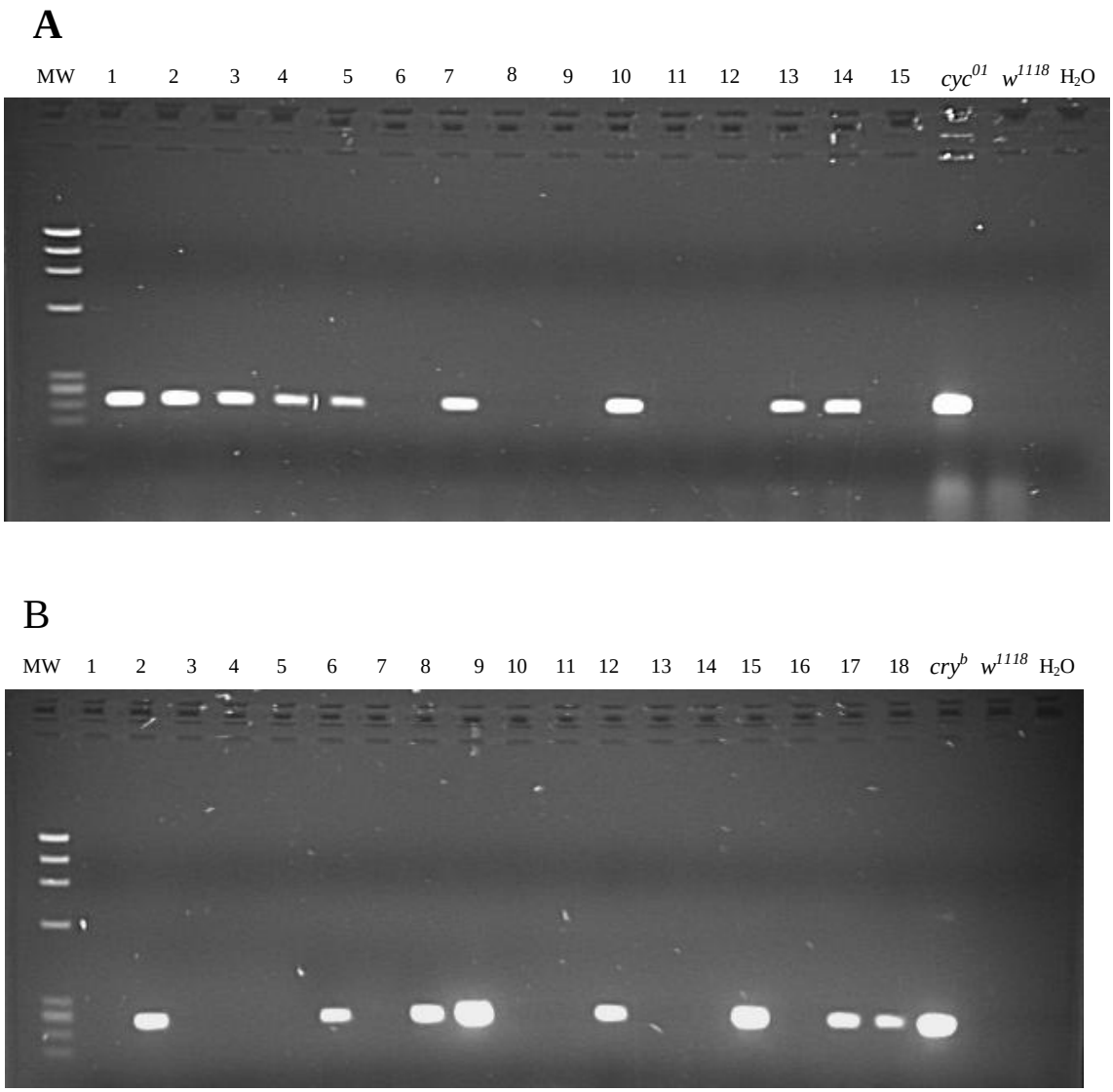


Figure 4.6. Molecular genotypying by PCR with allele-specific primers. A) *cyc*^{01}. B) *cry*^{b}. C) *Pdf* ^{01}. The amplified DNA fragments were resolved on a 2% agarose gel. The numbers above each lane represent different individuals. *w*^{1118}, negative control. H_20, water control (no DNA). MW, molecular weight marker ΦX Hae III.

C

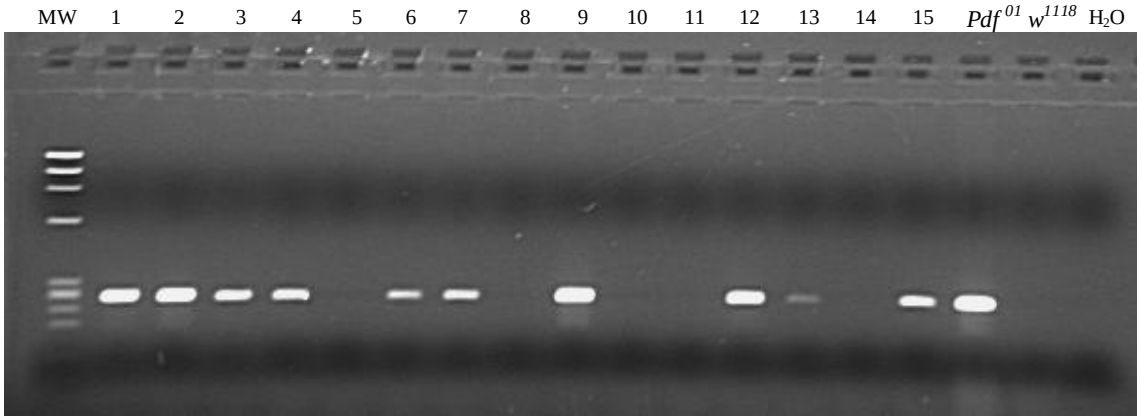


Figure 4.6. (continued)

For the transgenic constructs (i.e. GAL4, GAL80 and UAS elements), the backcrossing was done by simply following the presence of the mini-white marker. Initially, the loss-of-function w^{1118} mutation was backcrossed into the "Houten" genetic background in order to create the standard $w^{1118}{}_{HU}$ strain (Figure 4.7). Subsequently, all the transgenic constructs were backcrossed to $w^{1118}{}_{HU}$ for 8 generations. The progeny of each cross was screened based on the eye colour, which ranged from red to yellow depending on the position effect of each insertion.

4.3.2. Behavioural analysis

Following the standardization of the genetic background, the "Houtenized" clock mutants were analysed with respect to circadian locomotor behaviour. Although similar analyses have been exhaustively performed before, and the behaviour of each mutant is well defined, this is the first time that a congenic background has been used. In any case, the behavioural analyses provide an important checkpoint to verify the

reliability of the "Houtenized" flies stocks, reinforcing the presence of the mutations phenotypically.

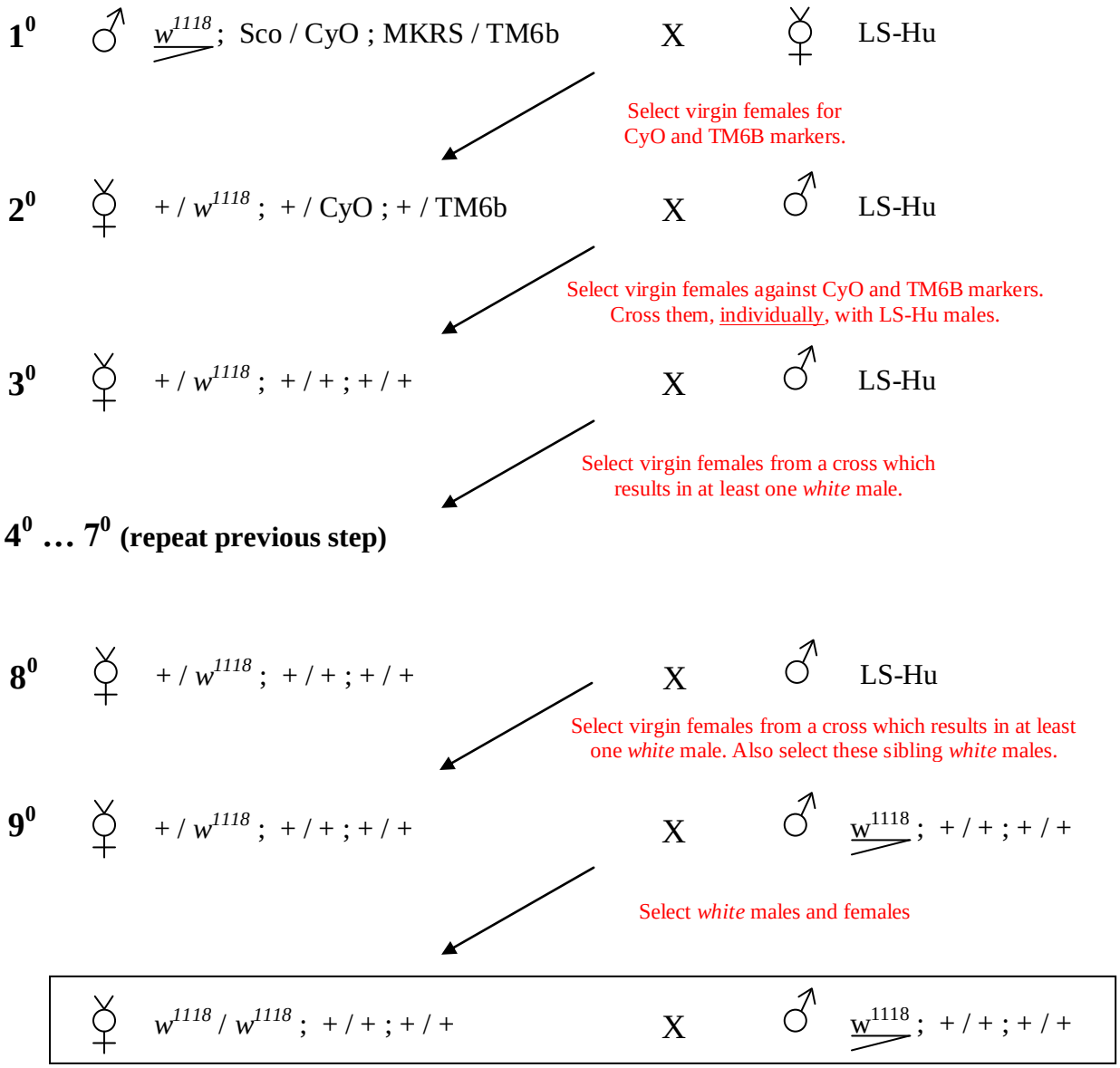


Figure 4.7. Backcrossing the w^{1118} mutant allele to a LS-Hu genomic background.

The behaviour of the clock mutants was analysed under entraining (LD) and free-running conditions (DD). In order to reveal putative genetic interactions, the effect of each mutation was assessed in both *ls-tim* or *s-tim* backgrounds (except for tim^{01} due to obvious reasons). If an interaction is revealed at the behavioural level, it might (or not) be used to explain similar results with other phenotypes (i.e. photoperiodic response), reported in the next section (4.3.3).

The locomotor activity rhythms were assayed in 3 days of LD (12:12) and 7 days of DD (Figure 4.8). A thorough observation of the activity patterns reveals that both *ls-tim* and *s-tim* controls entrain well to LD cycles and maintain robust rhythms in DD, with periods (τ) slightly longer than 24 h (Table 4.1). Importantly, statistical analysis (one-way ANOVA) revealed no significant differences in their periods ($F_{1,53}$ = 3.3, P = 0.075).

As one could have expected, per^{01}, tim^{01} and Clk^{jrk} flies exhibited arrhythmic behaviour soon after the flies were transferred to DD (Figure 4.8; Table 4.1). Overall, Pdf^{01} flies were also arrhythmic, however, this effect seems to be dependent on the *tim* haplotype. On an *s-tim* background, 37% of these mutants were still rhythmic, exhibiting a ~2 h shortening of its free-running period (Table 4.1). In fact, on the first 2 days of DD, most of these flies appear to sustain rhythms, which rapidly dampen after that. On an *ls-tim* background, Pdf^{01} flies are mainly arrhythmic and the presence of any rhythmic behaviour on the first days of DD is severely attenuated. Regarding $norpA^{p24}$ and cry^{b} mutants, robust rhythms were observed with a period very close to that of controls (Table 4.1). In this case, the *tim* allelic variant made no difference to the behaviour output in DD.

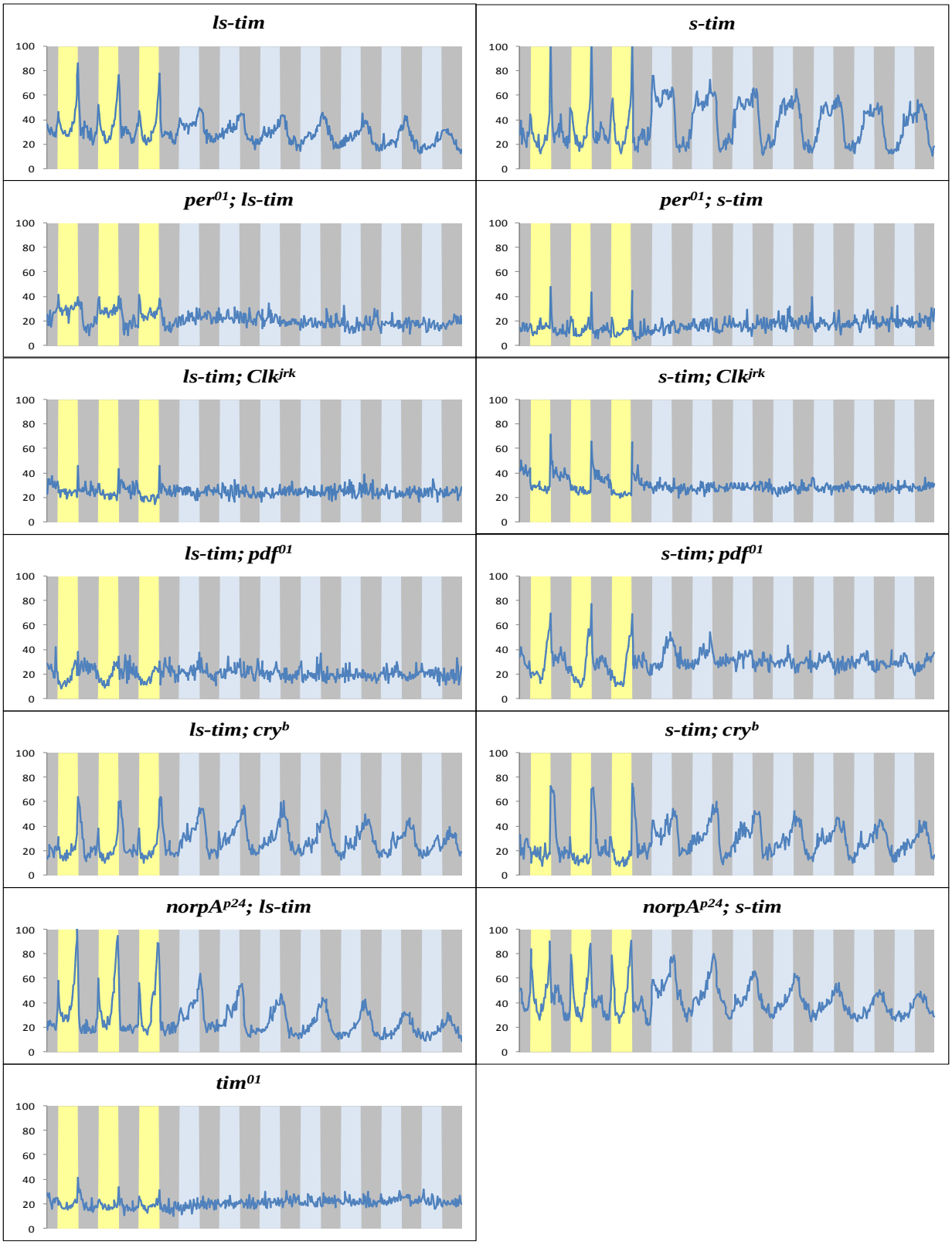


Figure 4.8. Overall activity patterns for "Houtenized" clock mutants. The plots represent the average of activity counts (in 30 min bins) under 3 days in LD (12:12) and 7 days in DD. Highlighted in yellow, light phase, in grey, dark phase, and in blue, subjective light phase.

Table 4.1. Free-running rhythms of "Houtenized" clock mutants.

Genotype	N	Rhythmic flies (%)	Mean period (± SEM)
ls-tim	30	83	24.58 ± 0.16
s-tim	30	100	24.89 ± 0.08
tim^{01}	29	3	Arrhythmic
per^{01}; *ls-tim*	26	4	Arrhythmic
per^{01}; *s-tim*	28	11	Arrhythmic
$norpA^{p24}$; *ls-tim*	31	77	24.88 ± 0.14
$norpA^{p24}$; *s-tim*	31	94	24.36 ± 0.45
ls-tim; cry^{b}	30	83	24.77 ± 0.13
s-tim; cry^{b}	31	94	24.49 ± 0.45
ls-tim; Clk^{jrk}	29	3	Arrhythmic
s-tim; Clk^{jrk}	31	0	Arrhythmic
ls-tim; Pdf^{01}	25	8	Arrhythmic
s-tim; Pdf^{01}	32	37	22.54 ± 0.17

The period (τ) was determined after 7 days in DD. Those genotypes that exhibited less than 20% rhythmic flies were classified as arrhythmic. N, number of flies tested.

In addition to the free-running behaviour, the locomotor activity of the clock mutants was also analysed in entraining conditions. By averaging the activity counts of two consecutive days for each fly, the LD activity patterns for all the genotypes were plotted and observed in detail (Figure 4.9). In wild-type control flies, *ls-tim* and *s-tim*, most of the activity is centred on the light-dark transitions (the so-called "morning" and "evening" peaks), resulting in a nice bimodal pattern. Importantly, the onset of activity starts before the transitions and are not merely responses to environmental conditions. This anticipatory nature is an important indicative of a functioning circadian clock. Even so, although both genotypes exhibited a marked anticipation for the evening (lights-off) transition, a subtle difference was observed for the morning (lights-on). In *ls-tim*, this phenomenon seems to be triggered some hours in advance and resulted in a steady rise of activity before lights-on, while in *s-*

tim it mainly occurs just before the transition (i.e. the last 30 min bin of the dark phase, Figure 4.9).

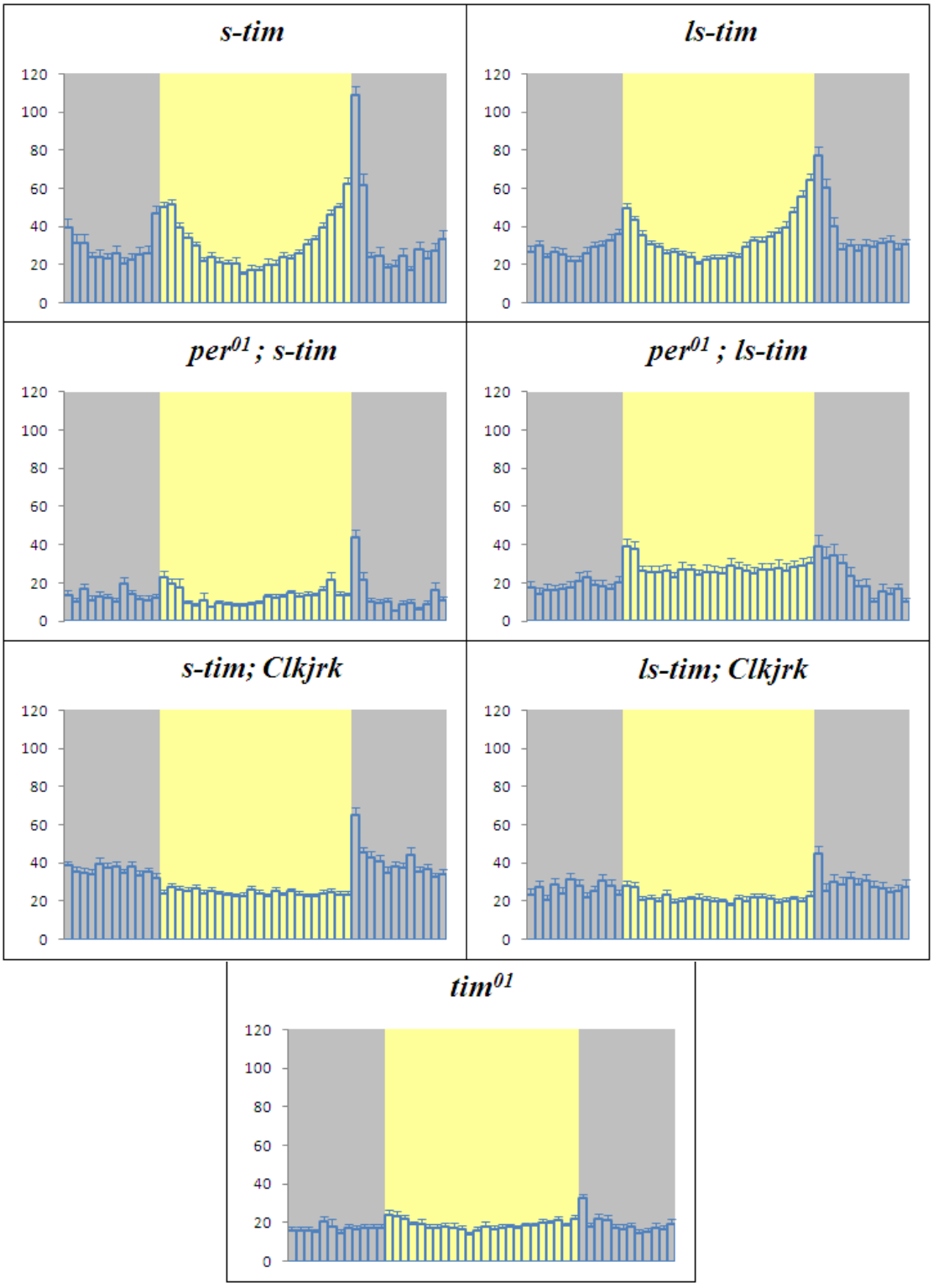


Figure 4.9. Activity patterns of "Houtenized" clock mutants in entraining conditions. The data correspond to two consecutive cycles of LD (12:12). Blue histograms represent average activity counts (± SEM) in 30 min bins. In yellow, light phase. In grey, dark phase.

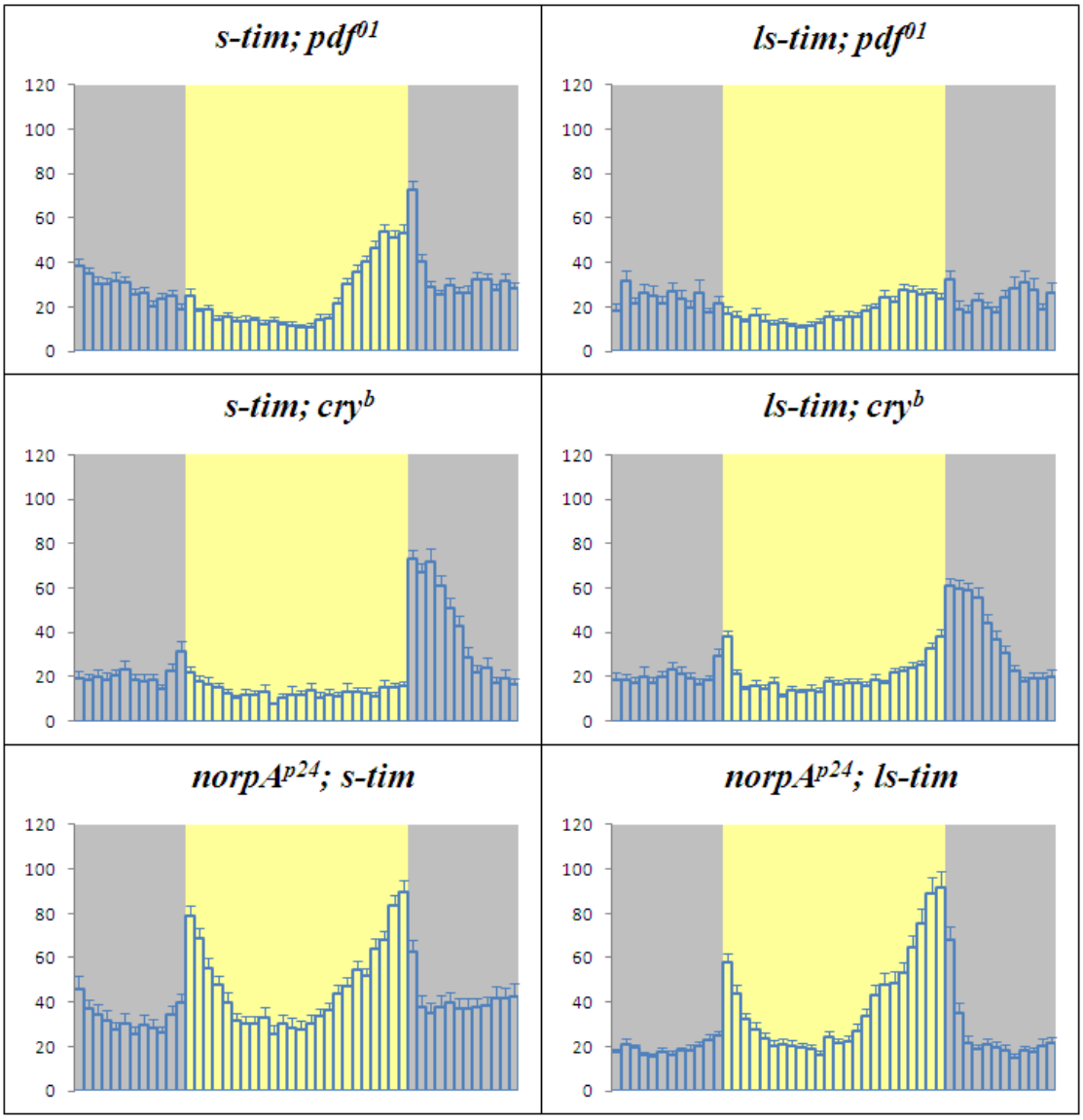


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Figure 4.9. (continued)

Reflecting the arrhythmic phenotype observed in free-running conditions, morning and evening anticipation events are significantly disrupted in the core clock mutants *per*^{01}, *tim*^{01} and *Clk*^{jrk}. Nonetheless, these genotypes still react to the light-dark transitions, becoming more active soon after the conditions change. In *Clk*^{jrk}, exceptionally, the reaction to the lights-on transition, also referred to as startle response, is also abolished.

In *Pdf* ^{01} mutants, the anticipatory behaviour is disrupted for the morning, yet conserved for the evening bout of activity. As another characteristic feature of this mutant, the phase of the evening peak is advanced towards the middle of the day. Curiously, it is also notable that the strength of the evening component depends on the *tim* natural allele, being largely reduced in the *ls-tim* background. The influence of *tim* natural variants was also observed in *cry*^{b} flies. Although the morning anticipation was not disturbed in these mutants, the evening anticipation appears to be attenuated, especially in flies carrying the *s-tim* polymorphism. As for the *norpA*^{p24} mutants, the activity profiles in LD conditions revealed conserved morning and evening anticipatory behaviours. The position of the evening peak of activity, however, seems to be slightly advanced in relation to the control flies.

4.3.3. Photoperiodic response

The circadian behaviour analyses of "Houtenized" clock mutants confirmed their characteristic phenotypes, providing additional proof that the fly stocks are ready to be assayed for the photoperiodic response (diapause). This physiological output is structured at many levels, including a photoreceptor (to capture environmental cues), a clock (to measure and interpret seasonal time) and an effector mechanism to trigger hormonal events leading to an arrest in vitellogenesis. It is widely accepted that many genes, which might not even be directly related, contribute to the final ovarian phenotype.

The current investigations with *tim* natural variants (Tauber *et al*., 2007; Chapter 3) have highlighted the importance of this gene in the control of diapause. Because its natural alleles exhibit a very robust difference in diapause levels, the

effect of any genetic manipulation needs to take this into consideration (see also Peschel *et al*., 2006). For that reason, the analysis of the photoperiodic response of "Houtenized" clock mutants was carried out on an *ls-tim* background. Based on the data reported in Chapter 3, the experimental design was fixed at 12°C and two different photoperiods: one short (LD 8:16) and one long (LD 16:8). At this temperature, *ls-tim* control flies should show intermediate diapause levels, which would allow the identification of mutants that either promote or reduce this response. As one could have noticed, the short photoperiod condition was slighted changed when compared to that adopted in Chapter 3, where an LD 10:14 was used. The intention was to magnify the observed difference in diapause levels between short and long-days (i.e. the amplitude of the photoperiodic effect).

The photoperiodic response of the "Houtenized" clock mutants is summarized in Figure 4.10. The statistical analysis of the data (Two-way ANOVA) is shown in Table 4.2. Interestingly, my results revealed that, when compared to the natural allelic variant *ls-tim*, the tim^{01} loss-of-function mutation promotes higher diapause incidence, at both short and long photoperiods, completely abolishing the photoperiodic effect (Figure 4.10A). These results corroborate preliminary data obtained on a "domesticated" laboratory background (Tauber *et al.*, 2007). The statistical analysis revealed significant effects for Genotype and Photoperiod, as well as a positive interaction between these variables. Resembling tim^{01}, the photoperiodic response of cry^{b} mutants (Figure 4.10B) appears to be also high and constant, irrespective of the photoperiod. However, despite significant effects for Genotype and Photoperiod, there was no significant interaction (Table 4.2).

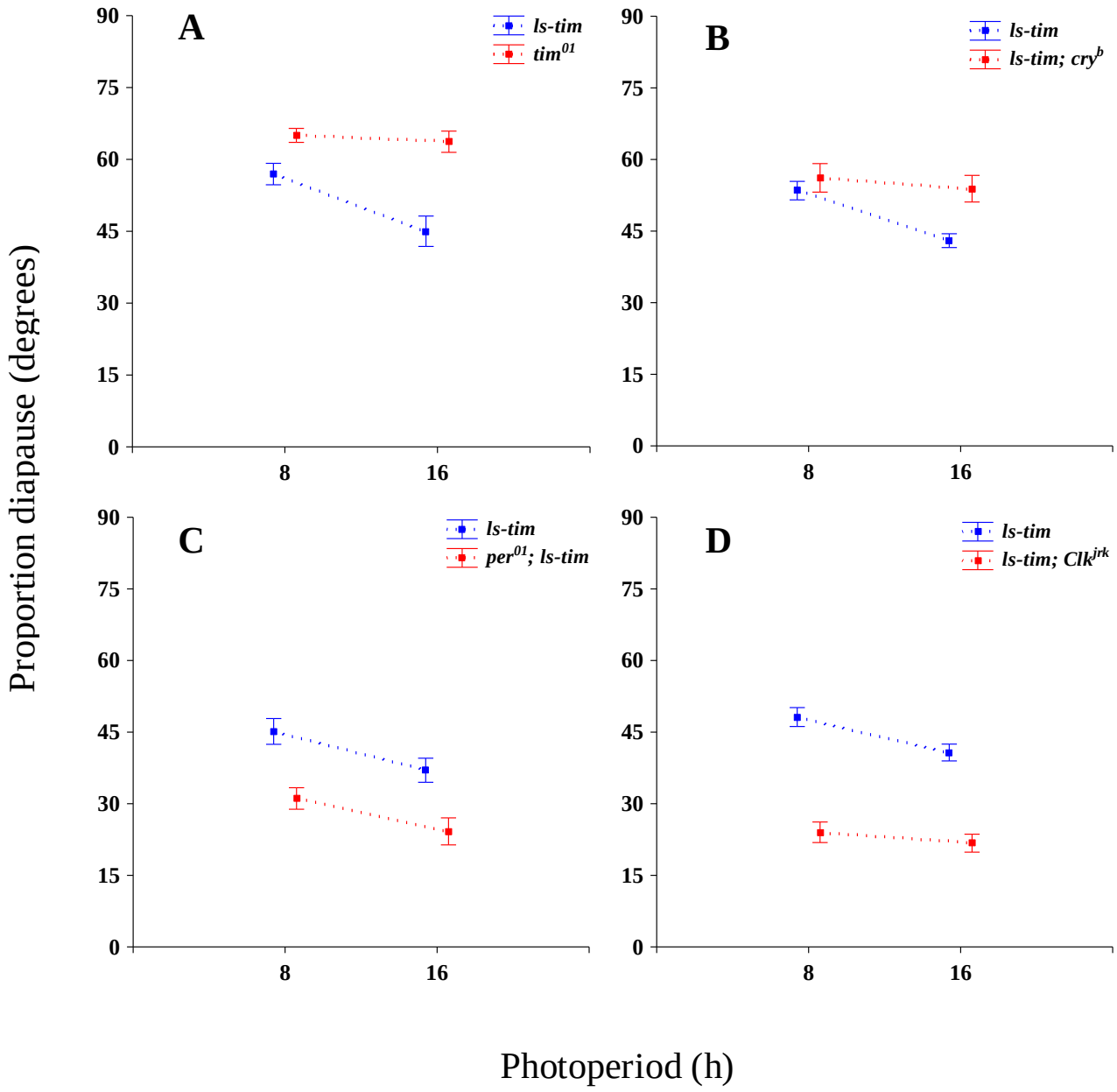


Figure 4.10. The photoperiodic response of canonical clock mutants. A) *ls-tim* X *tim*^{01}(N=400); B) *ls-tim* X *ls-tim*;*cry*^{b} (N=1040); C) *ls-tim* X *per*^{01};*ls-tim* (N=1160); D) *ls-tim* X *ls-tim*;*Clk*^{jrk} (N=1160); E) *ls-tim* X *norpA*^{p24};*ls-tim* (N=720); F) *ls-tim* X *ls-tim*;*Pdf*^{01} (N=1800). X axis, photoperiod (in hours of light). Y axis, diapause incidence in degrees (mean proportions, arcsin ± SEM).

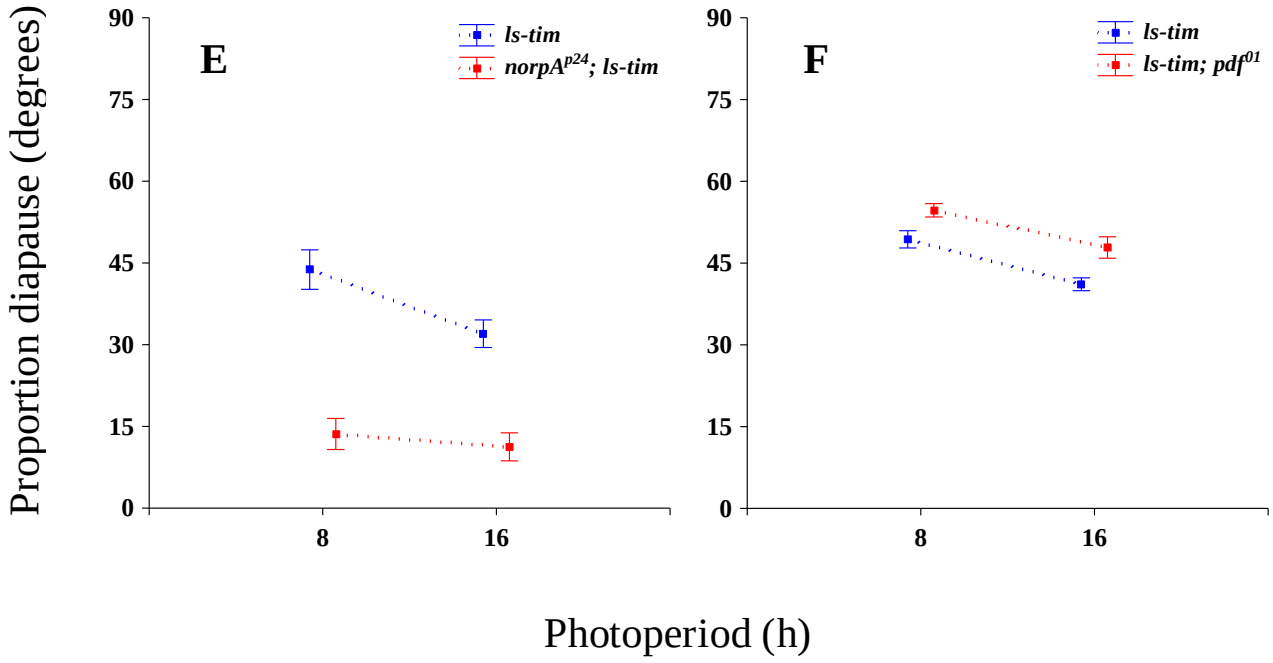


Figure 4.10. (continued)

Table 4.2. Statistical analysis (two-way ANOVA) of the photoperiodic response of clock mutants on an *ls-tim* background.

A. *ls-tim* X *tim*^{01}	**SS**	**d.f.**	**MS**	***F***	***P***
Genotype	894.51	1	894.51	32.327	$< 10^{-4}$
Photoperiod	220.04	1	220.04	7.952	0.012
Genotype X Photoperiod	140.69	1	140.69	5.085	0.038
Error	442.73	16	27.67		

B. *ls-tim* X *ls-tim;cry*^{b}	**SS**	**d.f.**	**MS**	***F***	***P***
Genotype	596.9	1	596.9	8.156	0.006
Photoperiod	527.9	1	527.9	7.213	0.010
Genotype X Photoperiod	219.4	1	219.4	2.998	0.090
Error	3513.1	48	73.2		

C. *ls-tim* X *per*^{01}; *ls-tim*

	SS	d.f.	MS	*F*	*P*
Genotype	2584.10	1	2584.10	26.4401	$< 10^{-5}$
Photoperiod	809.20	1	809.20	8.2797	0.006
Genotype X Photoperiod	5.61	1	5.61	0.0574	0.812
Error	5277.63	54	97.73		

D. *ls-tim* X *ls-tim;Clk*^{jrk}

	SS	d.f.	MS	*F*	*P*
Genotype	6669.49	1	6669.49	117.917	$< 10^{-6}$
Photoperiod	337.29	1	337.29	5.963	0.018
Genotype X Photoperiod	94.92	1	94.92	1.678	0.201
Error	3054.28	54	56.56		

E. *ls-tim* X *norpA*^{p24}*;ls-tim*

	SS	d.f.	MS	*F*	*P*
Genotype	5763.61	1	5763.61	76.2812	$< 10^{-6}$
Photoperiod	444.18	1	444.18	5.8787	0.021
Genotype X Photoperiod	196.54	1	196.54	2.6013	0.117
Error	2417.84	32	75.56		

F. *ls-tim* X *ls-tim;Pdf*^{01}

	SS	d.f.	MS	*F*	*P*
Genotype	816.3	1	816.3	15.344	$< 10^{-3}$
Photoperiod	1271.4	1	1271.4	23.898	$< 10^{-5}$
Genotype X Photoperiod	11.2	1	11.2	0.210	0.648
Error	4575.3	86	53.2		

All the other genotypes analysed did not cause an attenuation of the photoperiodic effect, but rather modulated the levels of diapause incidence. In *per*^{01} (Figure 4.10C), *Clk*^{jrk} (Figure 4.10D) and *norpA*^{p24} (Figure 4.10E) mutants, this

physiological response appears to be downregulated. In *Pdf* ^{01} mutants, on the other hand, it seems to be slightly upregulated (Figure 4.10F). As one expected, the statistical analyses assigned significant values to Genotype and Photoperiod, but not to Genotype X Photoperiod interaction.

To investigate putative genetic interactions, the photoperiodic response of *cry*^{b} and *Pdf* ^{01} mutants was also examined on an *s-tim* background (Figure 4.11). Surprisingly, both mutations exhibited very low diapause levels, contrasting with the results obtained on an *ls-tim* background. That suggests that *tim* has a dominant effect over *cry* and *Pdf* in the determination of diapause incidence. In addition, a careful observation of the data indicates, once again, that *cry*^{b} might cause an attenuation of the photoperiodic effect, although new statistical analysis has revealed an only marginal Genotype X Photoperiod interaction ($F_{1,28}$ = 4.097, P = 0.052) .

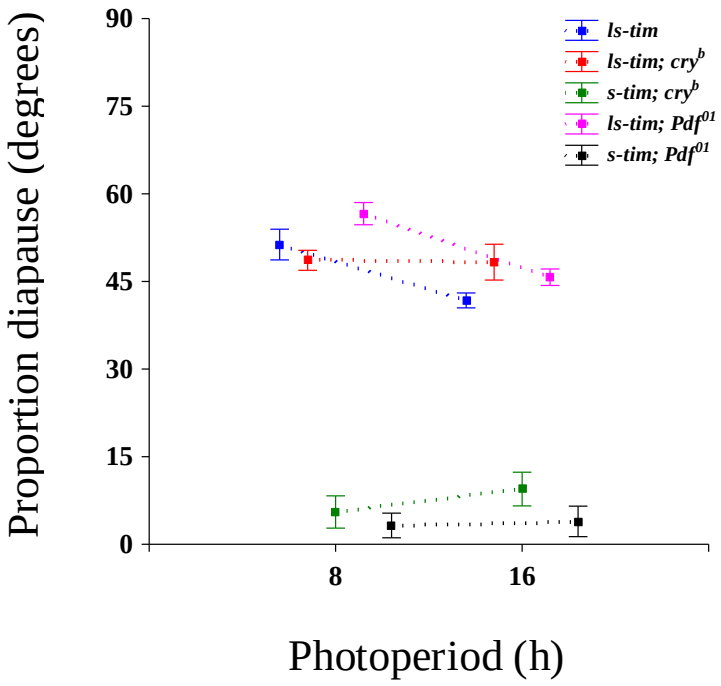


Figure 4.11. *tim* natural polymorphisms determine diapause levels in *cry*^{b} and *Pdf*^{01} mutants. X axis, photoperiod (in hours of light). Y axis, diapause incidence in degrees (mean proportions, arcsin ± SEM). N= 1600.

4.4. Discussion

In this chapter, I described the creation of a new set of Drosophila tools to study seasonal adaptation of circadian related phenomena. For the first time in the field, a number of classical clock mutations and transgenes, as well as balancer chromosomes, were transferred to a common genetic background, derived from a wild population of flies collected in Houten (Holland). This extensive work involved repeated generations of backcrossing and molecular genotyping methods based on PCR. The primers used and the PCR parameters were optimally adjusted to identify the particular molecular lesion of each mutation.

Recent studies have reported striking differences between the *tim* natural alleles, *ls-tim* and *s-tim*, with respect to its circadian light sensitivity and photoperiodic response (diapause) (Tauber *et al.*, 2007; Sandrelli *et al.*, 2007). Although the allelic variants do not drive any obvious effect on standard circadian behaviours, exhibiting wild type free-running rhythms and anticipation of morning and evening peaks in LD cycles, one cannot discard the possibility that their influence might be masked by epistatic interactions with other genes. As a result, the role of each clock mutation, for both circadian rhythmicity and diapause, was assigned in a controlled background for the *tim* polymorphism.

The locomotor activity assays of the "Houtenized" clock mutants in either entraining (LD) or free-running conditions (DD) allowed us to confirm characteristic phenotypes in a completely new genetic background. As previously reported, the core clock mutants per^{01}, tim^{01} and Clk^{jrk} were arrhythmic in DD and lacked anticipatory behaviour in LD (Konopka and Benzer, 1971; Sehgal *et al.*, 1994; Allada *et al.*, 1998; Grima *et al.*, 2004). High occurrence of arrhythmicity and the impairment of morning

anticipation were also observed for the output mutant *Pdf* ^{01} (Renn *et al.*, 1999; Stoleru *et al.*, 2004). Interestingly, a careful examination of this mutant's locomotor activity data reveals that a good proportion of the flies maintain weak rhythms on the first 2 days of DD, which is especially marked in the *s-tim* background. In LD, *Pdf* ^{01} mutants exhibited the expected advance in the evening peak, however, the amount of activity underlying it is clearly reduced in flies carrying the *ls-tim* allele, which might explain the higher degree of arrhythmicity in this background.

With regards to *norpA*^{p24} and *cry*^{b} mutants, robust free-running rhythms with normal period (τ) were revealed, indicating that the molecular clock machinery was not altered (Stanewsky *et al.*, 1998). However, as these mutations disrupt entrainment genes, phenotypic effects would be reserved for the behaviour in LD conditions. In fact, *norpA*^{p24} flies were still able to anticipate both light transitions, although the evening peak of activity appeared to be a bit advanced when compared to the wild-type controls. That is to be expected since this mutant behaves as if the temperature was colder, promoting high splicing levels in *per* transcripts, which causes an earlier upswing in PER protein levels and, as a result, shifts the evening peak (Collins *et al.*, 2004; Majercak *et al.*, 2004). As for *norpA*^{p24}, the analysis of *cry*^{b} mutants in LD cycles revealed the persistence of morning anticipatory behaviour. Surprisingly, though, the anticipation of the evening activity bout was present in *ls-tim*, but greatly reduced, or even absent, in *s-tim* background, suggesting the both *cry* and *tim* interact and contribute to this phenotype.

The influence of *cry* and *Pdf* on the activity increase before lights-off has been recently studied (Cusumano *et al.*, 2009). It appears that the evening cell oscillator (i.e. 3 LNds + the 5th s-LNv) synchronizes to LD cycles either through on-site CRY expression or PDF-modulated visual system inputs. The contribution of both genes is

further noticed in *Pdf* ^{01}*cry*^{b} double mutants, which do not exhibit any evening anticipatory behaviour. A similar phenotype was observed in *Pdf* ^{01} mutants subjected to winter-type environmental conditions (i.e. low-light intensity, short photoperiod and cold temperature), suggesting that PDF's role in the visual system-mediated light entrainment is dominant when CRY function is reduced. Interestingly, my data not only confirmed that this phenotype is affected by these genes, but also outlined the importance of the *tim* natural variants. The relative inhibition of the evening peak in *Pdf* ^{01}flies carrying the *ls-tim* allele might be explained by its reduced light sensitivity (Sandrelli *et al.*, 2007), which could be mimicking the effect of low-light intensity (Cusumano *et al.*, 2009). Since in *cry*^{b} mutants most of the entraining signalling cues come from the eyes, the differences observed with respect to the *tim* allelic background probably stem from this tissue through PDF function. Should this hypothesis prove to be true, then *ls-tim* and *s-tim* might also drive important differences in the eye photoreception physiology.

The photoperiodic response analyses of the "Houtenized" clock mutants also revealed very interesting results. My data showed that mutations in the core clock genes *per*, *tim* and *Clk* considerably affect the diapause output, but not in the same way. While *per*^{01} and *Clk*^{jrk} mutations appear to inhibit diapause incidence, *tim*^{01} seems to promote it. Interestingly, *tim*^{01} appeared to be unable to distinguish short from long-day rearing conditions, confirming previous data obtained on a semi-homogeneous lab background (Tauber *et al.*, 2007). The same phenomenon was not observed in *per*^{01} mutants, which still reacted to photoperiodic changes just like it was initially reported by Saunders *et al.* (1989). In relation to *Clk*^{jrk}, considered by many as the most severe circadian clock mutation, the current data is not entirely conclusive. Although statistical analyses did not detect any significant difference

between mutant and control genotypes over photoperiod, the observation of the data suggests that the photoperiodic effect might be slightly attenuated. Future experiments, with more experimental replicates, might be necessary to further investigate this issue.

The phenotypic analysis of cry^{b} mutants revealed a significant increase in diapause levels, especially in long-day rearing conditions. As a result, this mutant also showed an attenuation of the photoperiodic effect, resembling that of tim^{01}. An increase in diapause incidence was also observed in Pdf^{01} mutants, although this effect was equal for both day lengths and did not result in any disturbance of the photoperiodic effect. Surprisingly, when Pdf^{01} and cry^{b} were analysed on an *s-tim* background, the resulting diapause levels were very low, indicating a dominant role for *tim* over both genes.

In contrast to the results obtained with Pdf^{01} and cry^{b}, diapause incidence was severely reduced in $norpA^{p24}$, surpassing the effects promoted by the *ls-tim* allelic variant and implicating the PLC signalling pathway in this response. The ability of these mutants to distinguish day length changes, nevertheless, seems to be unaltered (no Genotype X Photoperiod interaction).

Even though one could certainly observe the effects of each gene on diapause levels, the determination of their role in the ability to modulate the photoperiodic effect was compromised when a very low response was obtained with the mutants, such as in Clk^{jrk} and $norpA^{p24}$. Because diapause is influenced by small variations in environmental temperature (Chapter 3), future analysis could be performed at slightly colder conditions in order to increase its levels and reveal the true photoperiodic effect of such mutants. However, as we do not know how these mutations respond to

temperature changes, this strategy might not work and they may never achieve higher diapause levels.

The data presented here encourages us to create a simplified molecular model of the photoperiodic response based on clock-related genes (Figure 4.12). In such model, the proteins TIM, CRY and PDF would be acting as negative regulators, whereas PER, CLK and NORPA would function as positive regulators of diapause. Should all these genes interact on a common network or act independently to drive this phenotype is not clear and subject to speculation.

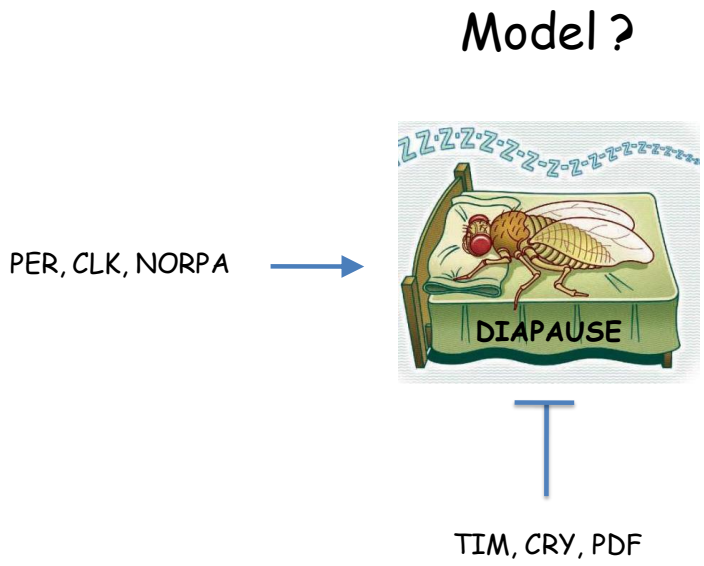


Figure 4.12. Simplified model of diapause regulation by clock genes.

CRY and TIM, for instance, exhibited similar responses and could be operating together to determine the photoperiodic response, as they normally do for the circadian rhythms. Because its loss-of-function mutations appear to attenuate the photoperiodic effect, the role of these genes might be accredited either to the photoreception pathway or to the photoperiodic clock itself. With respect to the

determination of diapause levels, however, *tim* is completely dominant over *cry*, suggesting that the latter is probably acting upstream, as a photoreceptor to the clock mechanism.

The role of CLK in this genetic network could be many, including its direct influence on *tim* expression. If that is the case, then the reduced diapause levels of Clk^{jrk} mutants could be simply explained by a downregulation of *tim* transcripts. Alternatively, CLK could contribute to diapause by activating the expression of one (or more) of its many targets genes (McDonald and Rosbash, 2001; Claridge-Chang *et al.*, 2001; Ceriani *et al.*, 2002), as some of these could be involved in stress related phenotypes, which are closely related to aging and diapause (Williams *et al.*, 2006). One of the CLK direct targets, *Pdp1ε*, induces the expression of *takeout* (Benito *et al.*, 2010), which was recently implicated as a major regulator of life-span (Bauer *et al.*, 2010). In addition, *takeout* could act as a carrier protein for juvenile hormone (JH) (Lerro and Prestwich, 1990; Meunier *et al.*, 2007), which has been associated with diapause in *D. melanogaster* (Saunders *et al.*, 1990).

The particular way through which NORPA could be affecting diapause could either involve its role in the circadian network or, alternatively, an undefined pleiotropic action. As previously mentioned, under entraining conditions, *norpA* mutants advance the evening peak towards the middle of the day and, therefore, behaves as if the temperature was colder (Collins *et al.*, 2004; Majercak *et al.*, 2004). In addition, the circadian clock of these flies is less photoresponsive, which leads to an attenuated synchronization when exposed to a new light regime (i.e. LD cycle) (Stanewsky *et al.*, 1998). Based on this information, one could speculate that *norpA* mutants would exhibit higher diapause levels at both photoperiods, as if they were sensing the upcoming winter before the wild-type flies. However, my data revealed an

opposite scenario, with very low diapause incidence, suggesting that the effect of NORPA in this phenomenon is probably driven by an alternative pathway to the one used to modulate the circadian clock.

Considering the current model for the circadian clock, the output factor PDF could be promoting its effect on diapause by affecting the communication between neurons involved in this response. In fact, in *D. melanogaster* and many other insect species, PDF positive fibers project to dorsal regions of the brain, in close proximity to neurons directly connected with important neuroendocrine complexes (i.e. *corpora allata* and *corpora cardiaca*), which have been implicated as putative diapause effector centres (Saunders *et al.*, 2002; Shiga and Numata, 2000, 2007; Hamanaka *et al.*, 2005; Shimokawa *et al.*, 2008;). Interestingly, the surgical ablation of these fibers in *P. terraenovae* resulted in intermediate diapause levels for both short and long-day rearing conditions, suggesting that the photoperiodic timing, rather than the ability to induce diapause, was impaired (Shiga and Numata, 2009). In *D. melanogaster*, this scenario appears to be different. As my data shows, PDF appears to downregulate diapause, but does not influence the photoperiodic effect. Moreover, its effect is largely influenced by *tim*, as it was shown for *s-tim Pdf* ^{01} *flies*. Therefore, the role of PDF in the cellular network underlying the photoperiodic response in *D. melanogaster* is secondary, and not essential for the determination of seasonal timing, although one should consider that the PDF expressing neurons (i.e. LNvs) might still play a crucial part. Future ablation experiments will be necessary to test this hypothesis.

The core clock protein PER could be exerting its function through interaction with CLK. In *per*^{01} mutants, *Clk* mRNA levels are low (Glossop, 1999), which would probably result in the downregulation of CLK direct targets as well, some of which, as

mentioned above, might be involved in diapause. This could possibly explain why PER phenocopies CLK by promoting low diapause levels.

In view of the current data, it is reasonable to expect that any model to illustrate the effect of clock genes on diapause may be over simplistic. The putative genetic pathways described above are highly speculative and need to be validated in future studies. This is the reason why some inconsistencies may have arisen. For example, if it is assumed that Clk^{jrk} drives low diapause incidence by downregulation of *tim* expression, then one could predict that the null mutant tim^{01} would exhibit a similar phenotype and not a constitutively high response instead. Another apparent contradiction emerges when diapause incidence is attributed to *Clk* mRNA levels, which are greatly diminished in both per^{01} and tim^{01} mutants (Glossop, 1999) and, nevertheless, generate opposite outcomes.

Even the conventional feedback loop model, structured around PER and TIM protein interaction and translocation into the nucleus, where both act as negative regulators, may not be perfectly adequate to explain behavioural rhythmicity under certain environmental conditions. By manipulating the LD cycles and assessing PER and TIM dynamics inside clock cells, interesting fundamental differences between both factors could be observed (Shafer *et al.*, 2004). While PER nuclear accumulation was adjusted to fit the night length, providing a molecular correlate for the behavioural changes, TIM did not. The pattern of TIM nuclear localization was roughly the same over a wide range of night lengths (i.e. it was not accelerated or delayed), being only affected by the lights-on transitions. At considerably short night lengths (i.e. LD 18:6), no TIM staining was detected in the cell, while PER was present mostly throughout the day, especially in the nucleus. Since these proteins

reacted to changes in day length in particularly different ways, it is not so surprising to find that they assume diverging roles in diapause regulation as well.

Overall, my results showed that clock genes can indeed exert a strong influence on diapause by controlling its levels and also the photoperiodic timing effect. Although one should consider the possibility of gene pleiotropy (Emerson *et al*., 2009; Bradshaw and Hozapfel, 2010), I believe it is very unlikely since all clock genes tested showed an effect on diapause. Therefore, I propose not only that these genes are functionally linked, but also that the whole circadian clock network is somehow modulating the diapause response in *D. melanogaster*, although the nuances of this regulation are yet obscure.

Regarding the anatomical location of the photoperiodic mechanism, studies of several insect species have appointed the brain as the centre for processing calendar information (Saunders *et al.*, 2002). Its precise location, however, is still unknown. My collection of "Houtenized" transgenic lines could be extremely useful to investigate this issue, but due to a lack of time, I did not pursue this lead. Through the UAS/GAL4/GAL80 system, the expression of a pro-apoptotic gene (i.e. using *uas-hid* or *uas-rpr*) could be restricted to a very small subset of cells to determine their possible roles in diapause. This "ablation" strategy was successfully implemented to map the location of clock neuronal clusters, the so-called "morning" and "evening" oscillators (Stoleru *et al.*, 2004). Alternatively, since *tim* expression (especially *s-tim*) promotes vitellogenesis (or downregulates diapause), the construct *uas-tim* could be manipulated to selectively rescue gene function in a tim^{01} background. The relative contribution of the canonical visual pathway could be assessed with the driver *gmr-GAL4*, which targets eye photoreceptor cells. By using *Pdf-GAL4*, the expression could be driven to the "morning" oscillator, while the combination of *tim-GAL4/Pdf-*

GAL80 or *cry-GAL4/Pdf-GAL80* would restrict it to the "evening" oscillator (Stoleru *et al.*, 2004).

If the circadian system is indeed relevant for diapause, then the neuronal network behind both phenomena could also be shared. This hypothesis might implicate both "morning" and "evening" oscillators operating together, under a defined phase relationship, to drive the photoperiodic response. Depending on the results obtained with the ablation and *tim* rescue experiments, the construct *uas-sgg* might also be used to speed up these oscillators, changing their phase relationship. This might provide an elegant way to test the "internal coincidence" model of two oscillators proposed by Pittendrigh (1972), although my current data do not support that, since tim^{01} and Clk^{jrk} mutants impair both oscillators and exhibit opposite effects in diapause incidence. Whatever the case may be, the genetic manipulation of these cellular clusters could give us an indication about how time information is measured and integrated to drive photoperiodism in insects.

Chapter 5: N-terminal evolution of *timeless*

5.1. Introduction

Population genetic studies on *D. melanogaster timeless* have reported that a new, naturally occurring polymorphism, *ls-tim*, has spread relatively recently (~8000 years) throughout Europe by directional selection (Tauber *et al.*, 2007). This natural variant produces both short and long isoforms of the protein, S-TIM and L-TIM, respectively, in comparison to the ancestral allele, *s-tim*, whose main product is the short one (Rosato *et al.*, 1997; Sandrelli *et al.*, 2007). There are a number of circadian-related phenotypes affected by this polymorphism, either at the molecular, behavioural or physiological level, which may explain why it has come under selection (Tauber *et al.*, 2007; Sandrelli *et al.*, 2007). To begin with, *ls-tim* is more prone to diapause than the ancestral variant, exhibiting higher incidence levels at different temperatures and on a wide range of photoperiods (Chapter 3; Tauber *et al.*, 2007). This suggests that, in a seasonal environment such as Europe, *ls-tim* flies should anticipate the colder temperatures of autumn by inducing diapause earlier than *s-tim*. Higher levels of diapause are often associated with enhanced fitness under stressful conditions, as shown by Schmidt and co-workers in a series of studies (Schmidt *et al.*, 2005a,b, 2006). Secondly, the circadian clock of *ls-tim* is less light-sensitive, as measured by its behavioural responses to brief light pulses (Sandrelli *et al.*, 2007). Again, such an attenuated circadian light response has been argued to be adaptive in seasonal environments, where exotic photoperiods can lead to disrupted circadian behaviour (Pittendrigh *et al.*, 1991; Shafer *et al.*, 2004). Thus, the two altered phenotypes of *ls-tim*, diapause and circadian photoresponsiveness, have been both

understood in terms of a reduced light response of TIM, the light-sensitive clock component (Myers *et al.*, 1996). Finally, at the molecular level, the L-TIM isoform shows reduced dimerization with the circadian blue-light photoreceptor Cryptochrome (CRY) in yeast two-hybrid assays (Sandrelli *et al.*, 2007). *In vivo,* this leads to a more stable TIM product in *ls-tim* flies, which correlates well with their reduced circadian photosensitivity and might also explain its higher diapause incidence (Tauber *et al.*, 2007; Sandrelli *et al.*, 2007). Consequently, *ls-tim* provides a compelling example of a recently derived mutation that has come under natural selection due to the adaptive phenotypes it can confer at different biological levels.

In this study, the molecular evolution of *ls-tim* is further investigated by looking for features that might have been the substrate for natural selection. Since the phenotypic effects promoted by this allelic variant lies on the presence of L-TIM, the N-terminal fragment unique to this isoform, a stretch of 23 amino acids, was subject to structure/function analyses in both yeast and fly models. Following the strategy adopted in related studies (Tauber *et al.*, 2007; Sandrelli *et al.*, 2007), a number of engineered N-terminal TIM proteins have been tested for their ability to interact with CRY, *in vitro,* and to transduce light information to the circadian clock, *in vivo*.

5.2. Methods

5.2.1. Transgenic constructs

The manipulation of TIM N-terminal sequence was carried out following a PCR-based site-directed mutagenesis protocol (Section 2.3.1). Mutagenized *tim* constructs were cloned in appropriate vectors for fly transgenesis (Section 2.3.2) or

yeast two-hybrid assays (Section 2.3.3, "N-terminal mutants"). Details regarding the LexA-CRY (full-length) construct are described elsewhere (Ceriani *et al.*, 1999).

5.2.2. Two-hybrid assays

The protein interaction assay was carried out according to the protocol developed by Golemis and Brent (1997). Initially, as a prerequisite for the system, the mutagenized *tim* fragments were introduced into the yeast vectors pEG202 and pJG4-5, encoding the LexA binding domain and the B42 activation domain, respectively. By cloning TIM in both vectors the system has more flexibility, since some proteins work better when fused to one or the other domain (e.g. stronger interactions, diminished background levels, etc). In the experiments described here, however, only the TIM constructs made in pJG4-5 were used, and their interactions were tested with an already available CRY construct in pEG202 (i.e. LexA-CRY; Ceriani *et al*, 1999).

The yeast strain was first transformed with the reporter (pSH18-34) and the LexA-CRY plasmid. Among the various resulting colonies, one was picked and grown on selective media. This specific yeast clone was then made competent for another round of transformation, this time with each of the TIM mutant constructs or the pJG4-5 empty vector, which was used as a negative control. The strategy above was chosen in order to minimize clonal variation of plasmid copy numbers, which can affect the interaction between the proteins and result in misleading interpretations.

The functional analyses of each TIM construct were based on two independent transformations. In each experiment, at least 5 colony replicates were examined. The two-hybrid assay was performed by streaking the colonies on X-Gal containing media, which had its pH optimized for β-galactosidase activity. The progress of the

reaction was monitored every hour by recording the intensity of the blue staining (i.e. degradation of the X-Gal substrate). Because TIM and CRY should only interact in the presence of light (Ceriani *et al.*, 1999), replica plates were made and placed in dark conditions (i.e. wrapped in foil), thereby representing negative controls.

Detailed information regarding the yeast two-hybrid strain and vectors, as well as transformation protocols and culture media can be found in section 2.4.

5.2.3. Flies

The microinjection of *Drosophila* embryos and generation of stable transformants was done by BestGene Inc. (http://www.thebestgene.com). All transgenic lines were generated according to the site-directed ΦC31 integrase system developed by Bischof *et al.* (2007). The host line, ΦX-86Fb (*ywM{eGFP.vas-int.Dm}ZH-2A; +; M{RFP.attP}ZH-86Fb;* +), is available at the Bloomington Stock Center (Stock # 27479) or through personal request to Konrad Basler's group at the FlyC31 website (http://www.frontiers-in-genetics.org/flyc31/). This "combined" line contains an *attP* site located at the 3rd chromosome, cytosite 86Fb, and a construct expressing ΦC31 integrase at the 1st chromosome, cytosite 2A.

All *tim* transgenes were placed on a *w tim^{01}* background by standard crossing schemes, using balancer chromosomes. The genetic background of these lines was homogenized in such a way that the 1st and 2nd chromosomes were from the "Houtenized" *w tim^{01}* strain and the 3rd chromosome, which carried the transgenes, was not altered (the same as the original host line) (see Figure 5.13).

5.2.4. Behavioural analysis

The locomotor activity of the flies (3-7 days old) was monitored for 2 days in LD (12:12) followed by 5 days in DD, at 25°C. The details regarding the experimental set-up, data collection and analysis are described in section 2.5.

For the phase-shift assays, the transgenic flies were given a brief light-pulse (10 min) at ZT15 and then maintained in constant darkness (DD). The phase delays, corresponding to the third day in DD, were calculated as described in section 2.5.3.

5.3. Results

5.3.1. N-terminus *in silico* analysis

The *ls-tim* natural polymorphism is characterized by the insertion of a *G* nucleotide, which results in the recruitment of additional N-terminal 23 residues via an upstream *ATG* start codon (Tauber *et al.*, 2007). Conversely, *s-tim* has a frame shift, due to absence of this *G*, which truncates the upstream ORF after 19 residues but leaves the downstream start codon intact. In practical terms, while flies carrying the ancestral *s-tim* allele generate only the short isoform, S-TIM, those carrying the newly derived *ls-tim* generate two, S-TIM and the long isoform, L-TIM. Hence, the phenotypic differences between *ls-tim* and *s-tim* natural variants (Tauber *et al.*, 2007; Sandrelli *et al.*, 2007) stems from the presence (or not) of L-TIM. In order to investigate if a post-translational modification event could account for the L-TIM driven phenotypes, a number of bioinformatics prediction algorithms were applied on its unique N-terminal 23 residues.

The careful examination of the primary sequence revealed the presence of two serines (S), one at position 2 and the other at 22, which could serve as a substrate for phosphorylation (Figure 5.1.). However, when the sequence was compared to motif databases using the neural network-based algorithm NetPhos (Blom *et al.*, 1999), only the latter (S22) exhibited a significant score (Figure 5.2). Using similar prediction tools, such as NetPhosK (Blom *et al.*, 2004) and PhosphoMotif Finder (Amanchy *et al.*, 2007), the identity of a number of kinases that could be acting at this motif were revealed, including PKA, PKC, calmodulin-dependent protein kinase II, casein kinase II and GSK3. The latter is a homologue of SGG, a Drosophila kinase involved in the regulation of TIM nuclear translocation (Martinek *et al.*, 2001), and consequently with the pace of the clock (Stoleru *et al.*, 2005).

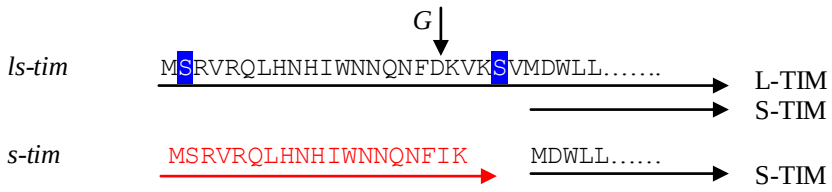


Figure 5.1. TIM isoforms. The insertion of a *G* nucleotide in *ls-tim* results in the translation of two protein isoforms (black arrows), while *s-tim* can translate only one. The two candidate phosphorylation sites (serines at positions 2 and 22) are highlighted in blue. The putative truncated 19-residue peptide from *s-tim* is shown in red.

A

>TIMELESS protein sequence
MSRVRQLHNHIWNNQNFDKVKSVMDWLLATPQLYSAFSSLGCLEGDTYVVNPNALAILEEINYKLTYEDQTLRTFRRAIG
FGQNVRSDLIPLLENAKDDAVLESVIRILVNLTVPVECLFSVDVMYRTDVGRHTIFELNKLLYTSKEAFTEARSTKSVVE
YMKHILESDPKLSPHKCDQINNCLLLLRNILHIPETHAHCVMPMMQSMPHGISMQNTILWNLFIQSIDKLLLYLMTCPQR
AFWGVTMVQLIALIYKDQHVSTLQKLLSLWFEASLSESSEDNESNTSPPKQGSGDSSPMLTSDPTSDSSDNGSNGRGMGG
GMREGTAATLQEVSRKGQEYQNAMARVPADKPDGSEEASDMTGNDSEQPGSPEQSQPAGESMDDGDYEDQRHRQLNEHGE
EDEDEDEVEEEEYLQLGPASEPLNLTQQPADKVNNTTNPTSSAPQGCLGNEPFKPPPPLPVRASTSAHAQMQKFNESSYA
SHVSAVKLGQKSPHAGQLQLTKGKCCPQKRECPSSQSELSDCGYGTQVENQESISTSSNDDDGPQGKPQHQKPPCNTKPR
NKPRTIMSPMDKKELRRKKLVKRSKSSLINMKGLVQHTPTDDDISNLLKEFTVDFLLKGYSYLVEELHMQLLSNAKVPID
TSHFFWLVTYFLKFAAQLELDMEHIDTILTYDVLSYLTYEGVSLCEQLELNARQEGSDLKPYLRRMHLVVTAIREFLQAI
DTYNKVTHLNEDDKAHLRQLQLQISEMSDLRCLFVLLLRRFNPSIHSKQYLQDLVVTNHILLLILDSSAKLGGCQTIRLS
EHITQFATLEVMHYYGILLEDFNNNGEFVNDCIFTMMHHIGGDLGQIGVLFQPIILKTYSRIWEADYELCDDWSDLIEYV
IHKFMNTPPKSPLTIPTTSLTEMTKEHNQEHTVCSWSQEEMDTLYWYYVQSKKNNDIVGKIVKLFSNNGNKLKTRISIIQ
QLLQQDIITLLEYDDLMKFEDAEYQRTLLTTPTSATTESGIEIKECAYGKPSDDVQILLDLIIKENKAQHLLWLQRILIE
CCFVKLTLRSGLKVPEGDHIMEPVAYHCICKQKSIPVVQWNNEQSTTMLYQPFVLLLHKLGIQLPADAGSIFARIPDYWT
PETMYGLAKKLGPLDKLNLKFDASELEDATASSPSRYHHTGPRNSLSSVSSLDVDLGDTEELALIPEVDAAVEKAHAMAS
TPSPSEIFAVPKTKHCNSIIRYTPDPTPPVPNWLQLVMRSKCNHRTGPSGDPSDCIGSSSTTVDDEGFGKSISAATSQAA
STSMSTVNPTTTLSLNMLNTFMGSHNENSSSSGCGGTVSSLSMVALMSTGAAGGGGNTSGLEMDVDASMKSSFERLEVNG
SHFSRANNLDQEYSAMVASVYEKEKELNSDNVSLASDLTRMYVSDEDDRLERTEIRVPHYH

Phosphorylation sites predicted: Ser: 63 Thr: 19 Tyr: 16

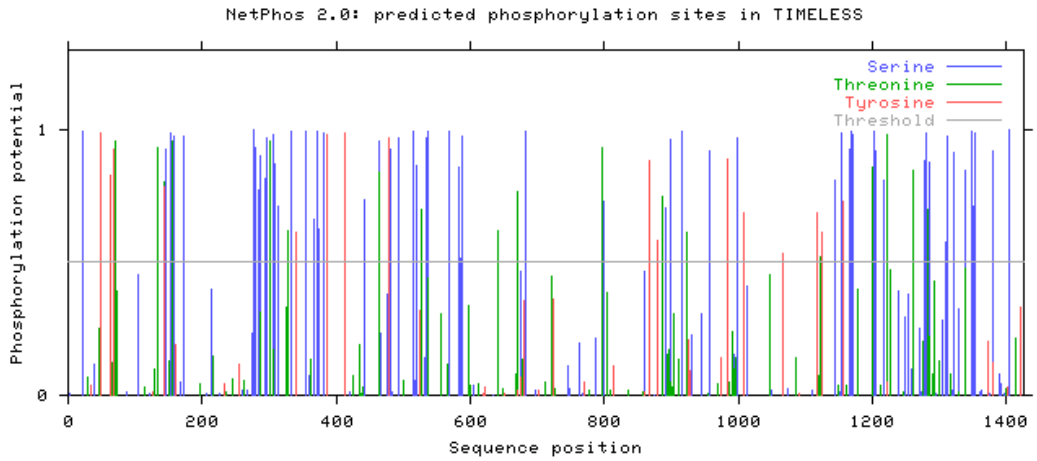
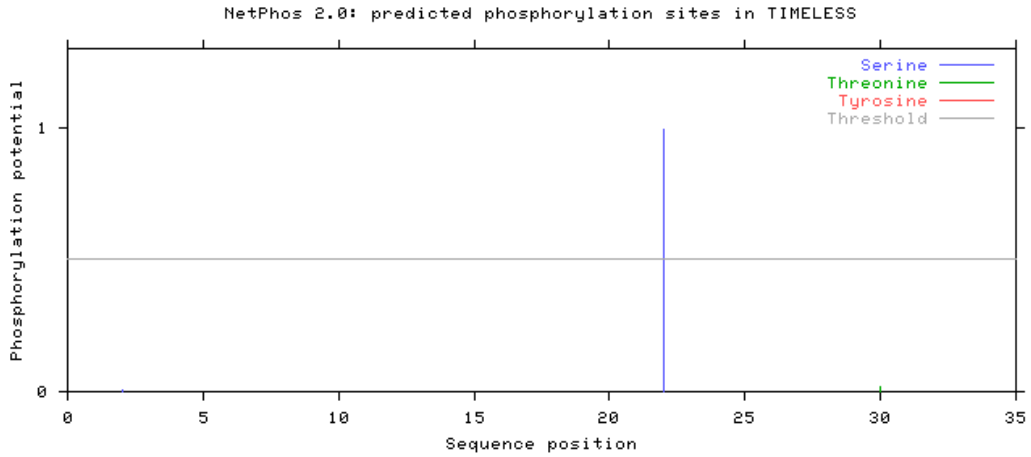


Figure 5.2. Prediction of putative phosphorylation sites in TIM protein sequence according to the NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/). A) The full-length TIM protein (1421 amino acids, isoform D; NCBI Accession Number NP_722912) was used as a query sequence for the identification of Serine (Ser, S), Threonine (Thr, T), and Tyrosine (Tyr, Y) phosphorylation motifs. The lower panel illustrates the phosphorylation potential of all the residues along the sequence, and selects those with high probability above the threshold line (cut-off value= 0.5). B) A closer inspection on the N-terminal residues present in L-TIM, but not in S-TIM, reveals the presence of two Serines (marked in blue), but only the one at position 22 exhibited a significant P-score (positive prediction).

B



Serine predictions

Name	Pos	Context	Score	Pred
TIMELESS	2	---MSRVRQ	0.009	.
TIMELESS	22	DKVKSVMDW	0.993	*S*
TIMELESS	35	PQLYSAFSS	0.029	.
TIMELESS	38	YSAFSSLGC	0.016	.
TIMELESS	39	SAFSSLGCL	0.117	.
TIMELESS	87	QNVRSDLIP	0.013	.
TIMELESS	104	AVLESVIRI	0.451	.
TIMELESS	121	ECLFSVDVM	0.008	.
TIMELESS	145	LLYTSKEAF	0.923	*S*

Figure 5.2. (continued)

There is a possibility that the N-terminal sequence could be modulating protein function by intrinsic properties of its amino acid composition. It has already been demonstrated that very small sequence fragments, often referred to as short linear motifs (i.e. SLiMs), can function as microdomains of fundamental importance in many biological systems (Neduva and Russell, 2006; Edwards *et al.*, 2007). These motifs are usually found in very flexible and unstable structures of the protein, also known as disordered regions, which seem to be involved in protein and DNA binding

(Ishida *et al.*, 2007). Interestingly, phosphorylation sites can be frequently found in these regions (Lakoucheva *et al.*, 2004).

To search for the presence of disordered regions, the N-terminal sequence was analysed by several prediction methods through a meta-approach (Figure 5.3) (Ishida *et al.*, 2008). By integrating the outputs from six different algorithms, which are based on different scoring matrices, a better prediction should be obtained. My results revealed that the first 4 residues (MSRV) had a significant disorder score. Although the first serine has not been initially implicated as a putative phosphorylation site, its presence in the disordered region was noted.

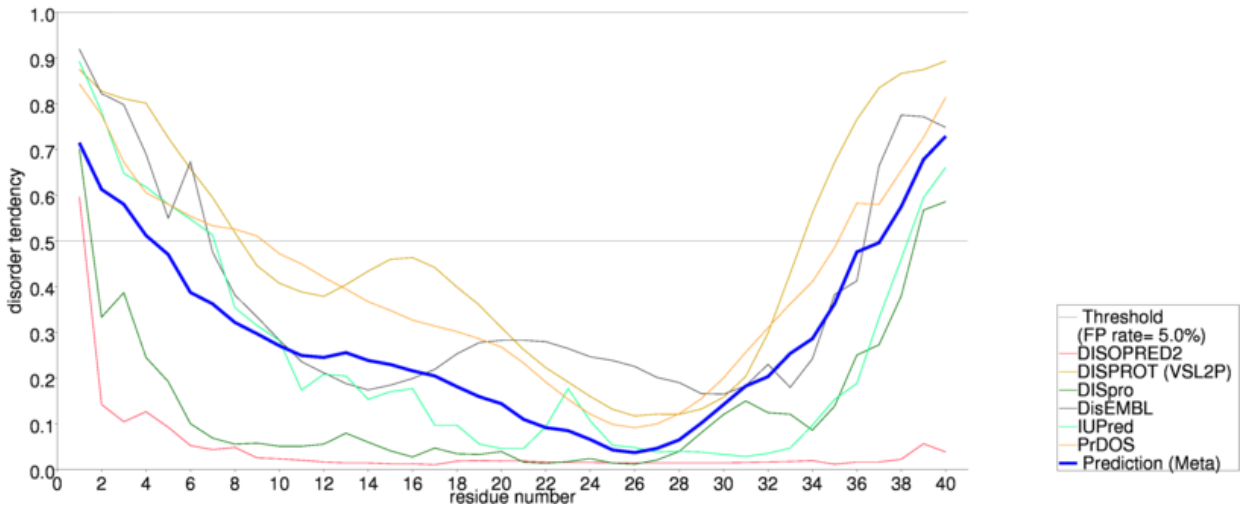


Figure 5.3. Meta-analysis of protein disorder in TIM N-terminal sequence. The plot represents the disorder tendency score for each residue according to different prediction methods (represented inside the right-hand box). The meta-prediction (metaPrDOS), in blue, integrates the results from all the methods. The false positive rate was set to 5%.

5.3.2. The experimental design

In order to perform structure/function analyses on L-TIM, I used a site-directed mutagenesis approach (Figure 5.4) to selectively manipulate some or even the whole 23 residues of the N-terminal sequence. The engineered TIM proteins can be subdivided in the following classes: "phosphorylation mutants", "sequence/length mutants", "G24 mutants" and "wild-type controls" (Figures 5.5 and 5.6). The effects promoted by each of the recombinant constructs were studied in yeast, by interaction assays with CRY, and in flies, by testing their circadian photoresponsiveness.

The "wild type controls" consist of three different constructs (Figure 5.5A). The first, L-TIM, corresponds to the *ls-tim* translated sequence. In the second, S-TIM (or simply S), the upstream *ATG* codon was substituted for a glycine codon (G), rendering the construct unable to generate the long isoform. This "artificial" S-TIM construct was successfully used in previous studies (Tauber *et al.*, 2007; Sandrelli *et al.*, 2007). The last control construct, SS-TIM (or SS), recreates the *s-tim* natural polymorphism, which produces the short isoform, S-TIM, and also a putative truncated N-terminal peptide of 19 residues. The functional comparison between both S-TIM constructs should provide an elegant way to analyse the role, if any, of this short N-terminal peptide.

The "phosphorylation mutants" includes a series of constructs to investigate if this type of post-translational modification event accounts for the phenotypic effects (Figure 5.5B). Although my *in silico* analyses have revealed significant scores for only one serine, I decided to include both in this study. Thus, the serines 2 and 22 were either substituted by an alanine (A), which avoids phosphorylation, or an aspartic acid (D), which mimics negatively charged phosphate residues (Basu *et al.*,

2003; Lin *et al.*, 2005; Ko *et al.*, 2010). Should I observe that the alanine (A) constructs drive any phenotype resembling that of S-TIM or SS-TIM controls, then I could conclude that the phosphorylation motif is indeed relevant. The opposite result is expected for the aspartic acid (D) constructs, which should reveal phenotypic similarities to the L-TIM control.

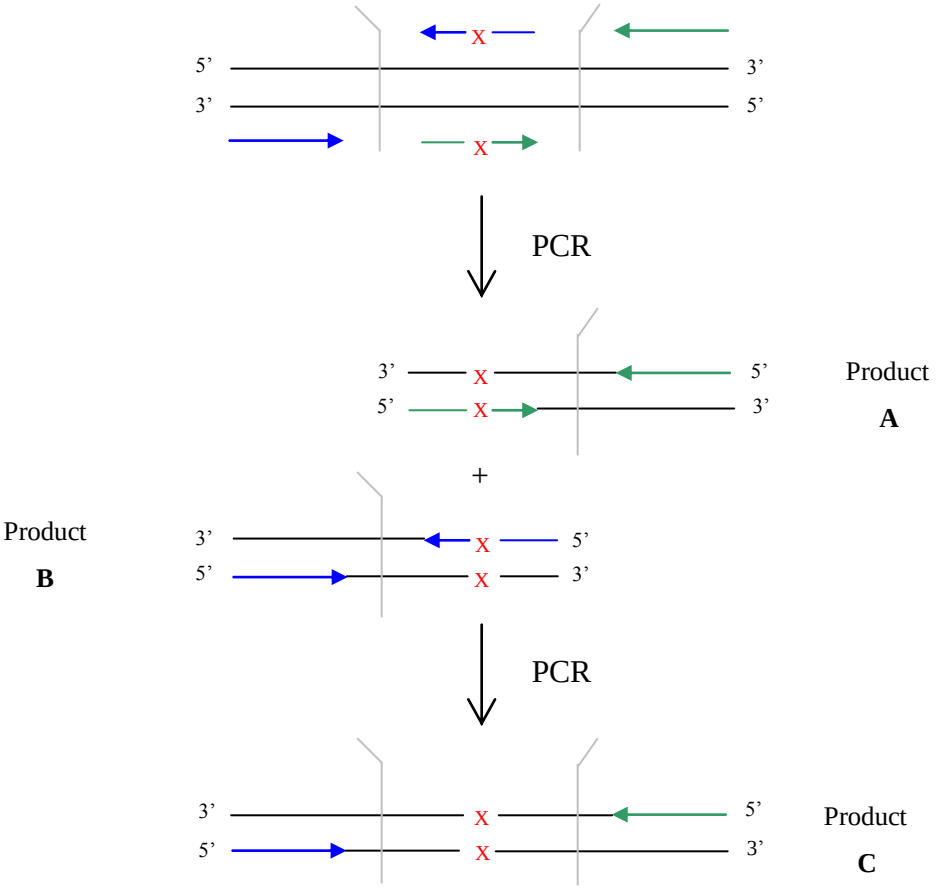


Figure 5.4. Schematic representation of a PCR-based site-directed mutagenesis assay. Initially, independent PCR reactions are performed, using different pairs of primers (illustrated in blue or green). The products of both reactions, A and B, are then mixed together to form a template for a new PCR round, which uses only the distally located primers. At the end, the resulting DNA molecule is identical to the original except for the mutation introduced by the primers (red X), and can be easily cut out at strategically located restriction sites (grey bars) and cloned into a host vector.

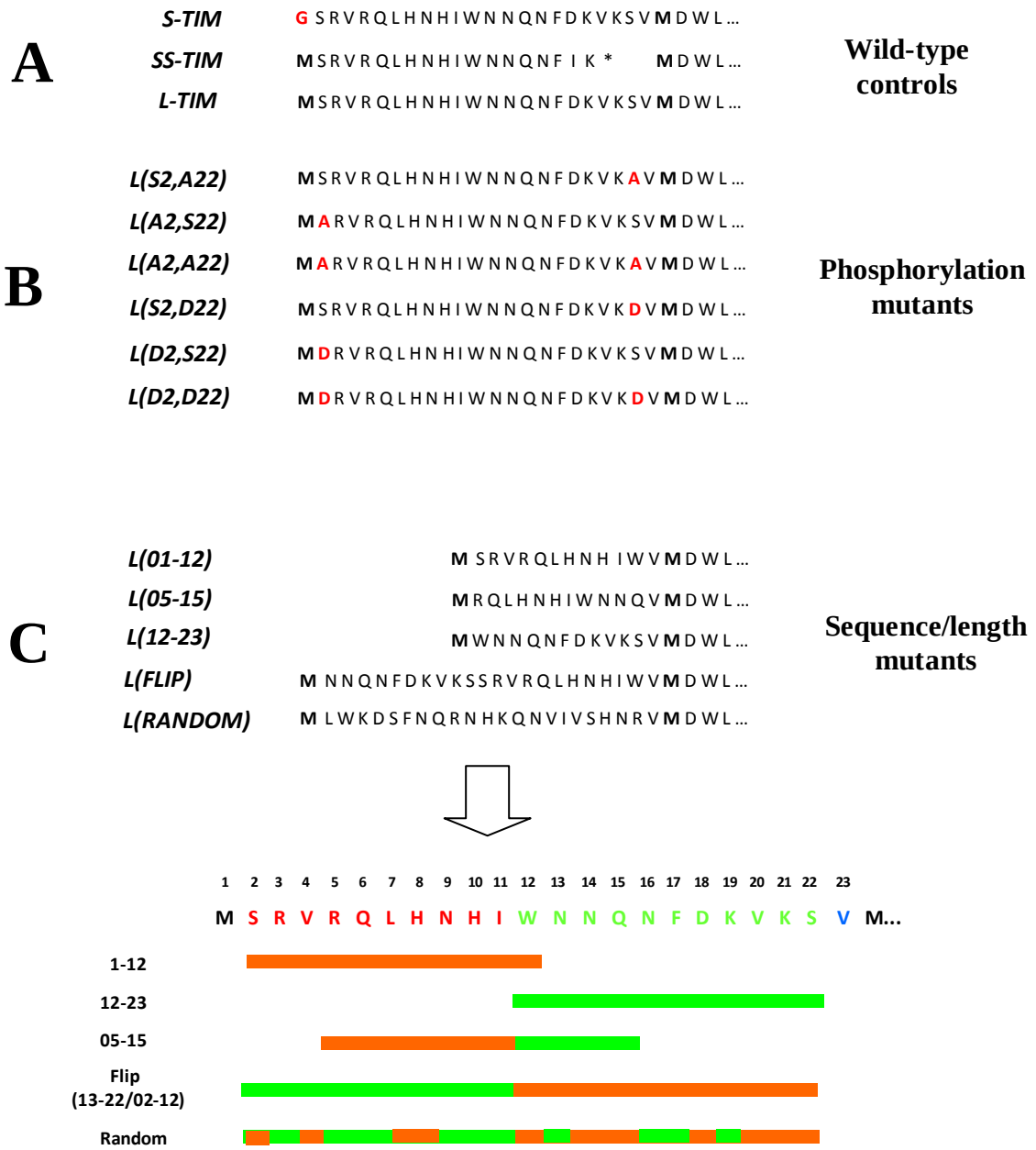


Figure 5.5. Mutagenized TIM constructs. A) Wild-type controls, B) Phosphorylation mutants, C) Sequence/length mutants. Manipulated residues are shown in red. The lower panel illustrates the experimental design for obtaining the sequence/length mutants.

The "sequence/length" mutants were designed to investigate the alternative hypothesis that the additional 23 amino acids are modulating TIM function

independently of post-translational modifications (Figure 5.5C). To check if the length of the N-terminus is an important parameter, I divided the 23 extra residues in three overlapping sequences, of 13 amino acids each, and cloned them in separate constructs: L(01-12), L(05-15) and L(12-23).

To check if the original sequence is important, the length was maintained but the order of the residues was altered in two different ways. While in one construct, L(FLIP), the two halves of the sequence were flipped, maintaining part of the linear structure, in the other, L(RANDOM), the order of the residues was completely shuffled. Importantly, the "sequence/length" manipulation preserved the methionine at position 1, in order to provide a translational start for the recombinant proteins, and the valine at position 23, so as to avoid the disruption of the Kozak sequence. In *D. melanogaster* and other eukaryotes, the Kozak consensus includes not only the start codon, but also some nucleotides located upstream, with special attention to the one at position -3 (relative to the translation start, +1) (Cavener, 1987; Cavener and Ray, 1991; Kozak, 1986). If mutations were unwittingly introduced into the Kozak sequence preceding the second methionine (i.e. the S-TIM start), then we could have altered the expression of this isoform, with profound consequences on our analyses.

Recapitulating a recent study by Tauber and co-workers (2007), I also generated a set of constructs where the methionine at position 24 (i.e. S-TIM start) was substituted for a glycine (G) (Figure 5.6), which results in the exclusive translation of the L-TIM isoform. Using this "artificial" L-TIM background, the putative phosphorylation site at serine 22 was again mutated to an alanine or aspartic acid. One should expect that these alternative "phosphorylation mutants", referred to as "G24 mutants" hereafter, may show a more marked phenotype than those described initially, which still keep the S-TIM translation intact. However, due to the fact that

my preliminary results in yeast (see Figure 5.9) demonstrated that this level of manipulation could lead to experimental artefacts, I decided to abandon this strategy. Moreover, the phenotypic studies on a genetic background that also produces S-TIM is more realistic, as in nature there is no allelic variant that generates L-TIM exclusively.

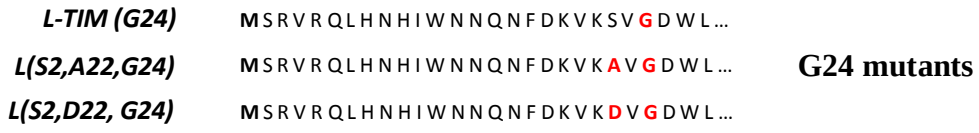


Figure 5.6. G24 mutants. The S-TIM translation start (methionine at position 24) was mutated to a Glycine (G) to create a construct that translates only the L-TIM isoform. This "artificial" background was used for further mutagenesis of a putatively phosphorylated serine (position 22), which was either replaced with alanine (A) or aspartic acid (D). The manipulated residues are marked in red.

Although very similar, and sharing an identical TIM coding sequence, the final constructs for fly and yeast studies differ in some aspects. For the fly work, the mutant DNA was cloned downstream of a *tim* promoter fragment, which was shown to rescue wild-type behavioural rhythms of tim^{01} flies (Ousley *et al.*, 1998; Wang *et al.*, 2001). As a result, the transgenic flies can alternatively translate both long and short isoforms of the protein, as do the wild-type. In addition, the fly constructs contain an *intron* (at the 3' end), which is retained at cold temperatures (Boothroyd *et al.*, 2007). This modification was adopted since the transgenic flies would be analysed in future diapause experiments, which are done at temperatures close to 12°C. I also generated

a fly line in which the *pattB* vector was transformed without any sequence (i.e. empty), which would provide an ideal tim^{01} control for the behaviour experiments. For the yeast studies, the TIM sequences are expressed as fusions to the LEXA or B42 domains, which provide the N-terminus for the recombinant proteins. In other words, unlike the fly ones, the yeast constructs are unable to generate two isoforms of the protein. For this reason, the SS-TIM control, which carries a stop codon in between the translation starts, could not be tested in the yeast system.

Altogether, the mutagenized constructs should contribute to a better understanding on how the N-terminal sequence modulates TIM function, either by the presence of a functional phosphorylation site or by intrinsic properties of the sequence, such as its length or amino acid composition.

5.3.3. CRY-TIM dimerization in yeast

To initiate the structure/function analyses, I used the yeast two-hybrid system to measure the interaction between CRY and the various N-terminal mutated versions of TIM (Figure 5.7). In this system, originally proposed by Fields and Song (1989), the interaction between two proteins can be studied by fusing one of them to a DNA binding domain (e.g. LexA) and the other to an activation domain (e.g. the peptide B52). If both the candidate factors, such as CRY and TIM, form a protein complex, them the activation domain will interact with the DNA binding domain promoting the expression of a reporter gene (e.g. *lacZ*), which can be encoded on another plasmid or integrated into the genome of the yeast host strain. Importantly, the reporter response (i.e. *lacZ* expression level) generally correlates well with the strength of interaction, which in practical terms can be observed as the intensity of the blue staining in X-

GAL plates. With respect to the experimental design, one should also consider that wild-type CRY and TIM should only interact upon light stimulation (Ceriani *et al.*, 1999). For this reason, each sample was analysed in both light and dark conditions, the latter representing a negative control.

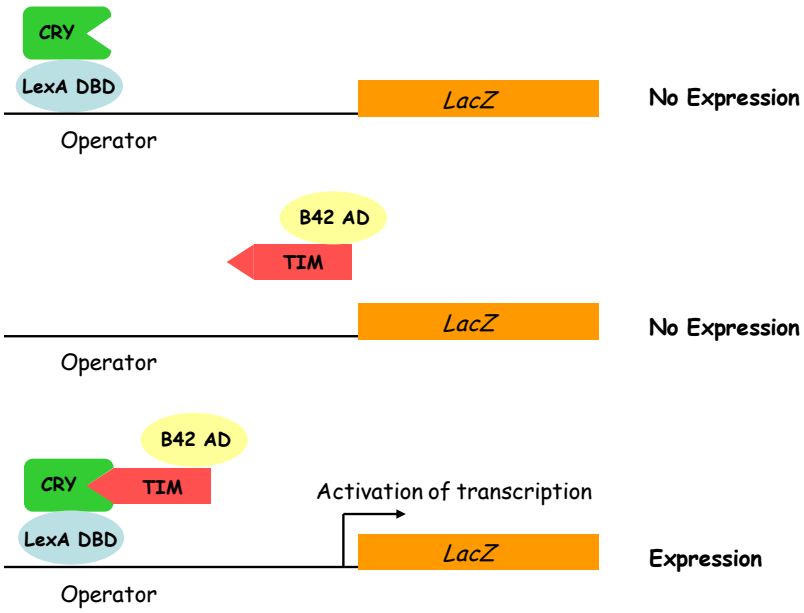


Figure 5.7. Yeast Two-Hybrid method. In the scheme, the expression of a reporter gene (*lacZ)* is only activated by the interaction of CRY and TIM, which are fused to the LexA DNA binding domain and the B42 activation domain, respectively. The dimerization strength between both factors correlates well with the expression levels of the reporter.

This approach was successfully used in a recent study to demonstrate that CRY's interaction with L-TIM is weaker than with S-TIM (Sandrelli *et al.*, 2007). That could possibly lead to a lower rate of TIM degradation upon light-stimulation, thereby providing an elegant explanation for the reduced photoresponsiveness of flies expressing the long isoform. Indeed, L-TIM appears to be more stable than S-TIM in fly heads (Sandrelli *et al.*, 2007; Peschel *et al.*, 2006, 2009).

As a complement to these studies, the molecular analysis of the N-terminal TIM mutants shall inform whether particular residues or sequence fragments are mediating the reduced interaction between L-TIM and CRY. Since post-translational modification events are very important for protein function, and appear to be widespread among clock components, my functional analyses initially focused on the "phosphorylation mutants" (Figure 5.8; Appendix Figure A1-1). Importantly, my results showed a pronounced difference between L-TIM and S-TIM controls, corroborating the previous finding (Sandrelli *et al.*, 2007) and indicating that the interaction assay was working. When the serine (S) at position 22 was substituted by an alanine (A), to avoid phosphorylation, or an aspartic acid (D), to mimic it, no apparent effect was observed in the strength of interaction with CRY (i.e. blue staining). A similar result was also obtained when the serine at position 2 was manipulated, although it appears that the alanine mutant (A2) exhibits a slightly stronger staining than the L-TIM control. The lack of a more substantial effect could be explained if the two serines are functional and the isolated effect promoted by each one might not be strong enough to detect. To overcome this issue, I also analysed constructs where both serines were manipulated simultaneously (Figure 5.8; Appendix Figure A1-1). However, this approach was ineffective, with the alanine (A2A22) and the aspartic acid (D2D22) constructs showing a comparable staining to the L-TIM control.

Following the phosphorylation hypothesis, a similar analysis was performed with another set of constructs: the "G24 mutants" (Figure 5.9; Appendix Figure A1-2). Surprisingly, in the "G24" background, the manipulation of the serine at position 22 (S22) significantly changed the interaction with CRY. Both alanine (A22) and aspartic acid (D22) constructs clearly exhibited a stronger staining than the L-TIM

control, though not as strong as S-TIM. This result was unexpected and does not fit the initial hypothesis, since the mutation D22 should mimic the effect of L-TIM by showing a weak staining. Nonetheless, the data clearly show that the combined manipulation of residues 22 and 24 clearly affects TIM protein function, indicating that those might indeed play an important role in CRY-TIM dimer formation. In face of these results, I decided not to include the glycine at position 24 in all the other mutagenized constructs described here.

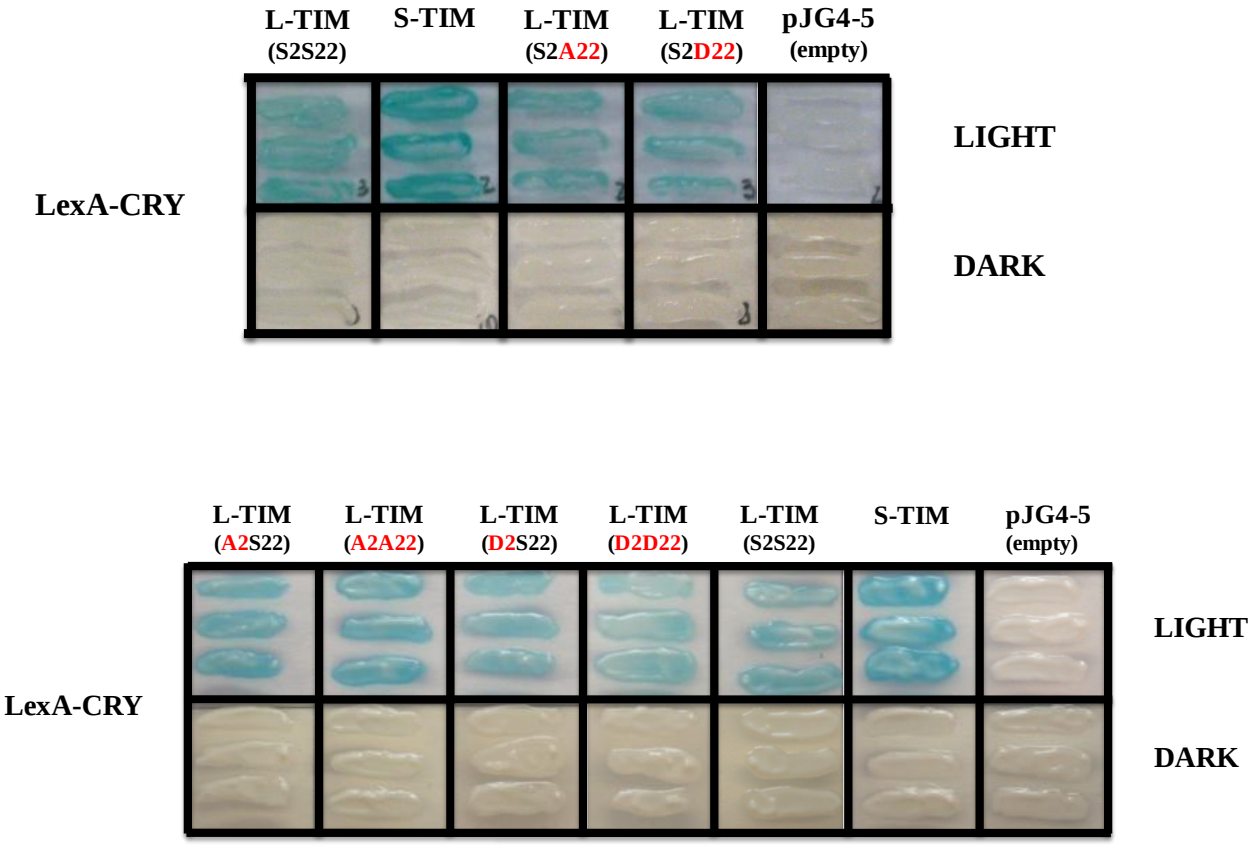


Figure 5.8. Yeast Two-Hybrid assay: "Phosphorylation mutants". Putative phosphorylation sites at serines (S) number 2 and 22 were mutated to alanines (A) or to aspartic acids (D) (mutations are shown in red). These mutant proteins were fused with the activation domain in pJG4-5 and challenged with LexA-CRY, under light or dark conditions (no interaction). pJG4-5, empty vector (negative control).

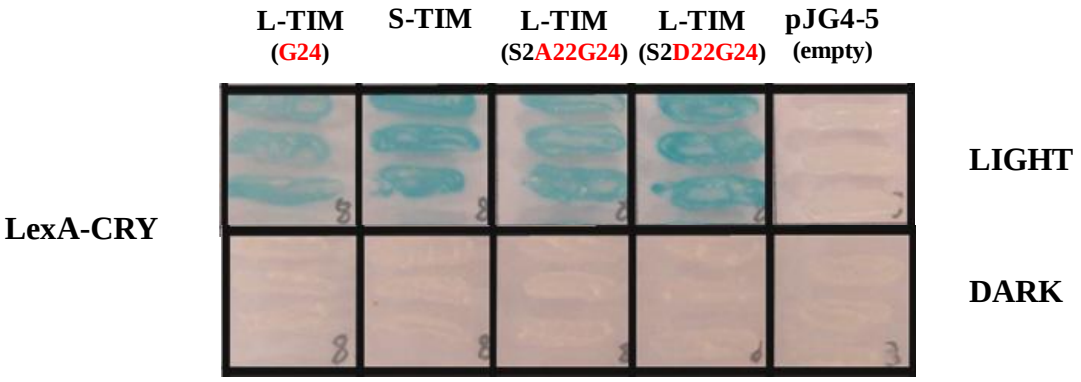


Figure 5.9. Yeast Two-Hybrid assay: "G24 mutants". A Putative phosphorylation site at serine (S) number 22 was mutated to an alanine (A) or to an aspartic acid (D), on a genetic background where the methionine (M) 24 was replaced with a glycine (G) (mutations are shown in red). These mutant proteins were fused with the activation domain in pJG4-5 and challenged with LexA-CRY, under light or dark conditions (no interaction). pJG4-5, empty vector (negative control).

To investigate the alternative hypothesis to phosphorylation, I performed an interaction assay with the "sequence/length mutants" (Figure 5.10; Appendix Figure A1-3). My results revealed that when the length of the N-terminus was shortened to include 13 out of 23 residues, an increase in the interaction strength was observed. That was particularly true for the construct L(05-15), suggesting that the amino acid composition also may also play an important part. In fact, if the length was kept intact and the sequence was partially or completely shuffled, such as in L(Flip) and L(Random) constructs, respectively, the interaction with CRY was again enhanced. Altogether, the results suggest that both the order of the residues and the length of the N-terminal fragment are important parameters that modulate TIM protein function.

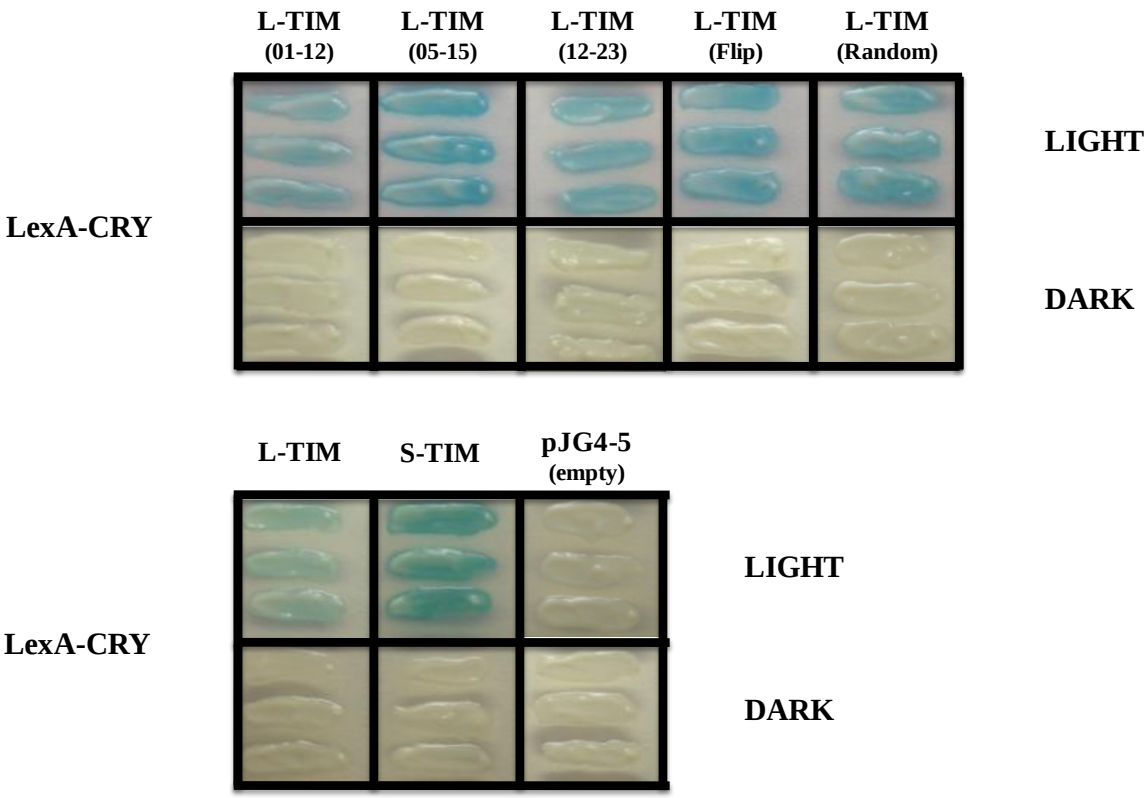


Figure 5.10. Yeast Two-Hybrid assay: "Sequence/length mutants". The additional N-terminal fragment of L-TIM was manipulated to reduce its length or change the order of its residues. The mutant proteins were fused with the activation domain in pJG4-5 and challenged with LexA-CRY, under light or dark conditions (no interaction). pJG4-5, empty vector (negative control).

5.3.4. Circadian light sensitivity in transgenic flies

Site-directed transgenesis

The molecular studies in yeast were extended to the behavioural level in flies. To achieve a suitable background for behavioural studies, the transgenic flies expressing the N-terminal engineered TIM constructs were generated according to a

system developed by Bischof *et al.* (2007), which is based on the bacteriophage ΦC-31 integrase. This method, unlike the random transposon-mediated transgenesis (e.g. through *P*-element, *piggyBac*, etc), places the transgenes at the same position and most importantly under the same genomic context of regulatory elements (Venken and Bellen, 2007). Consequently, the structural and functional analyses would not only be facilitated by the reduced number of transgenic lines (one for each construct), but also would be much more reliable since the expression levels of every transgene should be comparable.

The system proposed by Bischof *et al*. (2007) includes a collection of 25 "landing sites" spread all over the Drosophila genome (Figure 5.11A). Each one of these lines hosts a transposon carrying an *attP* site, which could be used as substrate for a recombination event with an *attB* sequence present in the transformation vector (e.g. our TIM constructs). This recombination event creates two hybrid sites, *attL* and *attR*, which are themselves refractory to the ΦC-31 integrase (Figure 5.11B). Importantly, all the 25 "landing sites" map to intergenically located sequences and are homozygous viable. Thus, the possibility of mutating or affecting the regulation of any other gene is greatly reduced when compared to conventional *P*-element transformation. Among all the "landing sites" available, the one referred to as 86Fb (i.e. with an *attP* located at cytosite 86F) was selected, since it has been shown drive strong gene expression when assayed with a *lacZ* reporter (Johannes Bischof, personal communication).

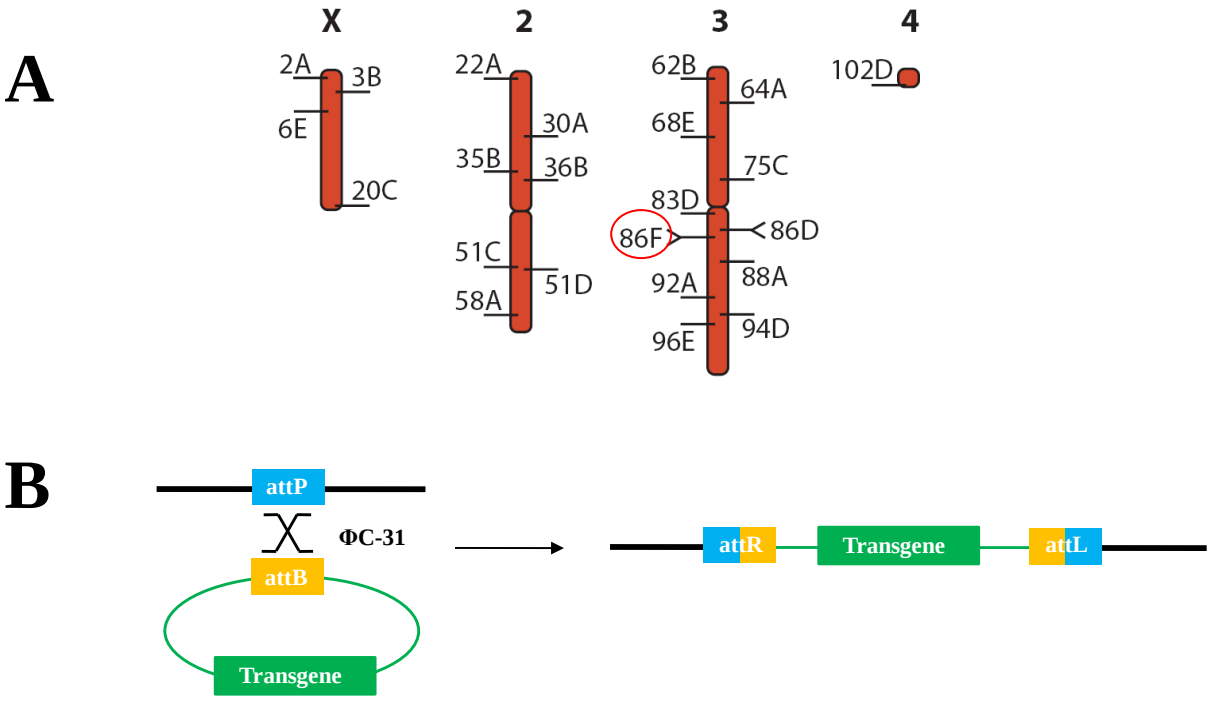
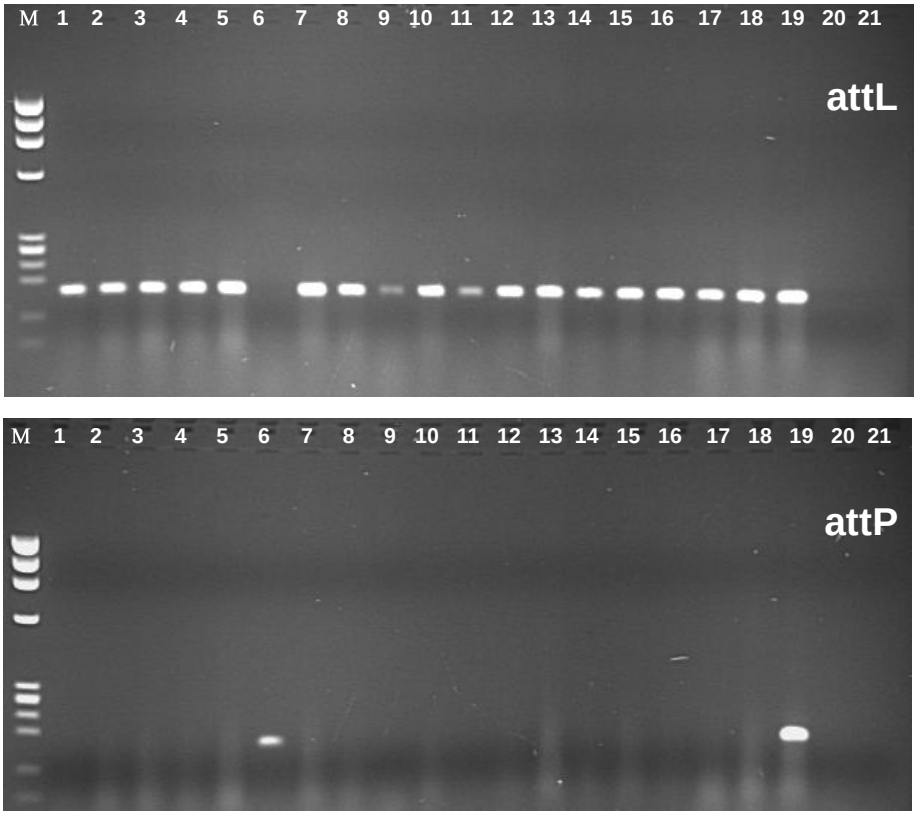


Figure 5.11. ΦC31-mediated site-directed transgenesis. A) FlyC31 collection of *attP* landing sites (Bischof *et al.*, 2007). The cytological location of each *attP* is marked. The site chosen to host the *tim* constructs is marked in red. B) Recombination scheme. In the presence of ΦC31 integrase, a plasmid carrying an *attB* sequence and a transgene (i.e. *tim* constructs) recombines, irreversibly, with an *attP* sequence present in the host line, creating two new sites: *attR* and *attL*.

Molecular confirmation of the integration sites

Although the ΦC-31 mediated site-directed transgenesis is a highly efficient technique (Bischof *et al.*, 2007), there are some pseudo *attP* sites across the *Drosophila* genome which could lead to non specific recombination (Groth *et al.*, 2004). For this reason, before I started the functional studies, the transgenic lines were checked by PCR for correct integration events (Figure 5.12), which are characterized by the loss of *attP* (specific for the original host line) and the appearance of both *attL*

and *attR* sequences. My results revealed that, except for SS-TIM, all the transgenes targeted the right genomic site. That implicates that the SS-TIM construct might not be suitable for my functional analysis, given the possibility of position effects.



1: L-TIM	8: S-TIM	15: L(A2,A22)
2: L(S2,A22)	9: L(01-12)	16: L(D2,S22)
3: L(S2,D22)	10: L(05-15)	17: L(D2,D22)
4: L(S2,A22,G24)	11: L(FLIP)	18: pattB(empty vector)
5: L(S2,D22,G24)	12: L(RANDOM)	19: attP-86Fb
6: SS-TIM	13: L(12-23)	20: w[1118]
7: L-TIM(G24)	14: L(A2,S22)	21: H_2O (no DNA)

Figure 5.12. Molecular confirmation of the site-directed transgenesis by PCR. All the transgenic lines were checked for a correct integration event, which is characterized by the loss of *attP* and gain of *attL* sequences at the landing site (cytosite 86F). Lanes 01-17: *tim* transformants; 18: *pattB* transformant (empty vector) ; 19: original host line, attP-86Fb. M, molecular marker ΦX Hae III.

Placing the transgenes on the tim^{01} background

Following the confirmation of correct integration events, the transgenic constructs were transferred to the arrhythmic *tim^{01}* background. The crossing scheme was designed in order that the 1st (or X) and the 2nd chromosomes were "Houtenized" and the 3rd (which is where all of the transgenes were inserted) was kept intact in the original laboratory background (Figure 5.13). By doing that, the phenotypic effect promoted by each transgene was very well controlled, with any possible influence of background greatly reduced.

My results revealed that all transgenic constructs were able to rescue the free-running behavioural rhythms of *tim^{01}* mutants (Table 5.1), indicating that the recombinant TIM proteins were indeed functional. The rescue efficiency ranged from 93%, in L(S2,D22,G24), to 77%, in SS-TIM. Corroborating previous findings (Tauber *et al.*, 2007), the average period values of S-TIM and L-TIM controls were about the same. However, when compared to L-TIM, the following constructs appeared to have approximately 1 h shorter periods (Table 5.1): SS-TIM, L(D2,S22), L- TIM (G24), L(S2,A22,G24), L(S2,D22,G24), L(12-23) and L(Random). The shortest period was observed for the construct SS-TIM, but one cannot attribute this effect to the construct alone, since this transgene targeted a different genomic position.

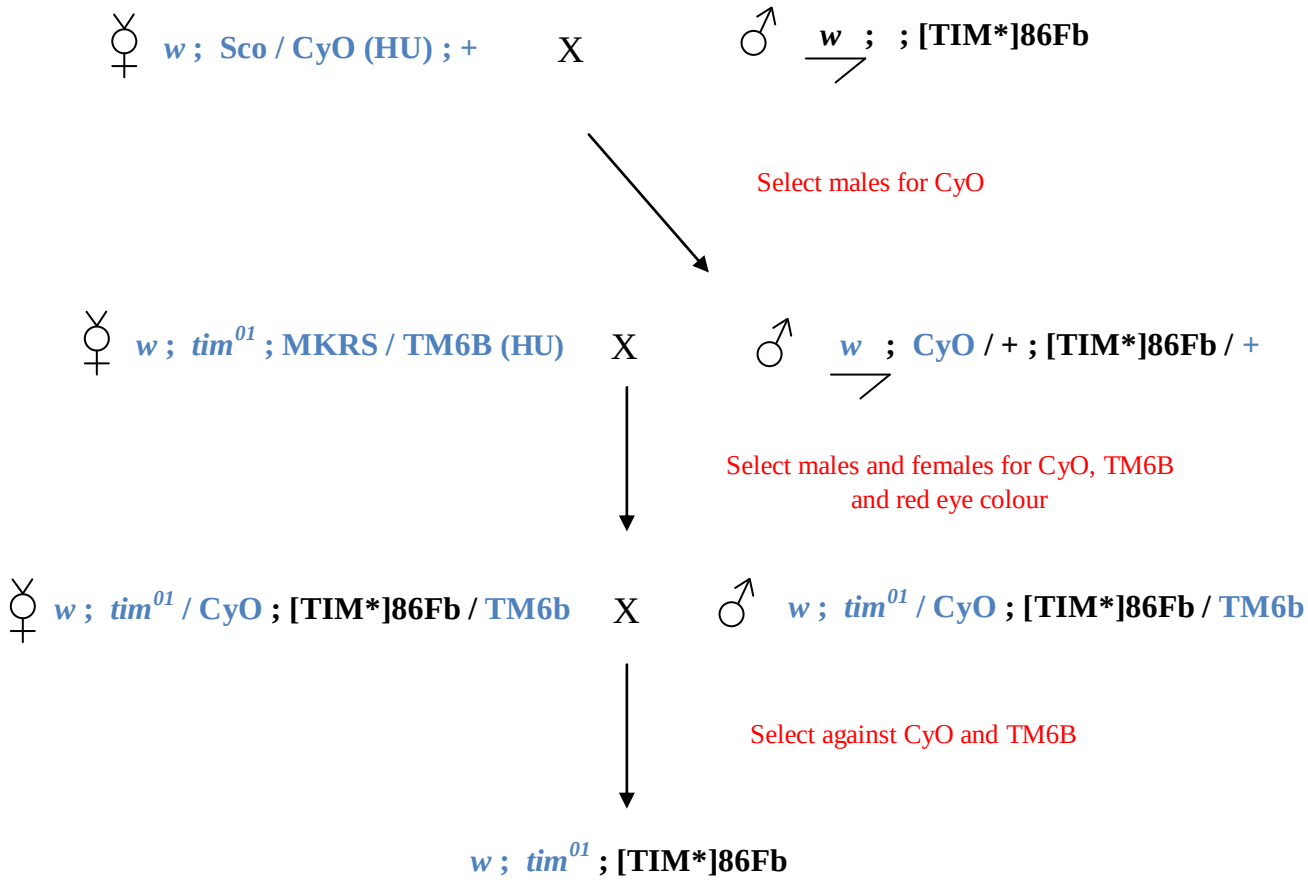


Figure 5.13. Placing the transgenes on a "Houtenized" *tim*^{01} genetic background. Represented in blue, "Houtenized" chromosomes (Chapter 4), and in black, chromosomes from the laboratory background.

Table 5.1. Rescue efficiency and free-running rhythms of the transgenic *tim* constructs.

Construct	N	Rhythmic flies (%)	Mean period (± SEM)	ANOVA
SS-TIM	53	77	22.52 ± 0.09	$F_{1, 86}$=148.97, $P<10^{-6}$
S-TIM	54	81	23.83 ± 0.06	$F_{1, 89}$=6.45, P=0.01
L-TIM	51	92	24.11 ± 0.09	------
L(S2,A22)	56	79	23.64 ± 0.08	$F_{1, 89}$=14.78, $P<10^{-3}$
L(A2,S22)	55	87	23.86 ± 0.09	$F_{1, 93}$=3.60, P=0.06
L(A2,A22)	51	86	23.88 ± 0.10	$F_{1, 89}$=2.91, P=0.09
L(S2,D22)	53	92	23.84 ± 0.08	$F_{1, 94}$=4.95, P=0.03
L(D2,S22)	51	84	23.20 ± 0.12	$F_{1, 88}$=37.83, $P<10^{-6}$
L(D2,D22)	54	91	23.59 ± 0.07	$F_{1, 94}$=21.20, $P<10^{-6}$
L- TIM (G24)	54	80	22.94 ± 0.09	$F_{1, 88}$=85.06, $P<10^{-6}$
L(S2,A22,G24)	48	85	23.06 ± 0.09	$F_{1, 86}$=63.51, $P<10^{-6}$
L(S2,D22,G24)	55	93	23.14 ± 0.06	$F_{1, 96}$=80.70, $P<10^{-6}$
L(01-12)	61	89	23.59 ±0.06	$F_{1, 99}$=24.25, $P<10^{-6}$
L(05-15)	54	91	23.90 ± 0.05	$F_{1, 94}$=3.96, P=0.005
L(12-23)	56	87	23.29 ± 0.06	$F_{1, 94}$=55.41, $P<10^{-6}$
L(Flip)	48	92	23.72 ± 0.08	$F_{1, 89}$=10.45, P=0.002
L(Random)	54	89	22.96 ± 0.08	$F_{1, 93}$=89.39, $P<10^{-6}$
tim^{01}	61	7	Arrhythmic	------

Phase-shift analysis

To study the circadian light sensitivity of the N-terminal mutagenized constructs, flies were given a brief pulse of light (10 minutes) at ZT15 and the resulting phase-shifts were calculated (Figure 5.14). Subsequently, the responses obtained from each genotype were compared to the controls, L-TIM and S-TIM, and the effects observed were analysed with ANOVA.

Since the analyses presented here strongly depend on the phenotypic differences between the controls, I decided to focus on this group of transgenes initially (Figure 5.14A). As expected, the statistical analysis revealed that the phase response of L-TIM is significantly smaller than S-TIM ($F_{1,94}$= 5.73, P= 0.019) or SS-TIM ($F_{1,94}$= 53.28, $P < 10^{-6}$). Interestingly, SS-TIM showed a larger response than S-

TIM ($F_{1,92}$= 10.93, P = 0.00135), which might implicate a role for the truncated 19-residue peptide, only present in the first. However, one cannot discard the possibility that this is merely position effect and, therefore, the SS-TIM control was removed from the subsequent analyses.

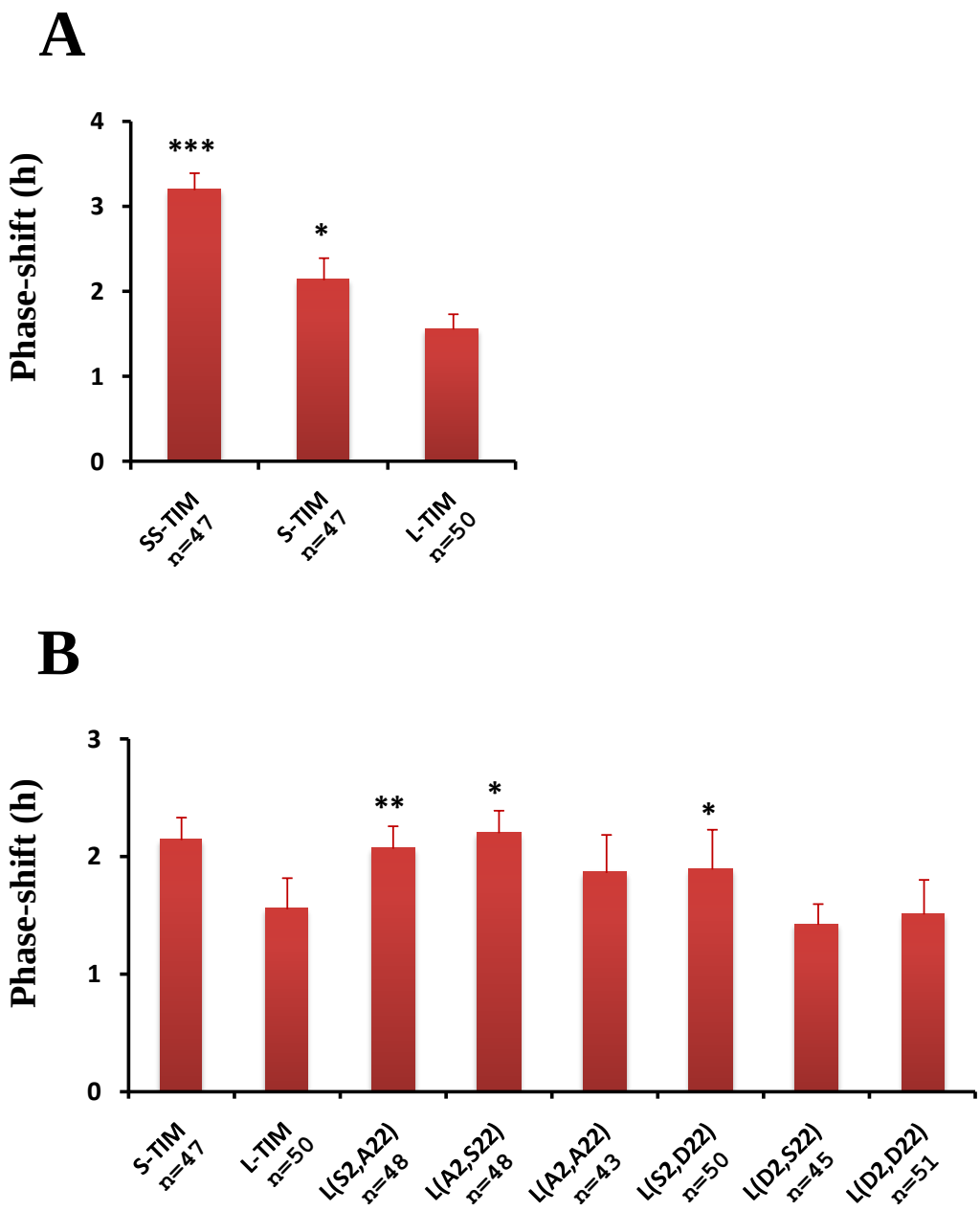


Figure 5.14. Phase response of transgenic *tim* constructs at ZT15. A) "Wild-type controls". B) "Phosphorylation mutants". C) "G24 mutants" D) "Sequence/length mutants". The data

represent mean phase-shifts (hours ± SEM) to 10-min light pulses delivered at ZT15. The black asterisks represent significance levels compared to the construct L-TIM, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. The red asterisk represent marginally significant effect, $P \sim 0.05$. The numbers of flies tested (n) are represented under the labels of each genotype.

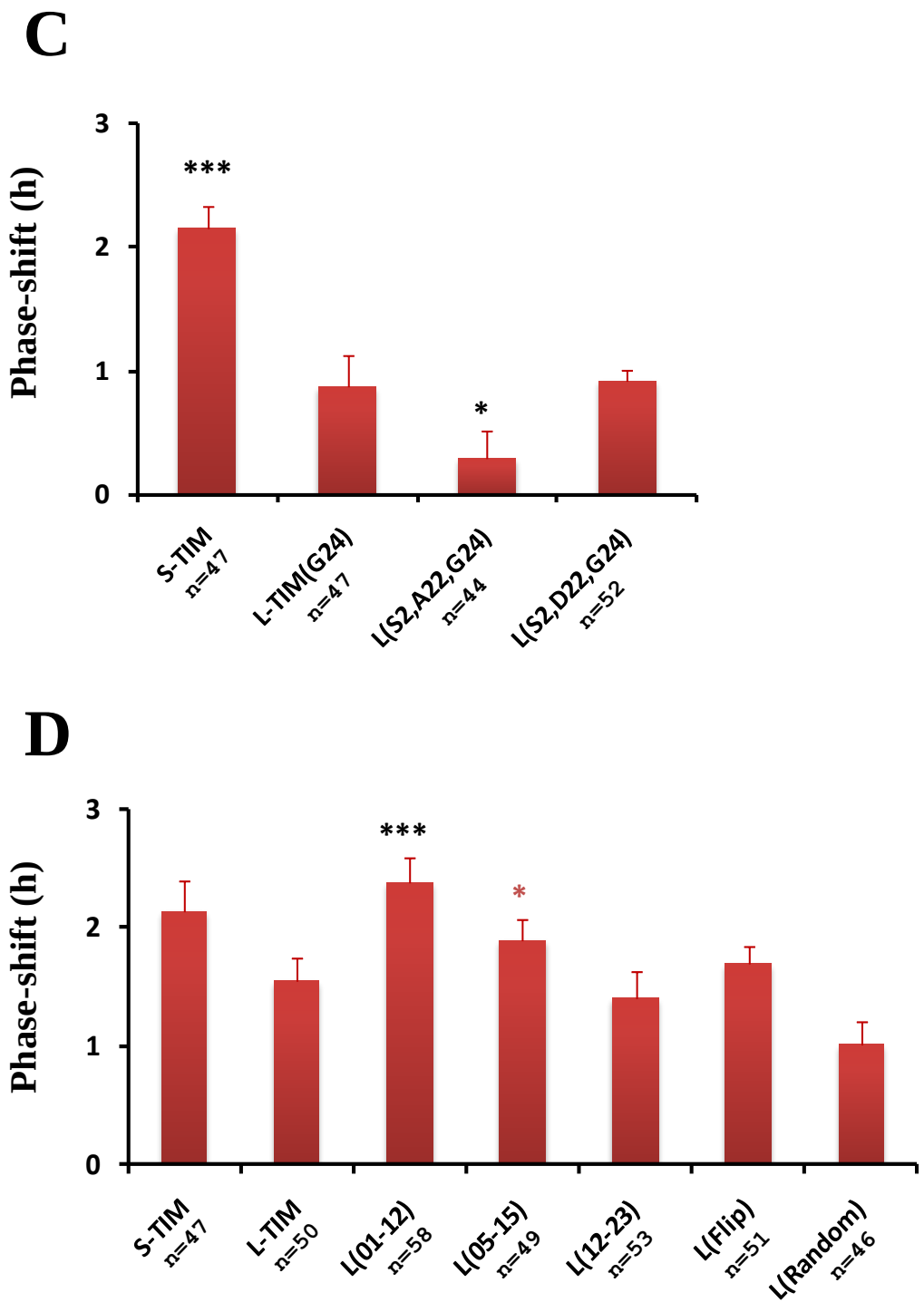


Figure 5.14. (continued)

The analysis of the "phosphorylation mutants" (Figure 5.14B) revealed that, when compared to the L-TIM control, a significant increase in the phase response was obtained for the constructs L(S2,A22) ($F_{1,95}$=7.06, P=0.00923), L(A2,S22) ($F_{1,95}$=4.73, P=0.03207) and L(S2,D22) ($F_{1,97}$=4.19, P=0.04332). As for the other constructs, L(D2,S22), L(D2,D22) or L(A2,A22), no significant difference from the control L-TIM was found. With respect to the alanine constructs, these data indicate that avoiding putative phosphorylation events on serines 2 or 22 result in an increase of phase-shift, resembling the S-TIM phenotype. However, this effect only happened when one position was manipulated. In other words, when both serines were simultaneously replaced by alanines, no increase in the phase response was seen. The same contradictory response can be observed with the constructs carrying aspartic acid, which should function as a phosphomimetic and resemble the L-TIM phenotype. Although no effect was found for two of these constructs, including the one in which both serines were manipulated, an unexpected increase in the phase-shift was observed when position 22 was manipulated alone. Therefore, it appears that the residues occupying positions 2 and 22 are playing an important role in the modulation of the phase-shift, but in an unpredictable way. And since the data were not consistent, it was impossible to define a putative role for phosphorylation.

The phase response of the "G24 mutants" was also analyzed (Figure 5.14C). My results revealed a difference between the controls, with the L-TIM(G24) construct exhibiting a significantly lower phase delay than S-TIM ($F_{1,92}$=13.59, P=0.00038). That is to be expected since nearly identical TIM constructs have already been tested in a recent study, coming to the same conclusion (Sandrelli *et al.*, 2007). The mutation of serine 22 to an aspartic acid did not induce any effect, whereas the mutation of the same residue to an alanine resulted in a slightly smaller response ($F_{1,89}$=5.47,

P=0.02158*)*. Thus, in "G24 mutants", the putative role of phosphorylation at these sites is minimal.

Should no post-translational modifications events be responsible for the reduced photoresponsiveness of L-TIM, then intrinsic properties of the N-terminal sequence, such as its amino acid composition or its length might be more relevant. To investigate this hypothesis, I compared the phase responses of L-TIM and the "sequence/length" mutants (Figure 5.14D). Interestingly, when the length of the N-terminal fragment was reduced, a highly significant increase in the response was revealed for the transgene L(01-12)($F_{1,105}$=12.48, *P*=0.00061). A marginally significant effect was also observed for the construct L(05-15)($F_{1,96}$=3.68, *P*=0.05808), but not for L(12-23)($F_{1,100}$=0.01475, *P*=0.90359, ns), which exhibited the same response as the control L-TIM. This suggests that reducing the N-terminus length might increase or not the phase-shift, depending on the remaining sequence. However, when the length was kept intact and the order of the residues was altered, such as in L(Flip) or in L(Random), no significant effect was observed. Therefore, it appears that it is not the order of the residues that is important, but its overall composition. It also suggest that those occupying the second half of the N-terminus (positions 12 to 23) are fundamental for alleviating the S-TIM pronounced phase responses.

5.4. Discussion

Recent studies with *tim* have demonstrated that a newly derived polymorphism is spreading throughout Europe by natural selection due to the various adaptive phenotypes it confers (Tauber *et al.*, 2007; Sandrelli *et al.*, 2007). The new variant, *ls-tim*, is characterized by a nucleotide insertion, which recruits an alternative upstream start codon. The outcome is the ability to express not only a short isoform of the protein, S-TIM, which is present in the ancestral allele *s-tim*, but also a longer one, L-TIM. When both alleles were compared in functional studies, *ls-tim* showed a reduced circadian light sensitivity. At the molecular level, this response could be explained by the reduced interaction between CRY and TIM, which ultimately leads to a more stable TIM (Sandrelli *et al.*, 2007). The reduced photoresponsiveness has also been extrapolated to the physiological level to explain the higher propensity of *ls-tim* to enter diapause. Importantly, all these potentially adaptive phenotypes were replicated in transformants that only express L-TIM, confirming that its presence is the determining factor.

In this study, an N-terminal sequence unique to L-TIM, which comprises 23 residues, is investigated for the presence of post-translation modification motifs and other features that could explain the characteristic phenotypes of this isoform. By manipulating one or more residues of this sequence, a number of N-terminal mutant constructs were created for structure/function analyses. The first group, referred to as "phosphorylation mutants", aims to define a functional role for two putative phosphorylation sites, comprising the serines at position 2 and 22. Also belonging to this class are the "G24 mutants", which investigate the role of serine 22 on a background where the methionine 24 was mutated to a glycine, disrupting the S-TIM

translation start. The last group of constructs are the "sequence/length mutants", whose N-terminal sequences were manipulated in order to reduce its size or change the order of its residues. All the constructs were investigated with respect to two phenotypes: CRY-TIM interaction and circadian photoresponsiveness.

Due to its relative success in previous studies (Sandrelli *et al*., 2007; Peschel *et al.*, 2009), the yeast two-hybrid system was the method chosen to analyse the CRY-TIM interaction. The N-terminal engineered TIM mutants were classified according to their strength of interaction, which correlates well with the reporter gene (i.e. *lacZ*) expression. The data from the "phosphorylation mutants" revealed that the manipulation of serines 2 or 22, or even both at the same time, resulted in very subtle or no effect in the interaction with CRY, suggesting that phosphorylation at those residues might not be important for this phenotype. In contrast, when the serine 22 was manipulated on a "G24" background, an increased interaction was observed for the alanine construct, which supports the phosphorylation hypothesis, but also for the aspartic acid, which rejects it. With regards to the "sequence/length mutants", it has been revealed that when the length or the order of the N-terminal residues was altered, a significant increase in the response was observed. Together, these results indicate that L-TIM reduced interaction with CRY is probably not mediated by a particular phosphorylation event. Instead, it appears to depend on the whole N-terminal amino acid sequence.

To investigate the circadian photoresponsiveness, transgenic flies carrying the TIM constructs were given a light pulse at ZT15 and the resulting phase delays were measured. The results with the "phosphorylation mutants" revealed that, when the serines at position 2 or 22 were individually mutated to alanines, significant increases in the phase-shift were obtained, suggesting that a functional phosphorylation site at

those residues might be involved. However, the same effect was not replicated when both residues were simultaneously replaced by alanines, as in the construct L(A2,A22). Moreover, a significant increase in the phase response was also obtained when the serine 22 was changed for the "phosphomimetic" aspartic acid, thus rejecting the hypothesis that phosphorylation explains the reduced L-TIM response. An unexpected result was also found with the "G24 mutant" background, where the replacement of the serine 22 for an alanine resulted in a slight decrease in the phase-shift. In relation to the "sequence/length" mutants, my results revealed that reducing the size of the N-terminal fragment can increase or not the phase response, depending on which region was removed. Interestingly, significant increases in the phase response were observed for the constructs L(01-12) and L(05-15), while no effect was reported for L(12-23). This suggests that the second half of the N-terminal fragment is more important for the L-TIM characteristic phenotype. However, it was also shown that when the order of the residues was partially or completely shuffled, no effect was observed, which contradicts the idea of a fixed sequence driving the phenotype. Therefore, combining the results obtained from all the constructs, it appears that the characteristic phase response of L-TIM can be attributed to the overall composition of the additional N-terminal fragment found in this isoform, with especial attention to residues 12 to 23. In addition, while the order of these residues do not seem to be important, it appears that isolated mutations that change its overall composition might result in unpredictable effects, either increasing or not the phase-shift.

Surprisingly, the functional analyses of the TIM constructs in yeast and in flies do not shown a correlation, suggesting that the original model proposed by Sandrelli *et al.* (2007) in which CRY-TIM interaction defines the amount of TIM degraded and, subsequently, determines the size of the phase-shift is perhaps over simplistic. An

example is the construct L(S2,A22), which showed an increase in the phase response, but not for the CRY-TIM interaction. The opposite applies for the construct L(Random), with an effect for the CRY-TIM interaction but not for the phase shifts. Speculating on these results, one could imagine that the N-terminus of TIM might be modulating not only its interaction with CRY but also with other proteins directly involved in its degradation, such as JET. If this is the case, then N-terminal engineered proteins, such as the ones described here, might interact better with CRY and nevertheless be more stable, or vice-versa.

Alternatively, the discrepancies found between the molecular and the behavioural analyses might be explained simply by the fact these phenotypes were studied in different model systems. It is possible that the interaction assay developed in yeast is not adequate to my studies, since both "prey" and "bait" proteins are expressed as fusions, which might affect protein function. In our particular TIM constructs, the translation start is encoded not by TIM but the activation domain B42. Thus, the N-terminal mutant fragments were not really "N-terminal". In addition, the detection of functional phosphorylated residues could have also been compromised in yeast, which may have a completely different "dictionary" of phosphorylation sites and associated kinases. To test for these possibilities, the CRY-TIM interaction studies could be performed in a more realistic Drosophila system, such as the S2 cell line.

The importance of the N-terminal residues seems to expand beyond its effect on circadian light sensitivity. Some of the constructs exhibited significantly shorter free-running periods, suggesting that the mutant proteins are affecting the pace of the clock. In these cases, it is possible that the N-terminal manipulation is affecting downstream regions of the protein which are known to bind PER (Saez and Young,

1996) and, as a result, the translocation of the complex to the nucleus. Even so, this effect if probably an experimental artefact, since both the L-TIM and S-TIM controls did not show period differences.

An interesting phenomenon was observed with the SS-TIM construct, which showed not only a significantly shorter free-running period but also a pronounced phase response. Since this construct simulates the original *s-tim* polymorphism, these effects could have been promoted by the presence of a truncated 19-residue peptide, which is not found in the S-TIM control. However, it could be also explained by position effects, as this was the only transgene to target a different genomic site and therefore was not reliable for functional studies.

The current structure/function analyses on the N-terminal sequence were not conclusive with respect to the presence of a functional phosphorylation site at serines 2 or 22, since the replacement of these residues for alanines or aspartic acids, which should avoid or mimic phosphorylation, respectively, resulted sometimes in the same phenotype. However, one cannot discard this possibility and should also consider that it might be involved in other phenotypes controlled by TIM, which are independent of CRY. Therefore, it might be important to investigate if the N-terminal sequence is indeed phosphorylated (or not) by mass spectrometry-based methods (Qin and Zhang, 2002). Afterwards, one should focus attention on the identification of the relevant kinase. Commercially purified kinases could be used to challenge an L-TIM peptide fragment in the presence of radioactive γ-labeled ATP. Subsequently, the radioactive phospho-peptides could be purified by thin layer chromatography (TLC) (Lou *et al.*, 1996) and, finally, quantified and detected through the use of a phosphoimager.

To complete the functional studies on the N-terminal sequence, new analyses need to be performed. Regarding the two-hybrid system, my current approach with X-

GAL plates is definitely not very quantitative. Smaller effects, such as those obtained with the "phosphorylation mutants" could have been easily underestimated. So, the same yeast colonies should be also assayed in liquid culture, using ONPG as a substrate and a spectrophotometer to read the β-galactosidase activity. Importantly, it is worth considering that the observed phenotypes may not necessarily be attributed to the strength of CRY-TIM interaction, but to the stability of the TIM protein instead. In other words, a weaker reporter activity in the two-hybrid assays could simply reflect a higher degradation rate of the corresponding TIM construct in yeast. To cancel out this hypothesis, which potentially changes the interpretation of the data, the colonies need to be assayed for TIM expression as well. In relation to the transgenic flies, the mutagenized constructs could be explored a little further to assay TIM light sensitivity *in vivo*, as it was already shown by Sandrelli *et al.* (2007). Western blots with head tissues harvested at ZT1 should give an indication on the stability (or the turnover rate) of TIM mutants following the light stimulus. Contrary to the yeast two-hybrid system, this approach might correlate well with the phase responses observed at the behavioural level. Last but not least, the transformants should also be assayed for the photoperiodic response (diapause), another phenotype which is enhanced in flies expressing the L-TIM isoform. Altogether, these future analyses will complement my current data and conclude an exhaustive analysis on the special and unique N-terminal fragment present in L-TIM, which modulates its function and provides its many adaptive phenotypes.

Chapter 6: CRY binding sites on TIM

6.1. Introduction

The interaction between the core clock components PER and TIM is an essential aspect of circadian rhythmicity in *Drosophila* (Rosato *et al.*, 2006). This interaction precedes the movement of both factors into the nucleus, where they act as negative regulators of their own expression (Saez and Young, 1996; Darlington *et al.*, 1998). Using a deletion-based mapping assay in Schneider 2 (S2) *Drosophila* cells, the sequence fragments that promote PER-TIM interaction, and subsequent nuclear translocation, have been identified (Saez and Young, 1996). A region comprising the PAS A domain of PER (residues 233-365) binds to a sequence that includes the Nuclear Localization Signal (NLS) of TIM (residues 537-610). Both proteins also interact through residues 448-512 of PER, which embrace its Cytoplasmic Localization Domain (CLD), and residues 747-946 of TIM.

In order to obtain some insights about the biochemical and conformational nature of TIM, a number of structure prediction analyses were performed (Vodovar *et al.*, 2002). The output of these analyses led to the identification of two putative domains, comprising residues 1-399 and 559-1029 (numbers are according to the S-TIM isoform), that belong to the Arm/HEAT family. Interestingly, the Arm/HEAT domains were originally described in importins α and β, which are involved in protein transport to the nucleus (Conti *et al.*, 1998; Cingolani *et al.*, 1999). Furthermore, the predicted second Arm domain of TIM falls in the same region that interacts with the CLD of PER and, as a consequence, promotes their translocation to the nucleus.

The PER-TIM regulatory loop is entrained by environmental cycles through light-induced degradation of TIM (Hunter-Ensor *et al.*, 1996; Lee *et al.*, 1996; Myers *et al.*, 1996; Young *et al.*, 1996; Zeng *et al.*, 1996). This event depends on the function of the circadian photoreceptor CRY (Stanewsky *et al.*, 1998; Ceriani *et al.*, 1999). Upon light stimulation, CRY binds and induces a conformational change in TIM, which is then recognized by the ubiquitin ligase JET for subsequent proteasomal turnover (Koh *et al.*, 2006; Peschel *et al.*, 2006, 2009). Despite the various functional studies of CRY and TIM, the protein sequences mediating their interaction are not known. It has been reported, though, that a C-terminal region of CRY is responsible to its light-dependent interaction with TIM and PER (Rosato *et al.*, 2001). The removal of this sequence, in the CRYΔ construct, stimulates the CRY-TIM interaction not only in light, but also in dark conditions.

It has been reported that the unique N-Terminal fragment of L-TIM drives a reduced interaction between this isoform and CRY (Sandrelli *et al.*, 2007). My results in Chapter 5 not only corroborate this finding but also reveal that the manipulation of the TIM N-terminal fragment can enhance the interaction with CRY. In this study, fragments of the TIM linear sequence are challenged with CRY to map binding sites between these two proteins. An illustration of the TIM structural domains and binding sites described so far in the literature (Saez and Young, 1996; Vodovar *et al.*, 2002) can be found in Figure 6.1.

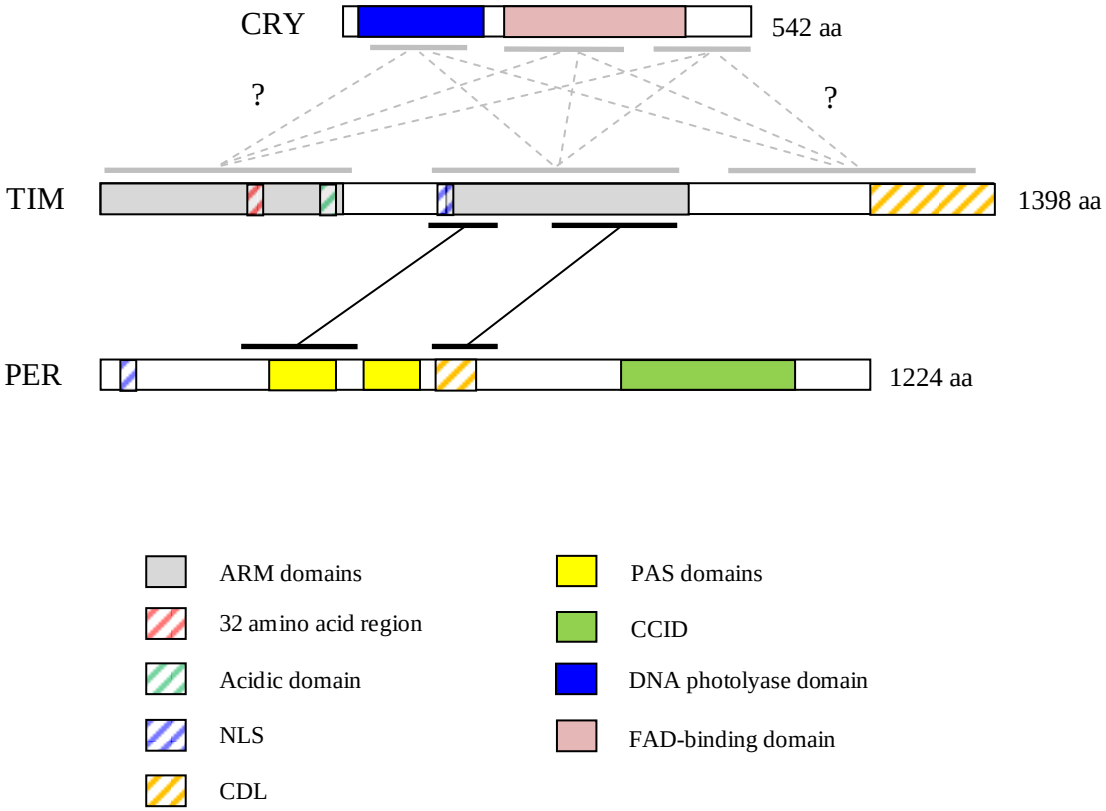


Figure 6.1.: Structural domains and binding sites of TIM, PER and CRY. The black lines connecting TIM and PER indicate interacting sites. Putative, yet unknown, binding sites between CRY and TIM are indicated in grey.

6.2. Methods

6.2.1. Yeast constructs

Six DNA fragments including different portions of *tim* cDNA were amplified by PCR and cloned in the yeast two-hybrid vector pJG4-5 to generate the TIMΔ constructs. Additional information about primer sequences, PCR conditions and cloning steps can be found in (section 2.3.3, "TIMΔ fragments"). Details regarding the LexA-CRY (full-length) construct can be found elsewhere (Ceriani *et al.*, 1999).

6.2.2. Two-Hybrid assays

The interaction assay was performed as described in section 2.4.4. Repeating the strategy described in Chapter 5, the yeast strain EGY48 was first transformed with the reporter (pSH18-34) and the LexA-CRY plasmid. Then, a single colony was picked and used for another round of transformation, this time with each of the TIMΔ constructs. The pJG4-5 vector, without any *tim* coding sequence (i.e. empty), was also transformed and the resulting colonies were used as negative controls (i.e. no interaction should be observed). The use of sequential transformation steps was chosen to minimize clonal variation of plasmid copy numbers, which can affect the interaction between the "bait" and "prey" proteins and lead to ambiguous data. The characterization of each TIM construct was based on two independent transformations. For every experiment, at least 5 colony replicates were examined, under light and dark conditions (i.e. negative control). More information about the yeast two-hybrid reagents and protocols can be found in section 2.4.

6.3. Results

To investigate the regions of TIM protein sequence that interact with CRY, I used a "deletion" strategy based on the yeast two-hybrid system (Figure 6.2). I opted to perform this mapping study on the S-TIM isoform, since it is the ancestral one. Six different constructs, containing fragments of TIM, were created in order to cover the entire protein sequence (Figure 6.2A). The first construct includes the N-terminal residues 1-446, where the first putative Arm domain is located. The second comprises residues 458-1078, spanning the second Arm domain. The third incorporates the C-

terminal residues 1056-1398. The fourth and the fifth cover the residues 1-1078 and 458-1398, respectively. The sixth and last construct includes the full-length protein sequence, with all 1398 residues, and served as a positive control for the assays.

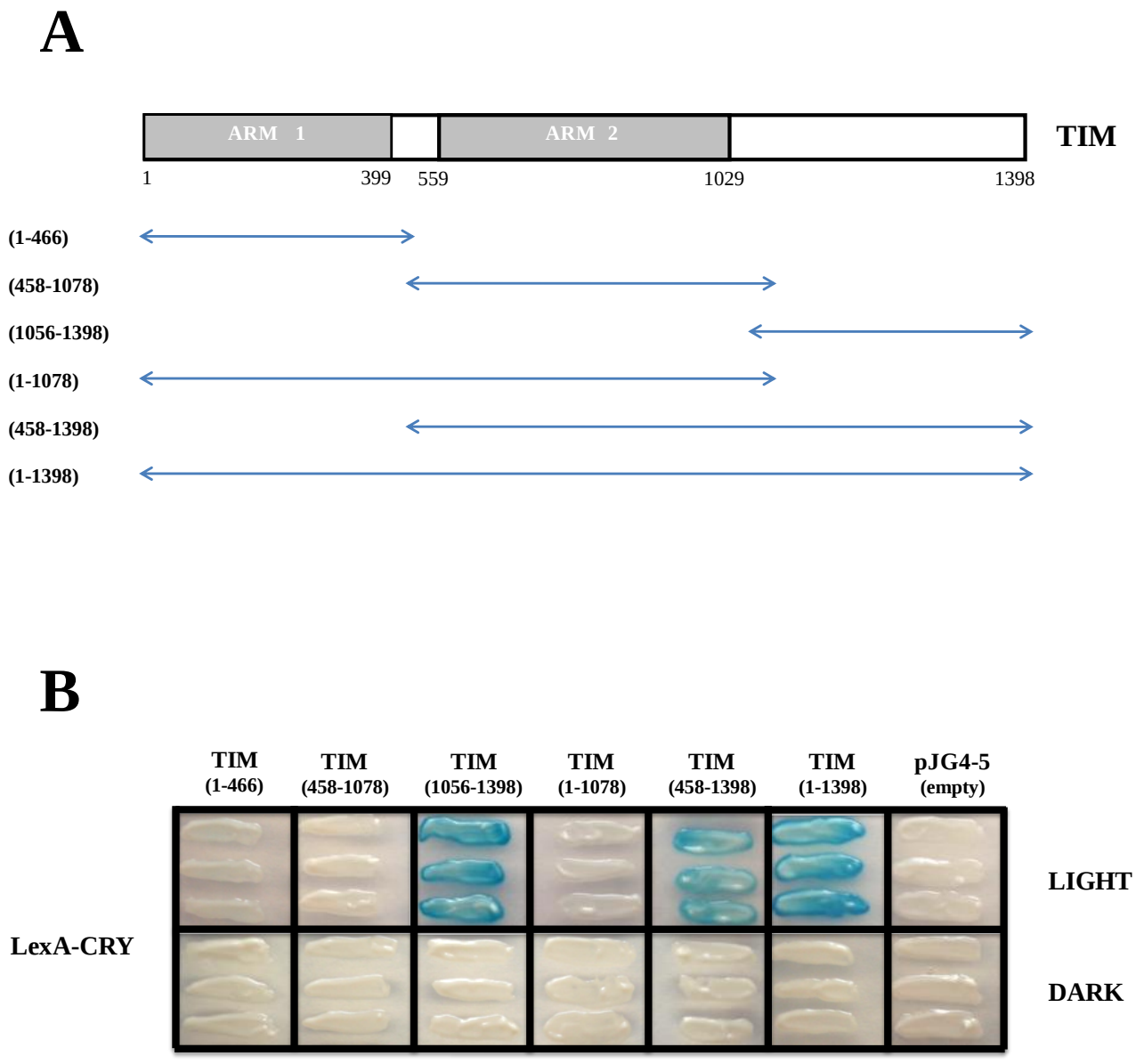


Figure 6.2.: Mapping of CRY-binding sites on TIM. A) Schematic representation of TIM protein sequence (S-TIM isoform, full length = 1398 residues, NCBI Accession number NP_722914). The putative ARM domains are marked, in grey, according to Vodovar *et al.*

(2002). Six constructs, containing different fragments of the TIM sequence, were generated (illustrated by blue arrows). B) The TIM constructs were challenged with CRY in yeast two-hybrid assays, under light or dark conditions (negative control). The numbers represent the range of residues included in each construct.

The TIM constructs were challenged with CRY in yeast two-hybrid assays (Figure 6.2B; Appendix Figure A1-4). Our results revealed a positive interaction for the constructs (458-1398) and (1056-1398), but not for the constructs (1-466), (458-1078) and (1-1078). These data indicate that CRY binds to a C-terminal region of TIM, somewhere within the residues 1056-1398. It also suggests that none of the predicted Arm domains (Vodovar *et al.*, 2002) appear to be involved in this interaction.

6.4. Discussion

Recent evidence revealed that the addition of an N-terminal fragment to TIM, as in the L-TIM isoform, can modulate its dimerization with CRY (Sandrelli *et al.*, 2007; see also Chapter 5). One could speculate that the N-terminal fragment might be affecting proximal regions directly involved in the interaction with CRY, although it has never been tested experimentally.

In this study, the TIM protein sequence was divided in six different fragments, which were challenged with CRY in yeast two-hybrid experiments. Two of these fragments covered the sequence of the ARM domains 1 and 2, in order to verify their role in the interaction between these two proteins. Our results revealed that CRY interacts with a sequence fragment located at the TIM C-Terminus, spanning residues

1056 to 1398. No interaction was observed for the fragment 1-1078, which encodes both Arm domains, but lacks the C-terminal portion.

Surprisingly, my data suggests that the N-Terminus of the protein does not directly bind to CRY. So, how can we explain that the addition of an N-terminal fragment in L-TIM provokes such striking effects in the interaction between the two proteins? One could speculate on a model where the presence of the N-terminal fragment is affecting TIM spatial conformation, which could reverberate in distally located C-terminal regions that bind to CRY (Figure 6.3). The N-terminus could either be forming a loop which partially hides the CRY binding site or, alternatively, mediating some indirect effect that slightly changes it. As a result, while the CRY-TIM dimerization dynamics would be optimal in S-TIM, the presence of an additional N-terminal fragment in L-TIM could be driving it to sub-optimal conditions.

Despite the positive evidence gathered so far, new analyses need to be performed to complement and validate the data of this study. As already discussed in Chapter 5, some proteins might not be very stable when expressed in yeast, which could potentially lead to wrong conclusions. From this perspective, it is possible that the lack of staining reported for some TIM fragments (i.e. 1-466, 458-1078 and 1-1078) are caused by their instability and fast turnover rate. To test this possibility, the same yeast colonies should be analysed for TIM expression using immunodetection methods (e.g. Western blotting). In case TIM is indeed detected in all constructs, with no significant degradation, then the two-hybrid data will get an extra support.

Further mapping experiments should be performed to restrict the interaction site, which currently extents for over 300 residues. The precise sequence would be extremely useful in phylogenetic studies, shedding light on the evolutionary history of the CRY-TIM partnership for light-mediated entrainment of the circadian clock.

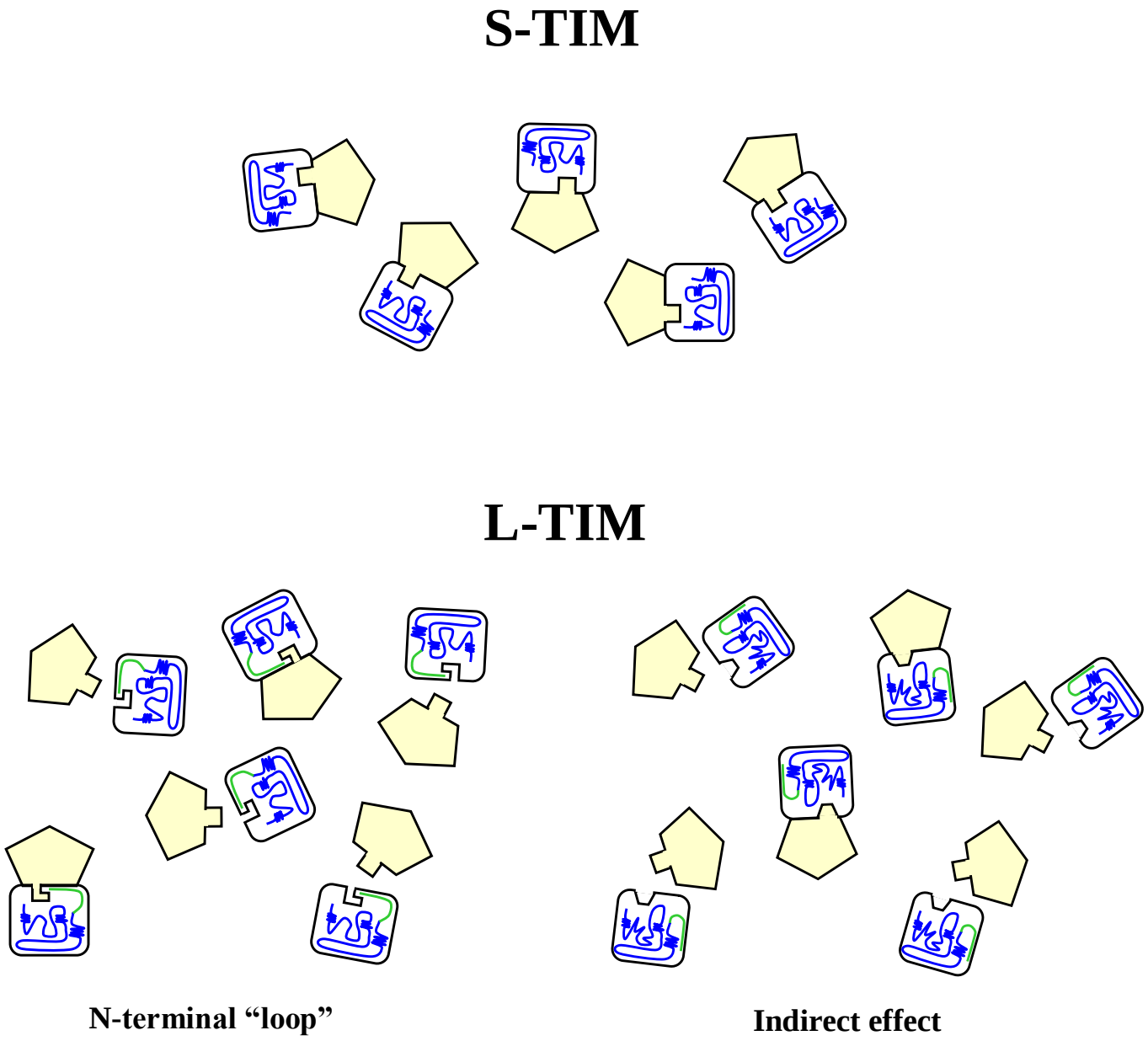


Figure 6.3.: N-terminal modulation of C-terminal CRY binding sites in L-TIM. Upper panel, S-TIM optimal interaction with CRY. Lower panel, L-TIM binds to CRY in sub-optimal conditions, caused by an N-terminal “loop” or an indirect effect. In yellow shapes, CRY. In white shapes, TIM. Represented in blue, the internal protein folding of TIM, and in green, the additional N-terminal fragment.

Moreover, one could generate a transgenic *tim* construct for functional analyses in which this portion is missing. This would allow the study of phenotypes driven by TIM and independent of CRY. For instance, flies expressing this version of *tim* could be assayed in diapause experiments. If CRY is indeed a photoreceptor for the photoperiodic clock, as it was speculated in Chapter 4, and if it is exerting its role through TIM, than flies expressing TIM without a CRY binding site would show no photoperiodic effect, with similar diapause levels under short and long-days.

Chapter 7: Discussion

In this work, circadian and photoperiodic aspects of clock genes were further investigated in the fruit-fly *Drosophila melanogaster*. A rare collection of congenic mutants was generated and provided the ideal background for comparative behavioural and physiological studies. The influence of circadian clock genes in the photoperiodic phenomena was explored, shedding light on an old debate in chronobiology. Special attention was paid to the canonical clock gene *tim* given its importance for adaptive phenotypes in seasonal environments such as Europe. Different evolutionary aspects of a newly derived *tim* variant were studied with detailed molecular and behavioural analyses, where putative substrates for natural selection were investigated. The results obtained in these studies (Chapter 3, 4, 5 and 6) are summarised and discussed below.

Temperature influence on diapause

Saunders and Gilbert (1990) have reported that temperature exerts a strong influence on diapause levels in *D. melanogaster*, thus modulating the critical day length in this species (Saunders and Gilbert, 1990). These observations, however, were obtained with the "domesticated" strain Canton-S, whose photoperiodic response may have been relaxed over more than half a century of domestication. Recent studies with *tim* natural variants revealed that the newly derived allele *ls-tim* exhibits higher diapause levels than the ancestral *s-tim*, at a wide range of photoperiods and a fixed temperature of 13°C (Tauber *et al.*, 2007). In Chapter 3, an isofemale line from Houten (Holland) was used to investigate the effect of

temperature on the diapause profiles of natural *tim* variants, *ls-tim* and *s-tim*, in order to observe how robust is the difference between these genotypes. My results have revealed that diapause, in natural populations, is induced on a restricted range of temperatures, which vary between 9°C and 13°C, and small changes of only 2°C can lead to significant effects. This observation perfectly agrees with that on Canton-S (Saunders and Gilbert, 1990), indicating that, at least for some aspects, wild populations respond to environmental stimuli in the same way as laboratorial strains. With respect to the genotype, *ls-tim* showed a more pronounced response than *s-tim* for all the temperatures tested, indicating that the phenotypic difference between them is indeed very robust. It is important to mention, though, that temperature appears to modulate the way the genotypes react to photoperiod changes, suggesting that *tim* not only affects diapause incidence but also the photoperiodic timing (this possibility was later confirmed with the tim^{01} null mutant). Overall, one could speculate that, in the natural environment, a combinatory effect of photoperiod, temperature and genetic background promotes diapause induction in flies.

Another implication of these results concerns the adaptive significance of the *ls-tim* polymorphism. While neutrality tests favour a directional selection scenario rather than a balancing one (Tauber *et al* 2007), they may not be sensitive to newly derived variants, as fewer neutral mutations can accumulate in the limited time since *ls-tim* occurred, and that is the substrate for these statistical tests (Tajima, 1989; Hudson *et al.*, 1987; Tauber and Kyriacou, 2005). So, it is formally still possible that perhaps *ls-tim* is favoured in the Mediterranean region, but further south or north, for some reason not yet determined, *s-tim* predominates, and thus a balancing scenario prevails. One possible reason could be that, in the colder north, temperatures fall quickly as winter approaches, and this could occur before the LD cycle reaches the

10-12 hours of light that is required to trigger the photoperiodic response. Thus in the north, *ls-tim* may not have an adaptive advantage over *s-tim*, if both alleles showed similar diapause levels at temperatures lower than 12°C. In the extreme south, if temperatures do not reach an average of 12°C, again *ls-tim* would have no diapause advantage over *s-tim*. My results clearly show that, irrespective of the lower temperatures used, *ls-tim* maintains significantly higher diapause levels than *s-tim*. Thus, this particular hypothesis cannot be supported by my results and, in turn, cannot underwrite balancing selection to explain *ls-tim/s-tim* distributions.

Indeed, another rational hypothesis concerning balancing selection has 'bitten the dust', when it was observed that *ls-tim* is more frequent in the north of Spain than in the south, the opposite of what is observed in Italy (Valeria Zonato, personal communication). If balancing selection was maintaining the *ls-tim/s-tim* distributions, then for the same latitudes, frequencies of these alleles might be similar and, if not, should still change in the same direction. Therefore, the Spanish results can simply be understood in terms of directional selection on *ls-tim* increasing its frequency from southern Italy to the north, across the Pyrenees and down again to southern Spain.

Another important aspect of the photoperiodic response that has not been studied so far in natural populations regards its maintenance and termination. It has been reported that Canton-S flies, reared under short days and 12°C, terminate diapause spontaneously after 6-7 weeks (Saunders *et al.*, 1989). If that holds true in the wild, it would result in the extinction of entire populations of flies, especially those living in northern environments, where harsh winter conditions last more than 3 months. However, since flies are found in such environments, diapause is probably maintained and terminated much later than expected. Saunders has set the standard for experiments that have followed, but it is critically flawed in terms of describing

diapause exit, because when flies trigger this response in the wild, temperature is still falling quite rapidly. Flies are then in 'deep' diapause simply because the temperature is low. By keeping the temperature at the bare minimum for diapause, it is small wonder that Saunders' females spontaneously reverse it, when they sense that temperatures are not falling any more. In the wild, as temperatures rise and photoperiods increase in the spring, flies will exit diapause. Whether this is a symmetrical exit (same temperature and photoperiod as they entered) remains to be seen.

The importance of changing temperatures and *tim* background for this exit phenotype could be explored in future experiments with natural populations, as the isofemale line used in my study. It would be interesting to see whether *ls-tim* not only induces diapause earlier, but also terminates it later.

Circadian clock mutants and diapause

The fact that diapause incidence is modulated by natural variants of the circadian clock gene *tim* was a surprising and unexpected finding (Tauber *et al.*, 2007; Chapter 3). Without a doubt, it stimulated discussions surrounding an old debate in chronobiology: the Bünning's hypothesis that the circadian system underlies photoperiodic time measurement (Tauber and Kyriacou, 2001; Emerson *et al.*, 2009). One of the biggest arguments against this hypothesis is that flies lacking a functional circadian clock, such as per^{01} mutants, are still capable of distinguishing long from short day lengths, indicating that the photoperiodic clock is an independent mechanism (Saunders *et al.*, 1989). In this context, a putative role for *tim* was also ruled-out. The main argument was that both *ls-tim* and *s-tim* natural alleles appeared

to be equally affected by variations in the photoperiod at 13℃ (Tauber *et al.*, 2007). Based on these data, the influence of clock genes on diapause has been interpreted as perhaps an isolated pleiotropic effect, with no causal relationship between circadian and photoperiodic timers (Emerson *et al.*, 2009). Indeed, Tauber *et al.* themselves suggested that the *tim* effect maybe be through peripheral photosensitivity, rather than affecting the photoperiodic timer. One should also add here that in *Chymomyza costata*, a natural mutation that removes a large part of the *tim* promoter produces a non-diapausing strain (Stehlik *et al.*, 2008).

To further explore this subject, a number of classical clock mutants were analysed in diapause experiments (Chapter 4). Importantly, these analyses were performed on a common genetic background, in order to allow an adequate characterization of the effects promoted by each mutation. Two aspects of diapause were investigated: the overall levels and the photoperiodic effect (i.e. the slope of the response; amplitude). With regards to the former, my results have revealed that all the mutants analysed were affected. Diapause was clearly downregulated in per^{01}, Clk^{jrk} and $norpA^{p24}$ and upregulated in tim^{01}, cry^{b} and Pdf^{01} mutants. In relation to the latter, it appeared that at least tim^{01} and probably cry^{b} were affected, being unable to detect day length variation. This particular phenotype might also be present in other clock mutants, such as Clk^{jrk} and $norpA^{p24}$, but it could not be investigated as precisely since their overall diapause levels were very low at 12°C. Future experiments with slightly lower temperatures could be useful.

The absence of photoperiodic effect in tim^{01} and cry^{b} could be explained by a disruption of either the photoreception pathway or the photoperiodic timer itself. Since *tim* exerts a dominant role over the *cry* mutant in regulating its diapause levels, one would surmise from classic genetic pathway analysis that *tim* is downstream of

cry. Indeed as CRY is a photoreceptor and TIM is its proximal target, this result makes sense. Moreover, since all the clock genes studied here showed an effect on diapause, either by modulating its levels or the photoperiodic effect, the simplistic hypothesis of gene pleiotropy is unlikely (Emerson *et al.*, 2009). Thus a more direct molecular link between circadian and seasonal timers and, consequently, are-evaluation of Bünning's hypothesis is required.

One could speculate that the effects on diapause could be driven by CLK-mediated expression of downstream genes (McDonald and Rosbash, 2001; Claridge-Chang *et al.*, 2001; Ceriani *et al.*, 2002). One of the CLK direct targets, *Pdp1*, activates the transcription of *takeout* (*to*) (Benito *et al.*, 2010), which has been accredited as a major regulator of lifespan (Bauer *et al.*, 2010), a trait closely related to diapause (Tatar and Yin, 2001; Williams *et al.*, 2006). Moreover, TO is a carrier protein for Juvenile Hormone (JH) (Lerro and Prestwich, 1990; Meunier *et al.*, 2007), which is also associated with diapause in flies and other insects (Saunders *et al.*, 1990, 2002). In Clk^{jrk} mutants, the whole signalling pathway leading to *to* expression might be reduced, negatively regulating the diapause response (or promoting vitellogenesis). The same hypothesis could also be used to explain the reduced diapause levels observed in per^{01}, where low levels of *Clk* transcript (Glossop, 1999) would probably lead to the downregulation of CLK direct targets as well.

How the PLC encoded by *norpA* affects diapause is obscure. Since the circadian clock in *norpA* mutants is less sensitive to light (Stanewsky *et al.*, 1998), one could speculate that diapause levels would be higher, as it was shown for the *ls-tim* allelic variant (Sandrelli *et al.* 2007; Tauber *et al.*, 2007). However, the mutant phenotype was exactly the opposite, suggesting that circadian light sensitivity does not always correlate with diapause incidence. Therefore, it is possible that NORPA

exerts its influence on diapause through a different signalling pathway. One should also add here that *norpA* mutants enhance the seasonally-relevant and temperature-dependent *per* 3' splicing (Majercak *et al.*, 1999, 2004; Collins *et al.*, 2004). Indeed, these mutants behave as if it was colder than it really is in terms of their locomotor activity profile (Majercak *et al.*, 2004; Collins *et al.*, 2004). Their lower diapause levels could thus be reflecting this thermal disruption, and dominate the light-sensing phenotype that this mutant conveys. It might be prudent to use the *per* 3' splice-locked mutants to see whether diapause is affected (Majercak *et al.*, 1999).

To understand how PDF affects the photoperiodic response, one should consider its contributions to the circadian neuronal network. It has been shown that PDF modulates the communication between clock clusters in the brain (Peng *et al.*, 2003), controlling different aspects of circadian behaviour, such as the morning (Stoleru *et al.*, 2004; Grima *et al*., 2004) and evening bouts of activity (Cusumano *et al.*, 2009). PDF fibers project into the dorsal brain and terminate very close to neurons directly connected to important neuroendocrine organs, the *corpora allata* and *corpora cardiaca*, which are considered to be diapause effector centres in many insect species (Saunders *et al*., 2002; Shiga and Numata, 2000, 2007; Hamanaka *et al*., 2005; Shimokawa *et al*., 2008). Interestingly, the ablation of these fibers in the blowfly *Protophormia terraenovae* led to average diapause levels at both short and long photoperiods, suggesting that the seasonal clock was disrupted (Shiga and Numata, 2009). These results raise the hypothesis that PDF signalling is mediating the transmission of seasonal time cues within the photoperiodic clock, or even connecting it to the effector centres. My results in *D. melanogaster*, however, do not fit this model. As previously mentioned, the Pdf^{01} mutation promotes diapause but does not influence the photoperiodic effect. In addition, it appears to be influenced by the *tim*

allele, since diapause was greatly repressed in *s-tim Pdf* ^{01} flies. Thus, it seems that PDF occupies a secondary role in the diapause response of *D. melanogaster*. Again, the epistatic effect of *s-tim* might suggest that it is downstream of *Pdf* for this phenotype. PDF could be modulating the neurons important for diapause (clock neurons or other targets), but this would be ineffective if those clock neurons were *s-tim*. Thus, we cannot discard the contribution of the PDF expressing cells in this phenomenon, which could be using other neurotransmitters to mediate more relevant effects.

Given the promising results with the mutants, the relationship between circadian and photoperiodic clocks could now be brought to the cellular level. My collection of congenic flies includes some *gal4/gal80/UAS* transgenic constructs which could be used to investigate this issue. The relative importance of the so-called "morning" and "evening" oscillators, as well as the contribution of eye photoreceptor cells, could be easily assessed. Overall, these studies will shed light on the way seasonal time is measured and integrated to drive the photoperiodic response in *D. melanogaster* and possibly other species. Unfortunately, although I spent a long time making these congenic lines, time ran out before I could do the experiments.

In addition to ours, a number of other studies have implicated circadian clock genes in the modulation of diapause in insects. In the drosophilid *Chymomyza costata*, this response was initially attributed to the *npd* locus, which was later identified as *tim* (Pavelka *et al.*, 2003; Stehlik *et al.*, 2008). In a more recent study with the bean bug *Riptortus pedestris*, diapause was associated with *cyc* and *per* (Ikeno *et al.*, 2010). Thus, it seems that circadian and photoperiodic clocks can share molecular pathways, suggesting that both mechanisms might have co-evolved.

Natural tim variants and circadian behaviour

In order to provide a behavioural check point prior to the diapause experiments, the congenic collection of clock mutants were also analysed in entraining (LD) and free-running conditions (DD) (Chapter 4). The mutant genotypes were analysed in either *ls-tim* or *s-tim* genetic backgrounds, in an attempt to reveal any genetic interactions. Two parameters that identify a functioning circadian clock were observed: the anticipatory behaviour prior to lights on/off transitions and the persistence of rhythms in constant darkness. Most of the mutant genotypes analysed behaved as previously reported. The core clock mutants per^{01}, tim^{01} and Clk^{jrk} were arrhythmic in DD and lacked the anticipatory behaviour in LD. On the other hand, $norpA^{p24}$ flies exhibited robust rhythms and normal anticipation.

Surprisingly, when Pdf^{01} and cry^{b} mutations were analysed in LD conditions, unexpected interactions with the natural *tim* variants were revealed. In Pdf^{01}, while the lack of morning anticipation was observed for both variants, the evening activity bout was severely reduced in *ls-tim* compared to *s-tim*. In addition, in the first two days of DD, one could notice that the majority of Pdf^{01} flies were still rhythmic when analysed on the *s-tim*, but not on the *ls-tim* background. With respect to the cry^{b} mutation, there was no effect of the *tim* natural alleles in the morning anticipation. However, the evening anticipation was completely impaired in the *s-tim*, but still present (perhaps slightly attenuated) in the *ls-tim* background. The role of *Pdf* and *cry* in the determination of evening activity has been recently investigated (Cusumano *et al.*, 2009). It seems that the evening cells mediate this behaviour through expression of CRY and PDF-mediated signals coming from the eyes. The contribution of both genes is highlighted in Pdf^{01} cry^{b} double mutants, which do not show any sign of

morning or evening anticipation. Interestingly, when CRY function is reduced by simulating winter conditions (i.e. low-light intensities, short photoperiods and cold temperatures), the behaviour of *Pdf* ^{01} resembles that of the double mutant with the lack of evening anticipation, suggesting a more prominent role for PDF when CRY is not present. In this context, one could speculate that the reduced evening activity observed in *ls-tim Pdf* ^{01} flies could be explained by the reduced light-sensitivity of this *tim* allele (Sandrelli *et al.*, 2007). As in *cry*^{b} mutants most of the entraining cues come from the eyes through PDF, the phenotypic difference between the *tim* variants might stem from this tissue.

N-terminal evolution of TIM

The newly derived *ls-tim* allele is spreading throughout Europe by natural selection due to adaptive advantages over the ancestral *s-tim* (Tauber *et al.*, 2007; Sandrelli *et al.*, 2007). The new variant expresses not only a short protein isoform, S-TIM, which is present in the *s-tim* allele, but also a longer one, L-TIM. It has been shown that flies carrying the *ls-tim* allele or the transgenic construct *P[L-TIM]*, which exclusively expresses the long isoform, exhibit particularly high diapause levels and a reduced circadian photoresponsiveness. Molecularly, these phenotypes could be explained by an attenuated interaction between CRY and L-TIM, which ultimately leads to a more stable TIM protein (Sandrelli *et al.*, 2007).

The phenotypic attributes of L-TIM are strictly related to the presence of an additional N-terminal fragment of 23 residues. Interestingly, bioinformatics analyses revealed the presence of two serines, one at position 2 and the other at 22, which could be putative phosphorylation sites. This raised the possibility that post-

translational modification motifs could be underlying the function of L-TIM. However, one should consider that it might not be the case and the overall properties of the N-terminus, such as its length or amino acid composition, could be more important parameters. To investigate these two possibilities, a number of N-terminal engineered versions of L-TIM were created for structure/function analyses (Chapter 5). Two phenotypes were investigated so far: the interaction with CRY, in yeast two-hybrid assays, and the circadian light sensitivity, in behavioural phase-response experiments.

My results revealed that, in yeast, both the length of N-Terminal sequence and the order of its residues are important parameters to define the strength of interaction between L-TIM and CRY. When the length was reduced or the residues were shuffled, an increase in the interaction was observed, resembling the S-TIM phenotype. The substitution of the serines 2 and 22 for alanines, which should avoid phosphorylation, or aspartic acids, which should mimic it, resulted in minor (or no) effects, suggesting that post-translation modifications might not be relevant.

A different picture emerged from the fly behavioural studies. Interestingly, a significant increase in the phase response was obtained when both serines were individually replaced by alanines, thus supporting the "phosphorylation hypothesis", but when they were manipulated simultaneously, this effect was not reproduced. Curiously, an increase in the phase response was also observed when an aspartic acid was placed at position 22, which is an unexpected finding, since constructs of this type should mimic phosphorylation and resemble the L-TIM control. When the length of the N-terminal fragment was reduced by half, a significant effect was revealed for some, but not all constructs. While L(01-12) and L(05-15) increase the phase response, L(12-23) still behaved as the L-TIM control. This suggests that the amino

acid sequence might also be playing a role. However, when the order of these amino acids was shuffled, no effect was found. Therefore, it appears that in flies the overall composition, rather than the order, of the residues is the parameter that defines the characteristic L-TIM phenotype. The manipulation of single residues can also lead to significant effects, which are difficult to predict. In this context, despite some positive evidence, the role of phosphorylation is not conclusive.

My interaction studies in yeast were performed in X-GAL plates. As previously mentioned, the intensity of the blue staining (i.e. *lacZ* reporter expression) correlates well with the strength of interaction. However, I appreciate that the "plate" assay is rather qualitative and does not allow precise determination of the phenotypes analysed. Therefore, a more quantifiable assay in liquid culture, using ONPG as a substrate for the reaction, will soon be performed with the same yeast colonies.

The contradiction between yeast and fly studies could suggest that the molecular interaction between CRY and TIM might not necessarily define the behavioural phase response, thus rejecting the model hypothesised by Sandrelli *et al*. (2007). One should consider that the N-terminal manipulation of TIM might not only affect its interaction with CRY, but also with other proteins directly involved in its degradation, such as JET (Koh *et al*., 2006; Peschel *et al*., 2006, 2009). That would explain the results obtained with the construct L(Random), which showed an increased CRY-TIM interaction, but a smaller behavioural phase-shift. Alternatively, these inconsistencies could be attributed to a not so adequate yeast approach to infer CRY-TIM interaction, which would implicate that my results are full of experimental artefacts. A third explanation would involve the circadian neuronal network, instead of the simplistic transcription-translation model for cell autonomous entrainment. It has been recently shown that a light-pulse in the early night leads to TIM degradation

in dorsally located neurons, but not in the s-LNvs pacemaker cluster. However, the expression of CRY in the s-LNvs is still necessary for the phase response, indicating that this behaviour is a network property (Tang *et al.*, 2010). Therefore, from this perspective, the strength between CRY and TIM interaction might not be as critical as previously thought.

To complete the functional studies with the fly constructs, a number of new analyses could be performed in the future. First, since my behavioural data concentrated on phase delays only (i.e. light pulses at ZT15), it would be necessary to define how is the phase advance (i.e. light pulses at ZT21). Secondly, TIM western blots could be used to assay the stability of this protein *in vivo*, which might provide a better molecular correlate to the behavioural data than the yeast analysis. I appreciate, though, that these results might reflect TIM state in the eyes, where clock products are more abundant, and not in the neurons driving the phase response. Finally, the constructs need to be tested in diapause experiments, as this phenotype is also enhanced by the presence of the L-TIM isoform. One should expect that higher diapause responses might not necessarily correlate so well with lower phase-shifts, as I have demonstrated for the *norpA*^{p24} mutant, which is known for the reduced phase-shifts (Stanewky *et al.*, 1998) and, nonetheless, exhibited low diapause levels (Chapter 4).

Mapping CRY interaction domains on TIM

Due to the importance of the N-terminal sequence in the modulation of TIM function, we ought to determine if this region is directly binding to CRY. To investigate that, a number of TIM protein fragments were challenged with CRY in

yeast two-hybrid experiments (Chapter 6). Surprisingly, my deletion mapping analysis revealed that CRY binds to a C-terminal sequence of TIM, somewhere between the residues 1056 and 1398. My results suggest that the N-terminal fragment found in L-TIM is not directly involved in the interaction with CRY. Instead, it might be affecting the tri-dimensional conformation of the protein and, as a consequence, modulating CRY binding. One could speculate that the N-terminus is either forming a loop that directly interferes with the C-terminal CRY binding site or, alternatively, it could be mediating some kind of indirect effect on this portion. Further deletions need to be performed for a fine mapping of the CRY binding region, which would shed light on the evolutionary history of TIM as the light-sensitive molecule of the circadian clock.

Appendix 1

LexA-CRY fusion was challenged with mutant versions of TIM in yeast two-hybrid assays. Five different colonies, C1 to C5, were analysed under light and dark (negative control) conditions. The experiment was repeated twice, using colonies from two independent transformations, and similar results were achieved.

Figure A1-1: “phosphorylation mutants”

Figure A1-2: “G24 mutants”

Figure A1-3: “sequence/size mutants”

Figure A1-4: “TIMΔ constructs”

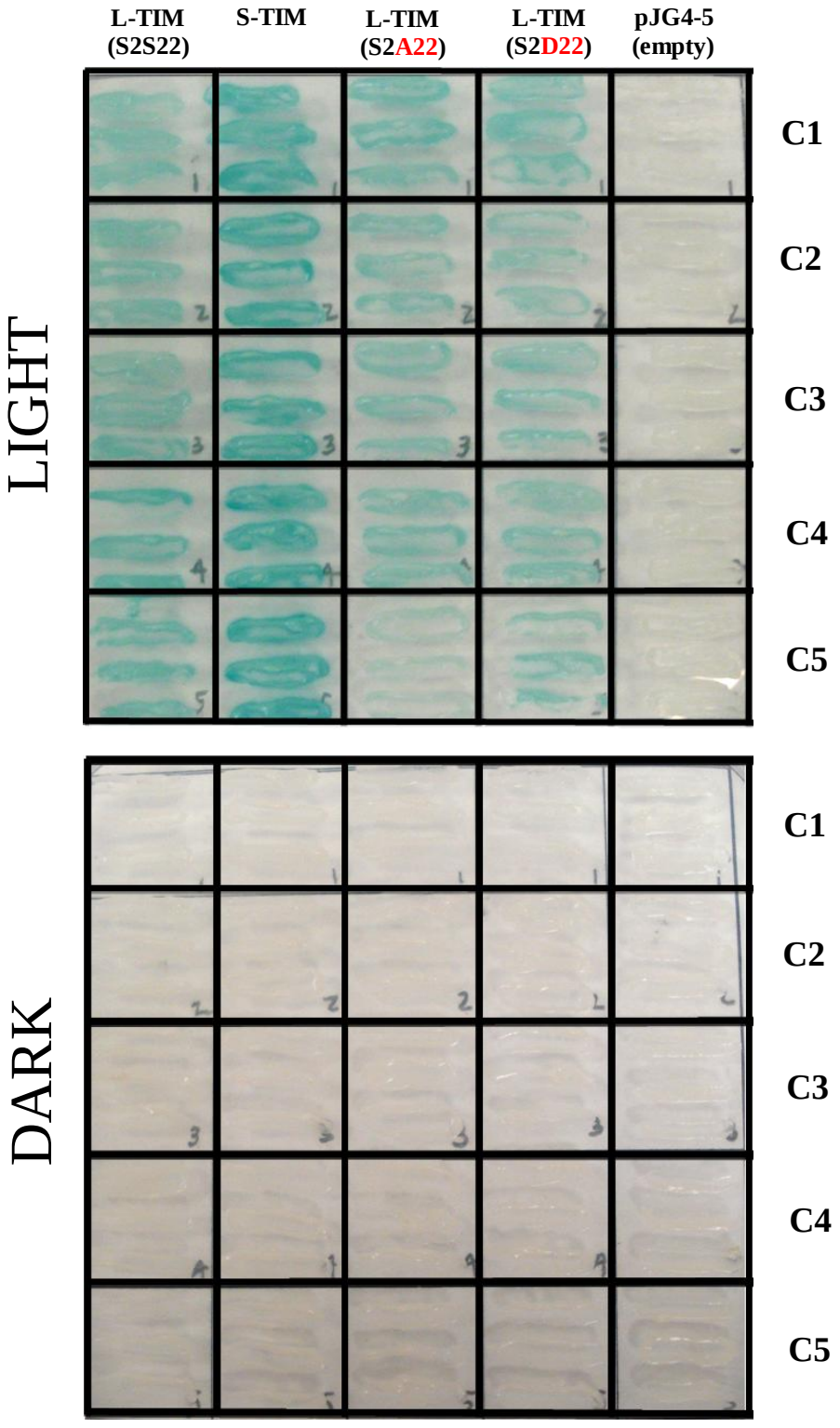


Figure A1-1: "phosphorylation mutants". Manipulated residues are shown in red.

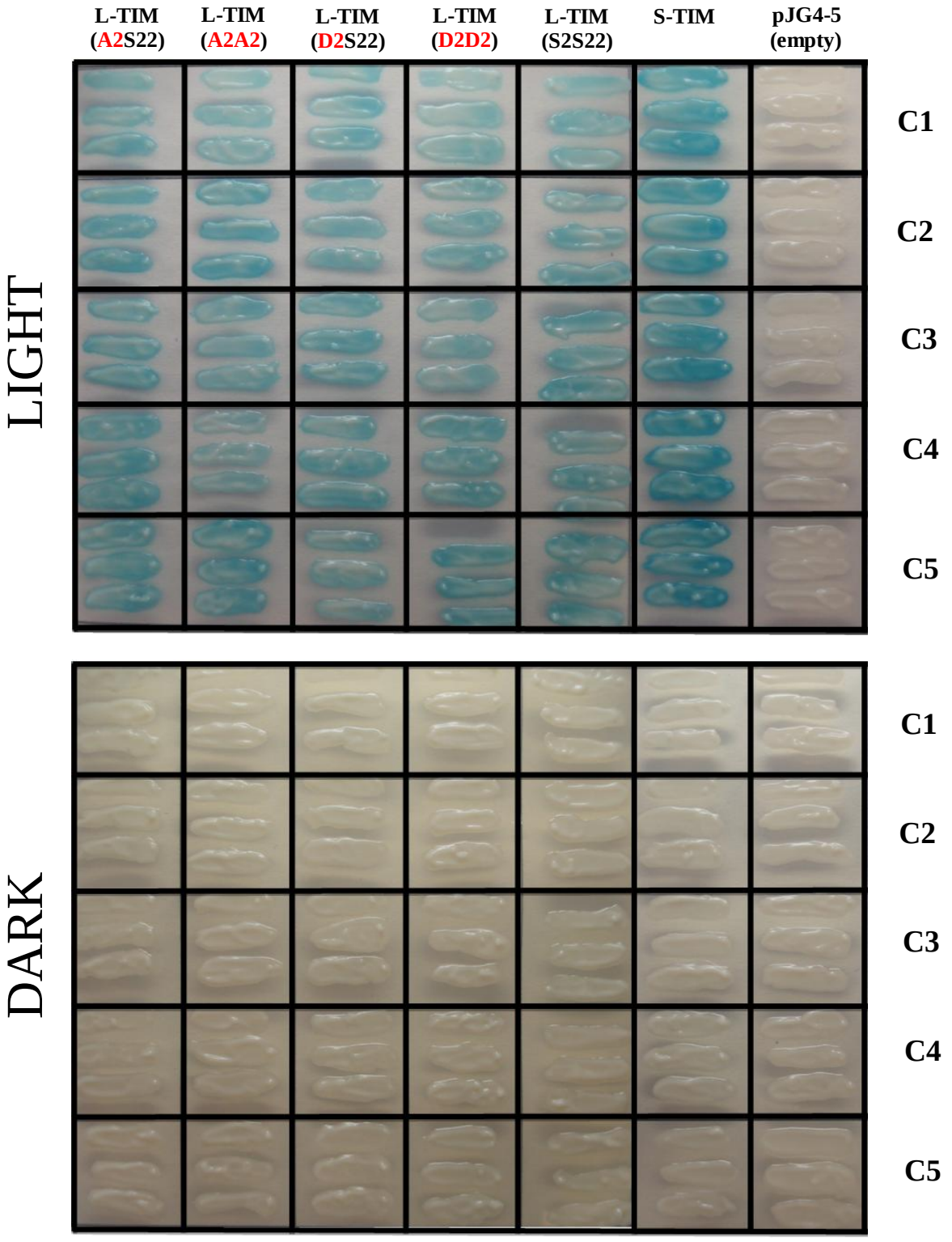


Figure A1-1: (continued).

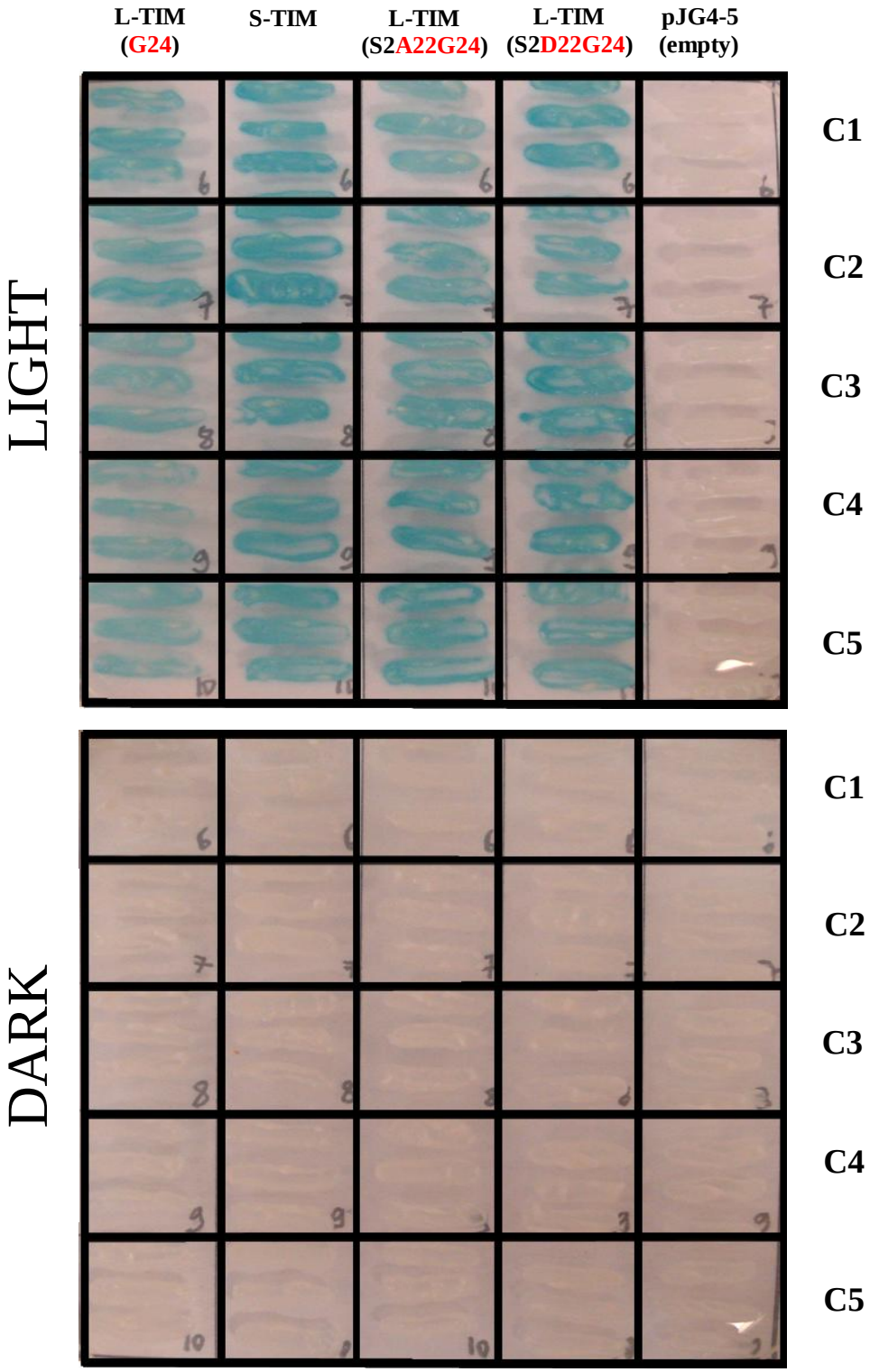


Figure A1-2: "G24 mutants". Manipulated residues are shown in red.

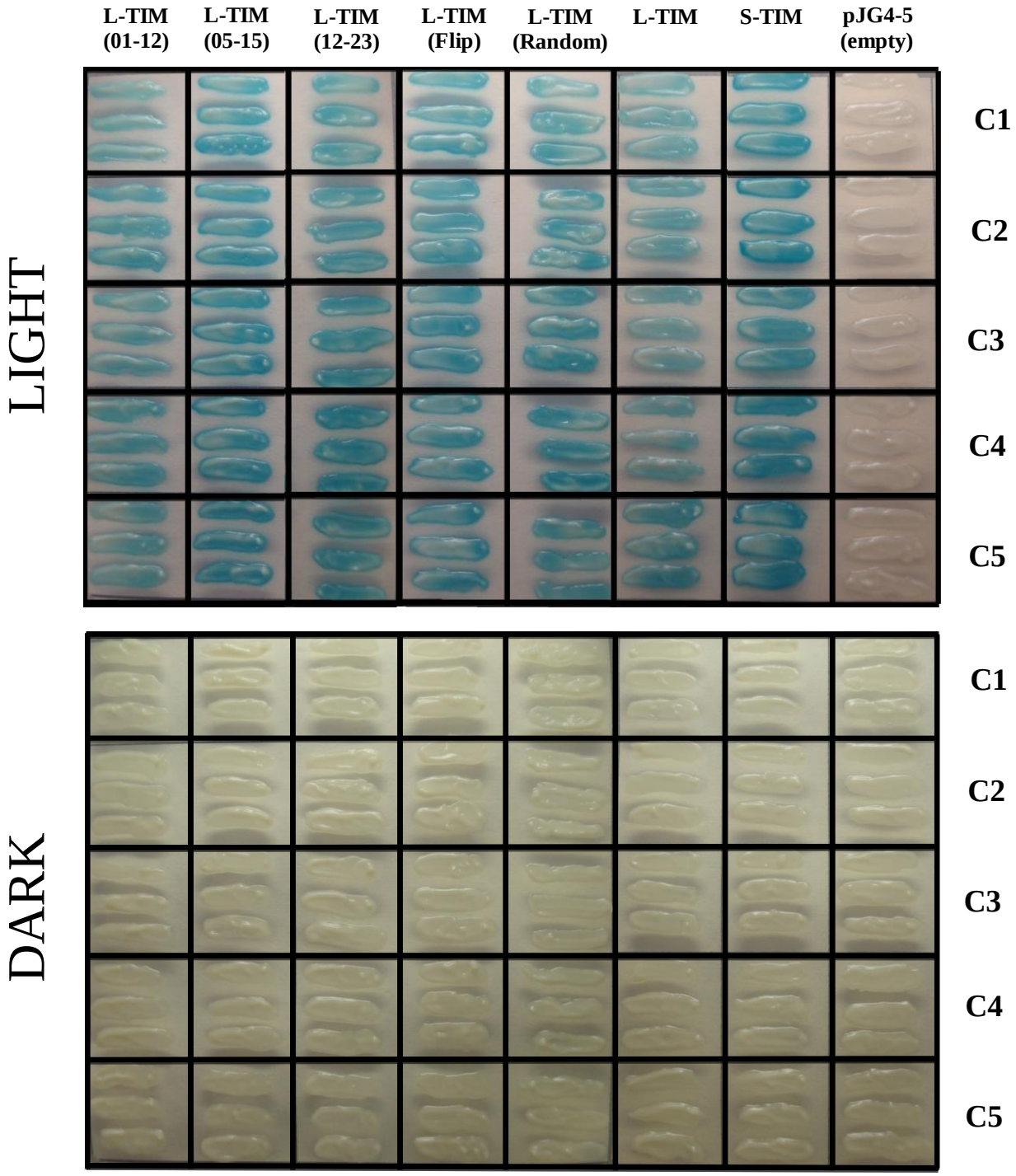


Figure A1-3: "Sequence/size mutants".

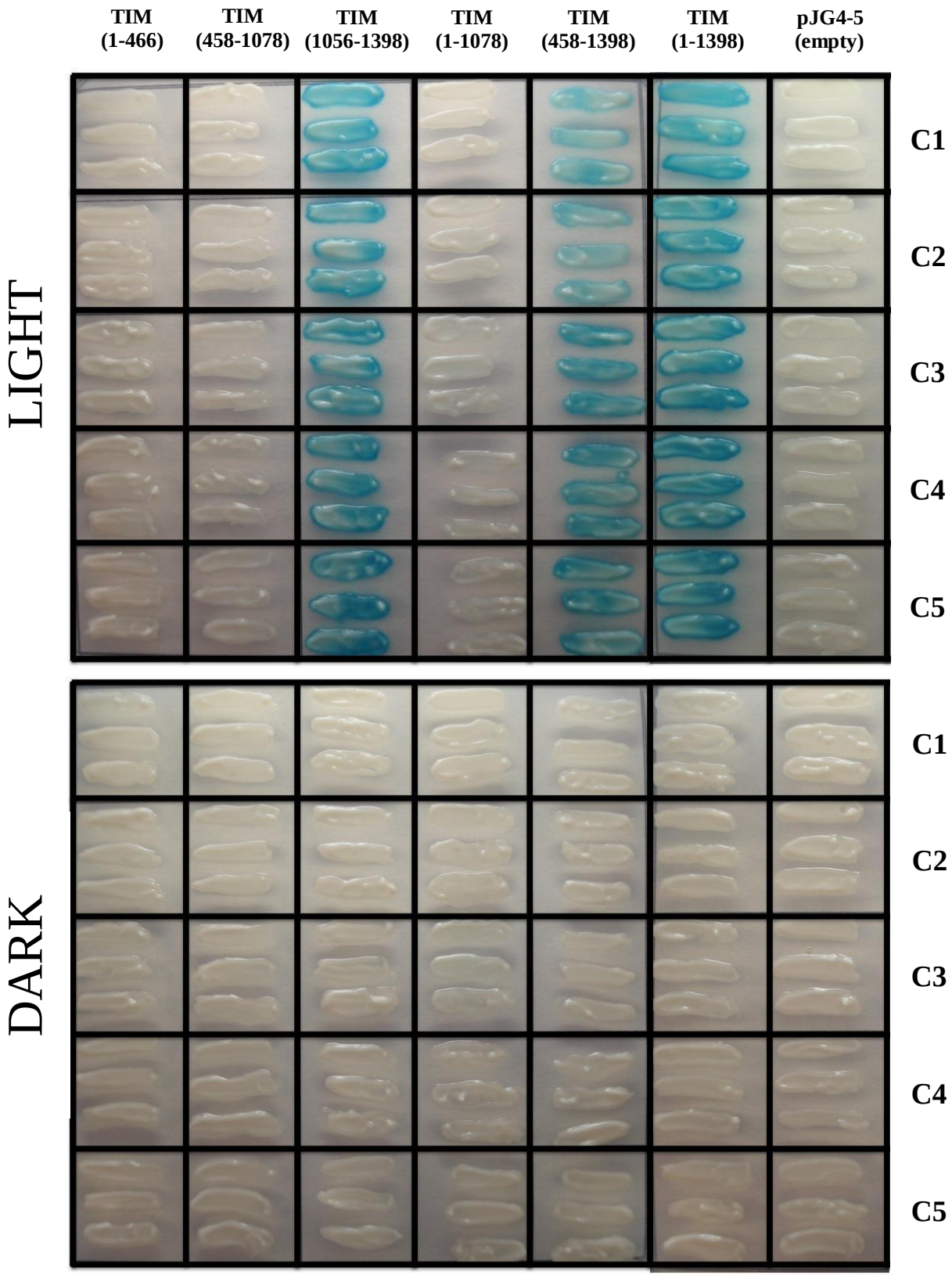


Figure A1-4: "TIMΔ constructs".

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