A comparative analysis of the effect of light and temperature on the *Drosophila* clock

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Abstract

Temperature controls the entrainment of *Drosophila melanogaster* locomotor activity to different seasonal conditions through the regulation of splicing of the intron within the 3' UTR of *period (per)* mRNA. This analysis of *per* 3' splicing and behaviour in different genetic backgrounds revealed that at low temperatures *per* splicing is regulated primarily by the light input received *via* the visual system. At higher temperatures, regulation is more stringent, requiring the visual system input during the day and the circadian clock at night. This is surprising as *cryptochrome* is assumed to be a circadian photoreceptor, yet regulates splicing primarily as a clock component. Conversely, the visual component, *no-receptor-potential-A,* appears to have an additional circadian role, probably in thermosensitivity, as a mutation generated levels of 3' splicing that could not be explained by its effect on the visual pathway.

This work also resulted in the identification of a unique circadian phenotype. per^{01}; cry^{b} mutants display an evening activity peak anticipating 'lights off' in light: dark cycles, which should be clock dependent, yet are arrhythmic under constant conditions, signifying the absence of a clock. This anticipation is not seen in tim^{01}; cry^{b} mutants, so is probably the result of the light dependent degradation of TIMELESS (TIM). Therefore, as per^{01}; cry^{b} flies have residual clock function even in the absence of PER, there must be a PER-independent role for TIM.

Finally, the *per* gene determines species-specific locomotor behaviour. This is not down to differential *per* splicing as an examination of *D.pseudoobscura* and *D.virilis* failed to discover regulated *per* splicing in either species. Instead, species-specific differences in behaviour seem to be determined by altered patterns of *per* mRNA expression. Thus, light and temperature sensitive *per* splicing has evolved in *D.melanogaster*, allowing it to 'fine tune' behaviour to the wide variety of habitats it occupies.

Acknowledgements

First off, I would like to thank Bambos for his supervision, providing the initial ideas from where this work derives (and some which I forgot along the way), and (eventually) understanding what I was trying to say in my interpretation of the data.

Without Jon Clayton, around for the first year or so, and Karen Garner for the whole three-year stretch, I would have had a much harder time getting started and learning new techniques. Then, of course, there are many other people in this lab and 226 upstairs who at some time (often many) have been useful - Ezio, Veryan, Stephane, Eran, Helen, Pat and Mark, this means you.

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Finally, to my Mum, Dad and brothers Simon and Nick - I hope that you read and enjoy what is written within.

So, was it worth it? Well, at least it got me a job in New York.

Abbreviations

AA	Amino acid
bp	Base pair
°C	Degrees Centigrade
dClk	*Drosophila clock*
cry	*cryptochrome*
CT	Circadian Time
cyc	*cycle*
dbt	*doubletime*
DD	Dark: dark (Constant Darkness)
h	hour
LD	Light: dark
LL	Light: light (Constant Light)
LN	Lateral Neuron
per	*period*
s	second
sgg	*shaggy*
tim	*timeless*
UTR	Untranslated Region
vri	*vrille*
ZT	Zeitgeber Time

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Chapter one: introduction

1.1

Almost all organisms have an internal clock which is set by daily changes in light and temperature, but is able to run with a more-or-less 24 h period even in the absence of external cues. This endogenous pacemaker is known as the circadian clock, and ensures that an organism wakes, sleeps and eats at the correct times. In this way, organisms tailor their behaviour so that they perform different activities at the most opportune times of day throughout the year. Despite the fact that the ubiquity of clocks indicates their functional importance, only recently has it been shown experimentally. The advantage of having a clock has been demonstrated most convincingly in cyanobacteria, in which bacteria with circadian periods matching their environmental LD cycle, were favoured in competition experiments Ouyang et al., 1998). More recently, in *D.melanogaster* it has been shown males without a functional clock have reduced reproductive fitness (Beaver et al., 2002).

An indication that a clock is also important in man comes from the common experience of 'jet lag'. Jet lag is more severe when travelling from west to east, and increases with distance travelled, indicating that humans can cope better with delaying their clocks rather than advancing them (Recht et al., 1995). A good demonstration of the detrimental effect of jetlag is provided by looking at the peculiar American pastime of baseball. Playing a baseball match against a team from the other side of the USA can entail crossing six time zones. The demand for baseball teams to play several games against each other over successive days, and the crammed fixture list, gives very little time for recovery on arriving on the other side of the country. The jet lag experienced by the travelling team means that home advantage is statistically significant. Acclimatisation reduces home advantage, with the probability of a team from the east winning a home match decreasing with the number of days since the western team arrived. As travel in the opposite

direction (east-west) has no significant bearing on the likelihood of the home team winning, baseball is biased against western teams. (Recht et al., 1995).

Serious research into circadian rhythms has been ongoing for nearly 60 years, and in the last 20 years it has become possible to dissect the molecular basis of the clock (Wager-Smith and Kay, 2000). The clock is a complex system involving many different genes, not only in the core oscillator, but also in the regulation of inputs and outputs. While many clock genes are conserved between species, more complex organisms have more complex clocks. Even in *D.melanogaster* where the core of the clock is well understood, many additional genes must be involved in ensuring that the clock runs with a 24-h period under a range of different conditions.

1.2 The *D.melanogaster period* gene

In *D.melanogaster,* most mutants in circadian rhythmicity are defined by their effect on the daily pattern of locomotor activity or adult pupal eclosion. Konopka and Benzer (1971) screened ethyl methanesulphonate- (EMS-) mutagenised flies for altered locomotor and eclosion rhythms and isolated three mutations in a single gene which they named *period (per).* One produced a longer period, one a shorter period, and the third was arrhythmic (Konopka & Benzer, 1971). Kyriacou and Hall (1980) observed that these mutations also affected the ultradian 60 s rhythm of the courtship song of *D.melanogaster,* demonstrating that one gene could control biological cycles acting over vastly different temporal domains.

By late 1984 period had been cloned, sequenced and used to rescue behaviour in arrhythmic per^{01} transformants (reviewed in Rosato and Kyriacou, 2001). *per* is expressed throughout the adult fly, with individual tissues containing their own oscillators, or 'peripheral clocks' (Liu et al., 1988; Plautz et al., 1997). It has become apparent that the *per* gene encodes a core clock component present across the animal kingdom (Stanewsky, 2003).

One of the most startling (but generally ignored) discoveries during this early period of molecular work was that the transformation of *per*^{01} *D.melanogaster* flies with the *per* gene from the closely related *D.pseudoobscura* species generated flies which had locomotor activity closely resembling that of *D.pseudoobscura* (Petersen et al., 1988). This demonstrates that species-specific differences in behaviour are encoded by *per*, the first example of a complex pattern of behaviour being controlled by a single gene. The consequences of this study are discussed further in the introduction to Chapter 6.

So, how does the PER protein generate circadian rhythms? The underlying oscillation of *per* mRNA is driven by a negative feedback loop involving its own protein (Hardin et al., 1990), that is maintained even under conditions of constant darkness (DD). Thus the core of the clock is a negative feedback loop where PER protein drives cycles of its own transcription. Fluctuations in *per* mRNA are controlled by the rate of *per* transcription, and the short half-life of *per* mRNA enables it to be part of a feedback loop (Hardin et al., 1992). Feedback loops such as this form the basis of all clocks identified to date (Dunlap, 1998).

A simple view of the clock might therefore be that of an input, central oscillator and output, as shown in figure 1.1. The *per* feedback loop then forms the basis of the central oscillator of the clock.

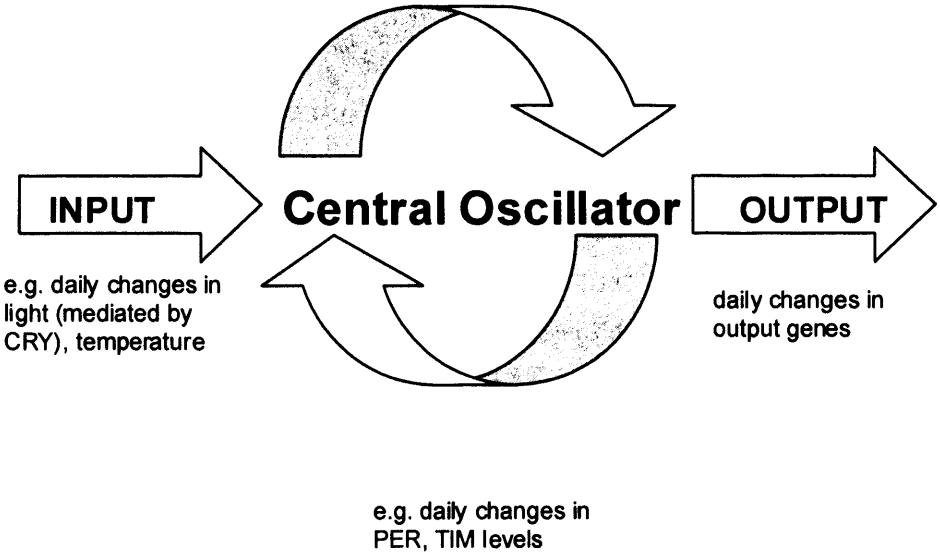


Figure 1.1: A schematic view of the circadian clock Inputs to the clock perturb the central oscillator which then alters the expression of output genes.

Although PER protein is also expressed in glia and in the eye of the fly, normal pacemaker function requires the expression of PER only within the lateral neurons (LN_vs) (Ewer et al., 1992; Vosshall et al., 1995; Shafer et al., 2002). The location of these clock neurons within the *Drosophila* brain is shown in figure 1.2.

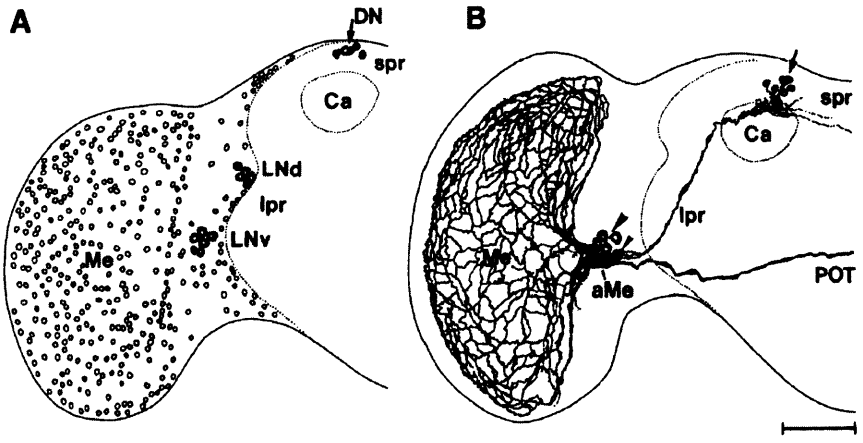


Figure 1.2 Location of *D.melanogaster* pacemaker neurons (from Helfrich-Forster et al., 1995). PER is expressed within the dorsal neurons (DN), dorsal lateral neurons (LN_d) and ventral lateral neurons (LN_vs). Other structures staining for PER expression are the glial cells. Figure shows left-hand side of the brain, with optic lobe outermost.

Within the *per* gene is a motif encoding 270 aa, known as the PAS domain, a dimerisation domain common to basic helix-loop helix transcription factors initially identified in **P**ER, **A**RNT and **S**IM. *In vitro*, mutations in the PAS domain of PER such as per^{L} alter the dimerisation efficiency either with PER itself, or its partner molecule TIMELESS (TIM), resulting in a longer period (Huang et al., 1993; Gekakis et al., 1995).

The original *tim* mutant tim^{01}, exhibits arrhythmic eclosion and locomotor rhythms, much like the previously characterised per^{01} mutant (Sehgal et al. 1994; Vosshall et al., 1994). *tim* mutants also show altered *per* expression, suggesting that *tim* acts upstream of *per* in the clock (Sehgal et al., 1994). The identification of TIM also helped elucidate how PER drives the clock, as in tim^{01} mutants the PER protein product fails to enter the nucleus. The PER and TIM proteins have been shown to dimerise both *in vivo* and *in vitro* (Rutila et al., 1996; Saez and Young 1996). Thus PER and TIM are believed to dimerise to enter the nucleus where they appear to down-regulate their own transcription (Vosshall et al., 1994). The association between PER and TIM could most easily be understood if both proteins carried PAS dimerisation domains, but TIM apparently does not (Sehgal et al., 1995; Vodovar et al., 2002).

1.3 PER/TIM dimerisation, nuclear entry and negative autoregulation

Both PER and TIM proteins are required to generate molecular rhythms as in per^{01} and tim^{01} mutants neither *per* nor *tim* RNA cycles (Myers et al., 1995, Sehgal et al., 1995). In both wild-type and short period mutant lines such as per^{s}, *per* and *tim* mRNAs cycle in phase with each other, several hours ahead of their protein products in both LD and DD (Myers et al., 1995; Sehgal et al., 1995). Initially it was thought that the delay between RNA and protein was fundamental to the clock as it would be required to allow the negative autoregulation of *per* and *tim* expression by their own protein products. If protein and RNA cycled in phase, it was assumed that the feedback loop would break down as the proteins would immediately inhibit

their own transcription (Hardin 1992). It turns out that the delay between transcription and translation, of *per* at least, is not fundamental to the clock, but is probably only necessary to generate a 24 h period (Suri et al., 2000).

In the tim^{01} mutant, the level of PER is reduced to a low level similar to that seen in wild-type flies late in the day or in constant light (Price et al., 1995). In contrast, TIM protein can still accumulate in per^{01} flies. This demonstrates that PER requires TIM for stability, but TIM is stable in the absence of PER. The stability of TIM is instead dependent on light, and TIM levels fall rapidly in response to both light pulses and 'lights on'. This light induced degradation of TIM is one way in which the environment regulates the clock and ensures that it is reset on a daily basis (Hunter-Ensor et al., 1996, Zeng et al. 1996, Myers et al., 1996).

The dimerisation of PER and TIM is essential before either can enter the nucleus (Saez and Young, 1996). In tim^{01} mutants, PER accumulation and nuclear localisation is blocked (Vosshall et al., 1995), suggesting that TIM is required for PER stability and transport into the nucleus (Sehgal et al., 1995). The light-degradation of TIM therefore limits the accumulation of PER and nuclear translocation of the PER/TIM complex. This means the PER protein accumulates over several hours in the cytoplasm of the pacemaker neurons, and can enter the nucleus only during a narrow time window. This prevents the premature inhibition of *per* RNA expression (Vosshall et al., 1994, Curtin et al., 1995, Kay and Millar, 1995, Reppert and Sauman, 1995, Sehgal et al., 1995).

Studies in *Drosophila* Schneider 2 (S2) cells show that PER and TIM accumulate in the cytoplasm if independently expressed, but co-expression causes immediate nuclear entry (Saez and Young, 1996). S2 cells are polyclonal, derived from an unknown subset of embryonic tissues, and do not normally express PER or TIM. Using this assay, it was therefore possible to determine the regions of TIM essential for the protein-protein interaction and nuclear translocation. Within both PER and TIM there are also cytoplasmic localisation domains (CLDs), which are encoded within the regions of

heterodimerisation, and become 'hidden' on dimerisation, thus regulating nuclear entry. The CLD allows the daily, regulated nuclear entry of PER and TIM to drive the expression of their RNAs in a rhythmic manner (Saez and Young, 1996). In per^{L} flies, nuclear entry is delayed as the association between TIM and PER^{L} is weaker than usual. This means that it takes longer for sufficient amounts of the PER/TIM complex to enter the nucleus, producing the longer period (Curtin et al., 1995; Gekakis et al. 1995).

1.4 bHLH transcription factors dCLOCK and CYCLE

Once within the nucleus, the role PER and TIM is to repress their own transcription. *per* and *tim* expression is regulated at both the transcriptional and post-transcriptional level. An *in vivo* luciferase assay was used to see which elements are required to generate the correct temporal expression pattern of *per* expression (Stanewsky et al., 1997). This revealed that both a promoter element and a transcript element are required for wild-type *per* expression. Although expression is rhythmic with only one of these elements, neither individual cycle matches wild-type *per*. Only in combination do they confer a normal *per* RNA cycle on the luciferase reporter gene in terms of phase and amplitude (Stanewsky et al., 1997). The promoter contains a 69 bp enhancer element, which includes a vital E-box (Hao et al., 1997), which gives a clue as to how PER and TIM regulate their own expression. The E-box is the binding site for basic-Helix-Loop-Helix (bHLH) transcription factors, and, as neither PER nor TIM contains a consensus bHLH domain, they must regulate their own transcription by disrupting associated transcription factors (Huang et al., 1993; Darlington et al., 1998).

These positive regulators of *per* and *tim* are the PAS-bHLH transcription factors, d*Clock* (d*Clk*) (Darlington et al., 1998; Allada et al., 1998; Lee et al., 1998) and *cycle* (*cyc*) (Rutila et al., 1998). *dClock* was initially identified as the mutation *Jerk*, heterozygotes of which display an altered period, and a relatively high level of arrhythmicity, whereas homozygous mutants are all arrhythmic, owing to low levels of PER and TIM. *Jerk* was shown to be a

homologue of the murine circadian mutant *Clock*, and therefore is now referred to as *dClock*^{Jerk} (*dClk*^{Jrk}) (Allada et al., 1998).

In order to regulate *per* and *tim* transcription, dCLK dimerises with CYC, a homologue of the mammalian BMAL1 transcription factor. *cyc*, like d*Clk,* is essential for rhythmic expression of *per* and *tim* (Rutila et al., 1998). In mammals BMAL1 and CLOCK associate to activate transcription from canonical CACGTG E-boxes. The *Drosophila* homologues also physically interact (Darlington et al., 1998; Lee et al., 1998) to regulate the expression of *per* and *tim*. As homozygotes, *cycle* mutants are arrhythmic owing to a lack of *per* and *tim* cycling and reduced levels of PER/TIM proteins. dCLK and CYC thus drive the expression of PER and TIM from E-box sequences within the *per/tim* promoters, a process inhibited by the PER/TIM dimer (Darlington et al., 1998; Lee et al., 1998).

While both *cyc* and d*Clk* are required for the clock to function, only the levels of dCLK, its phosphorylation and association with the PER/TIM complex are under circadian regulation (Lee et al., 1998; Rutila et al., 1998). CYC is constitutively expressed, yet *in vivo* most PER/TIM is not associated with either CYC or CLK. The primary role of CYC in the clock is probably as the preferred partner of dCLK, as within the adult head, most dCLK is found in association with CYC. Presumably CYC (and dCLK) have some other biological functions so there is no excess 'free' CYC interfering with the clock (Bae et al., 2000). *cyc* does not need to cycle as the fluctuating levels of dCLK limit its activity (Rutila et al., 1998).

Unlike *per* and *tim*, there is little if any delay between the RNA and protein cycles of *dClk* (Lee et al., 1998; Rutilla et al. 1998). The cycle of *dClk* RNA is clock controlled as in the absence of PER and/or TIM, d*Clk* levels do not fluctuate. The levels of d*Clk* RNA rise as PER/TIM enters the nucleus at night, and if this nuclear entry is delayed, then so is the increase in d*Clk* RNA and protein levels (Lee et al., 1998, Bae et al., 1998; Darlington et al., 1998). Therefore the clock requires that dCLK and CYC regulate PER and TIM

levels, and that PER and TIM also regulate dCLK levels (Bae et al. 1998; Lee et al., 1998).

The opposite phases of the *per/tim* and *dClk/cyc* RNA cycles and their mutual regulation ensures that when the levels of one pair of proteins are at their lowest, the others will be at their highest, maintaining perpetual daily RNA and protein oscillations. The negative feedback loop, and the resultant protein and RNA cycles are shown in figure 1.3.

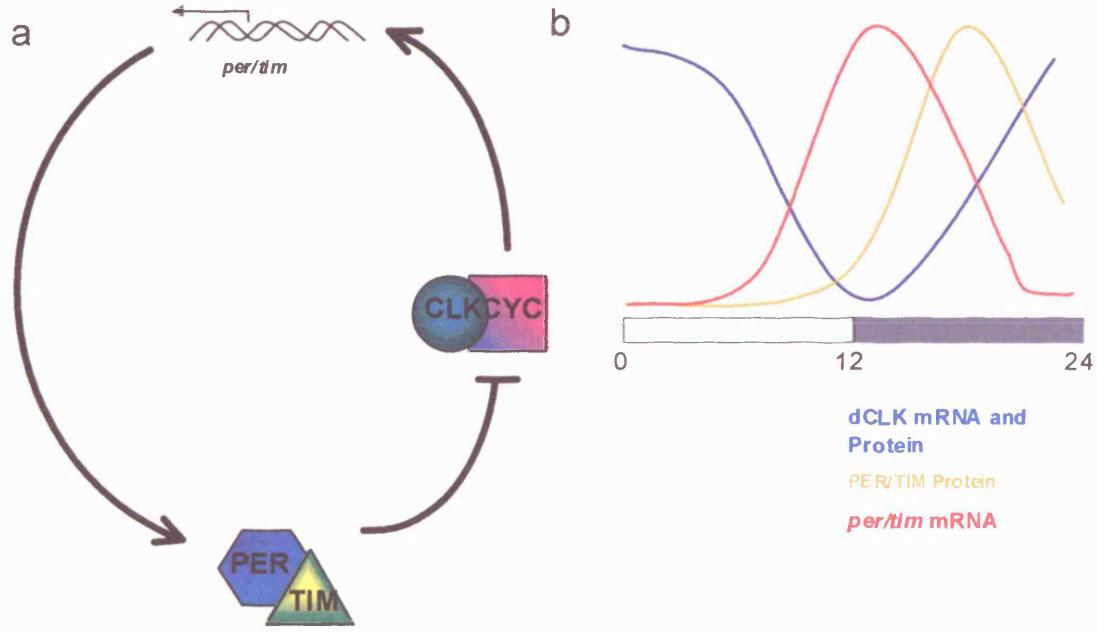


Figure 1.3. The dCLK/CYC and PER/TIM feedback loop. a). PER and TIM proteins regulate their own transcription by inhibiting the action of dCLK/CYC on the E-boxes of the *per* and *tim* loci. In this way, PER/TIM and dCLK levels cycle on a daily basis, out of phase with each other. b). In order for this to operate, when the levels of dCLK are at their highest, the levels of PER/TIM must be at their lowest. For further details, see text.

In vitro (Lee et al., 1999) and *in vivo* (Bae et al., 2000), PER and TIM, together or alone interact with the dCLK/CYC complex, impairing its E-box interaction without breaking the dCLK/CYC association. There is also evidence that the dCLK-CYC complex represses *dClk* expression (Glossop

et al., 1999). This means that when the PER/TIM complex interacts with the dCLK/CYC complex, it also produces an increase in *dClk* expression (Glossop et al., 1999), so in fly heads PER/TIM and CLK/CYC associate only during the night or early morning (Lee et al., 1998). The level of dCLK rises outside this time window, resulting in a rise in *per/tim* mRNA levels during the day.

Recent evidence suggests that once within the nucleus, PER no longer requires TIM to function (Rothenfluh et al., 2002). In S2 cells in the absence of TIM, PER lacking the CLD can enter the nucleus alone and repress CLK function. A construct encoding the PERΔCLD protein increases the incidence of arrhythmia in wild-type flies, which indicates the importance for rhythmicity of the delay in nuclear entry of the PER protein, and that PER alone is able to repress RNA expression (Rothenfluh et al., 2000). As a result, it may be that the primary role of TIM within the clock is to stabilise and transport PER into the nucleus, and to ensure that this process is regulated by light.

At dawn, the PER/TIM dimer is broken down, but monomers of PER persist within the nucleus for ~6 h. Once TIM has been degraded, there is not an immediate increase in *per* and *tim* levels, indicating that PER can repress transcription on its own. In the tim^{UL} mutant, the PER/TIM complex remains nuclear for an extended period of time which produces an ultra long free-running period of ~33 h. The levels of *per* and *tim* also remain high during this time, and only fall once the TIM^{UL} protein has been degraded by light. This indicates that the PER/TIM^{UL} complex is defective in repressive function, and once TIM^{UL} is degraded, the wild-type PER protein is able repress transcription alone (Rothenfluh et al., 2000).

The interaction between the PER/TIM and dCLK/CYC complexes generates the circadian feedback loop shown in figure 1.4.

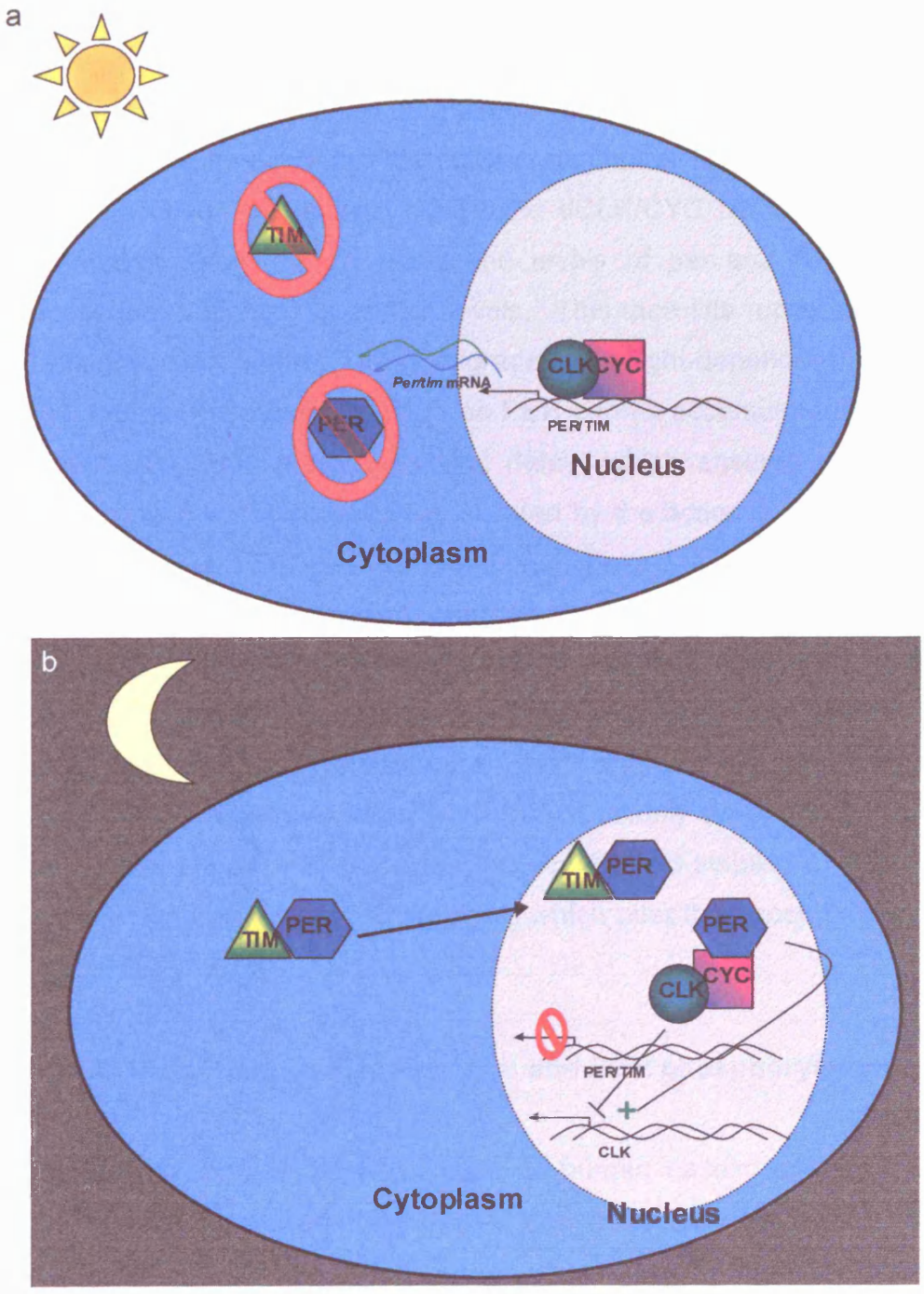


Figure 1.4 Nuclear localisation and the PER/TIM negative feedback loop. For details, see text

To conclude this section, the levels of *per* and *tim* transcription are under the control of the bHLH transcription factors dCLK and CYC (figure 1.4a). PER and TIM proteins begin to accumulate in the late evening and early night, where TIM stabilises PER and allows it to enter the nucleus (figure 1.4b). Once inside the nucleus, PER either with TIM or alone inhibits the induction of transcription of *per* and *tim* by the dCLK/CYC complex, and stimulates *dClk* expression. As a result, the levels of *per* and *tim* fall, leading to subsequent falls in the protein levels. This then lifts repression, and so the cycle can start again. TIM is degraded in a light-dependent manner, and is required for the stability of PER, so PER cannot accumulate until the evening under LD. This provides a vital delay, which ensures that *per* and *tim* transcription is not immediately inhibited by the action of the PER/TIM dimer. In DD, PER and TIM post-transcriptional controls take over and PER and TIM are degraded appropriately to generate the 24 h cycle.

In order to entrain this clock to a 24 h period, and adapt to seasonal changes in photoperiod and temperature, the core clock components are then regulated by a number of other elements, usually via phosphorylation, which can either increase or decrease the activity and stability of a given protein. Several kinases have been identified which alter the phosphorylation state of both PER and TIM.

1.5 DOUBLETIME/Casein Kinase Iε and PER phosphorylation

doubletime (*dbt)* is the homologue of human casein kinase Iε, and is the gene which is mutated in the *tau* hamster (Ralph and Menaker, 1988; Lowrey et al., 2000). *dbt* is also known as *discs overgrown,* which is an essential developmental gene, and as a result, null mutants of *dbt* are embryonic lethal (Zilian et al., 1999). *dbt* expression is not under circadian regulation at either the RNA or protein level, and DD has no effect on *dbt* levels or its phosphorylation (Kloss et al., 1998; 2001). Although null mutants are not viable, mutants which produce short and long periods have been identified

and shown to change the phosphorylation status of PER, altering its stability (Kloss et al., 1998; Price et al., 1998; Suri et al., 2000).

DBT is expressed within the adult brain, photoreceptors and LN_vs, and interacts with PER *in vitro* and in *Drosophila* S2 cells (Rothenfluh et al., 2000). Evidence suggests that DBT acts on PER and not TIM as the phosphorylation pattern of TIM in *dbt* mutants appears identical to that of wild-type flies (Suri et al., 2000). This is borne out by the analysis of the arrhythmic dbt^{ar} mutant, in which crosses to short *period* mutants restore rhythmicity in ~70% of flies (Rothenfluh et al., 2000). tim^{s} alleles do not rescue the rhythmicity of this line, indicating that the interaction is between DBT and PER not TIM. In this case, per^{s} must decrease and dbt^{ar} increase PER stability. Because PER is not degraded in dbt^{ar} mutants, the levels of monomeric TIM remain low as the PER/TIM dimer is always present to represses transcription (Rothenfluh et al., 2000).

The effect of DBT phosphorylation on PER stability can be determined by examining the effect of different *dbt* mutations. In pupal lethal dbt^{P} (low levels, but not loss-of-function DBT) mutants, PER is constitutively expressed in early pupae suggesting that DBT phosphorylation destabilises PER. In these mutants, *tim* RNA still cycles in LD as even though there is an excess of stable PER monomers, in LD cycles TIM is still degraded, regulating the nuclear entry of the PER/TIM dimer (Rothenfluh et al., 2000).

DBT is always associated with PER *in vivo*, and remains nuclear in per^{01} and tim^{01} mutants. This indicates that DBT follows PER in and out of the nucleus at the appropriate times of the day as part of a PER/TIM/DBT complex (Kloss et al., 2001). In dbt^{P} mutants in LL, both DBT and TIM are absent, but there is no increase in PER phosphorylation (Price et al., 1998). In tim^{01} mutants, or in tim^{UL} mutants after exposure to light, PER becomes phosphorylated through the PER-DBT association. This indicates that within the complex TIM prevents the phosphorylation of PER by DBT (Kloss et al., 2001). This then provides a mechanism for the daily resetting of the clock as at sunrise

TIM is degraded, which then frees PER for DBT phosphorylation, causing PER degradation at the beginning of the day (Kloss et al., 2001).

The regulation of the stability of PER by DBT is vital for the correct function of the clock. The dbt^s mutant delays the nuclear accumulation of PER, and in DD it produces a period 6 h shorter than that seen in wild-type flies (Bao et al., 2001). As nuclear accumulation is delayed even later in comparison to per^s flies, dbt^s presumably has an effect on period which differs from that of per^s. In dbt^s flies, the level of *per* mRNA increases later and decreases earlier than in wild-type flies. The most simple explanation is that dbt^s reduces nuclear PER levels earlier, prematurely derepressing its own RNA expression. This then accelerates *per* accumulation, and reduces the period (Bao et al., 2001).

In mammalian cell cultures it has recently been shown that the degradation of PER occurs after ubiquitination via the proteosome (Akashi et al., 2002; Yagita et al., 2002). F-box/WD40 proteins are known to target phosphorylated substrates in to the ubiquitin-proteosome pathway which is involved in the degradation of PER. There are three of these proteins within *Drosophila,* only one of which, SLIMB, has a clear circadian phenotype. SLIMB is found as a doublet within *Drosophila* cells, and there is evidence that a phosphorylated form of the protein associates with PER (Grima et al., 2002; Ko et al., 2002).

The role of *Slimb* seems to be in the degradation of PER once it has been phosphorylated by DBT. SLIMB preferentially interacts with, and *Slimb* overexpression increases the degradation of, DBT-phosphorylated PER, while RNAi knockout of SLIMB expression blocks PER degradation in the presence of DBT in S2 cells. This is mediated by the F-box as overexpression of SLIMB(ΔF), a mutant lacking the F-box, allows high levels of phosphorylated PER to accumulate in the presence of DBT. *Slimb* also alters the free running rhythm of the *D.melanogaster* clock with ~60% of flies overexpressing *Slimb* arrhythmic in DD. The overexpression of *Slimb(ΔF)* produces a similar phenotype to dbt^L mutants, with the evening peak not

occurring until after lights off, indicating there is a reduction in the degradation of PER (Ko et al., 2002).

dbt is only one of a number of casein kinases within *D.melanogaster* and it is not the only one with a clock function. The catalytic subunit of Casein Kinase 2α (CK2α) is expressed within the cytoplasm of circadian pacemaker neurons and phosphorylates PER *in vitro* (Lin et al., 2002). Heterozygous mutants of CK2 have lengthened periods due to delayed PER nuclear entry and levels of PER are increased. Like *dbt,* CK2α null mutants are homozygous lethal (Lin et al., 2002). Homologues of this gene play a role in the clocks of *Neurospora* and *Arabidopsis* (Suguano et al., 1998, 1999; Yang et al., 2001) and in the response to UV light in all eukaryotes, suggesting CK2α has a highly evolutionarily conserved function (Lin et al., 2002).

As well as being part of the clock, *doubletime, Slimb* and *casein kinase 2* are all components of the wingless developmental pathway. A fourth component of this pathway, *shaggy (sgg),* the orthologue of glycogen synthase kinase-3, also has a circadian role (Martinek et al., 2001). While DBT phosphorylation delays PER/TIM nuclear transfer, SGG has the opposite effect. SGG phosphorylates TIM and *sgg* overexpression advances nuclear entry, shortening the period of locomotor activity. SGG must either advance the PER/TIM association or nuclear localisation through TIM phosphorylation. As a result, the period is extended in *sgg* underexpressing lines as a higher level of PER/TIM is required before translocation occurs (Martinek et al., 2001).

1.6 Light entrainment of the clock

The initial response to light, the degradation of TIM, appears to be independent of visual photoreception (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). It seems instead to be dependent on a dedicated circadian photoreceptor, *cryptochrome* (*cry*), which has been demonstrated to associate with PER and TIM in a light-dependent manner (Stanewsky et al., 1998; Emery et al., 1998; Ceriani et al.,

1999; Rosato et al., 2001). CRY then seems to mediate the light dependent degradation of TIM, and also associates with the PER/TIM complex as it moves in and out of the nucleus (Stanewsky et al., 1998; Emery et al., 1998; Ceriani et al., 1999; Rosato et al., 2001). The role of CRY in the clock is fully discussed in the introduction to Chapter 5.

1.7 A model of the clock

A model of the core of the clock, and the roles of PER, TIM, dCLK, CYC, DBT, and CRY is shown in figure 1.5.

During the day light activates CRY, which leads to the degradation of TIM. In the absence of TIM, PER protein is unstable and is degraded by a pathway involving *Slimb* following DBT phosphorylation. This means that during the day dCLK and CYC are able to drive *per* and *tim* RNA transcription (figure 1.5a).

At night, CRY is no longer activated by light, so TIM is not degraded. As the levels of TIM increase, it dimerises with and stabilises PER. PER, TIM, DBT and CRY form a complex which is able to translocate into the nucleus. Within the nucleus, PER inhibits the action of dCLK and CYC, turning off the transcription of *per* and *tim* RNA. This produces a subsequent fall in PER and TIM protein levels. When TIM is degraded by light at dawn, this repression is lifted and the clock resets itself for a new day. While it is in the nucleus, PER also stimulates the transcription of d*Clk* mRNA, creating a second feedback loop (figure 1.5).

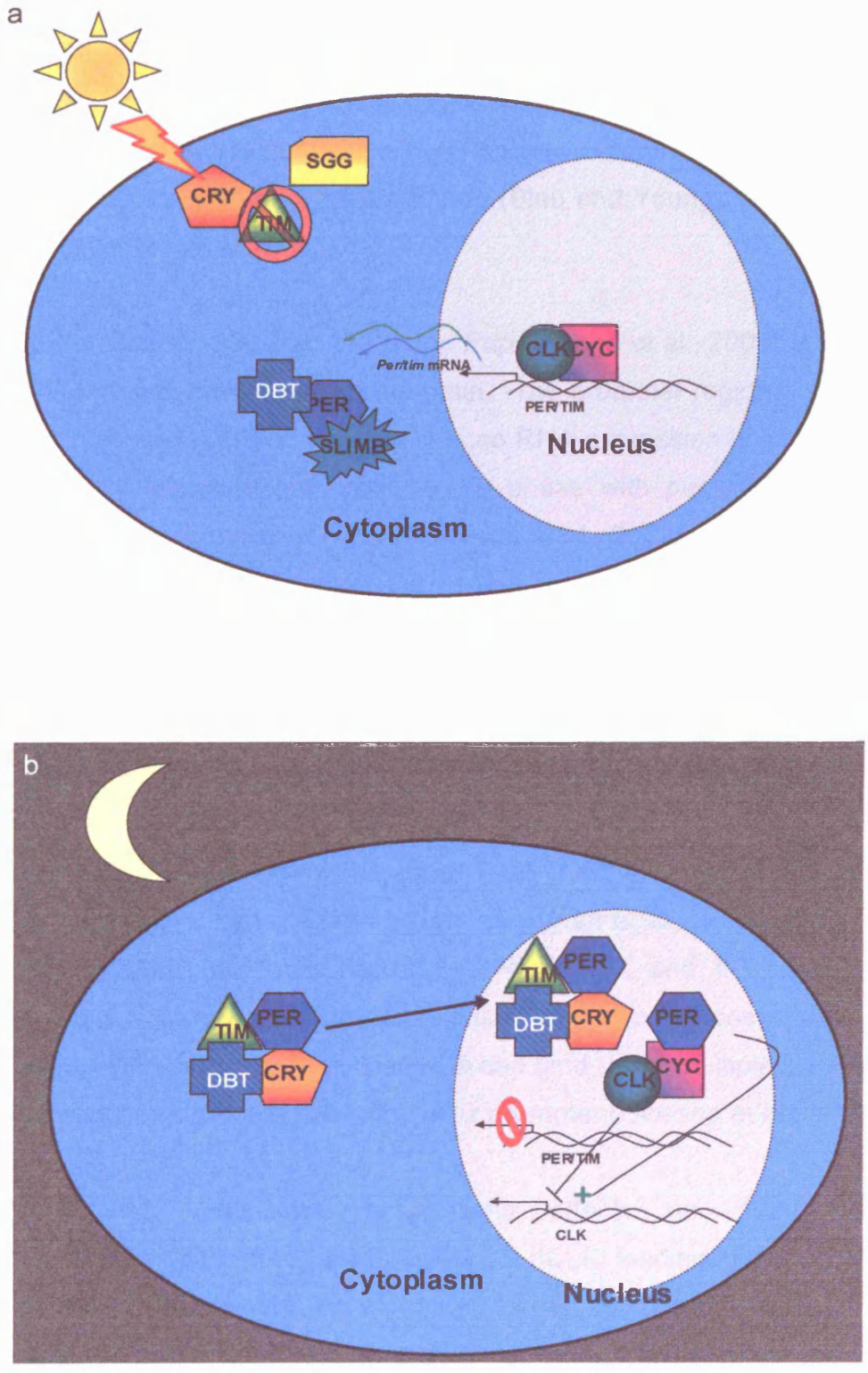


Figure 1.5 A model of the *D.melanogaster* circadian clock (see text)

1.8 VRILLE, PDP1 and the second circadian feedback loop

Recently two basic zipper (bZip) transcription factors *vrille* (*vri*) and *Par Domain Protein 1* (*Pdp1*) have been shown to control d*Clk* expression and form a second circadian feedback loop (Blau and Young, 1999; Cyran et al., 2003; Glossop et al., 2003).

Both *vri* (Blau and Young, 1999) and *Pdp1* (Cyran et al., 2003) are expressed within the pacemaker cells of the brain. The promoter regions of both genes contain consensus E-box sequences, so RNA expression is controlled by the dCLK-CYC complex and oscillates in phase with *per* and *tim* (Blau and Young, 1999; Cyran et al., 2003; Glossop et al., 2003). As the expression of both *Pdp1* and *vri* are under the control of dCLK/CYC, their mRNA's also cycle in phase with each other. However, peak levels of *Pdp1* are reached 3-6 h later, presumably owing to differences in RNA stability (Cyran et al., 2003). These differences are carried over into the protein cycle, where the peak level of VRI is reached earlier than that of PDP1 (Cyran et al., 2003).

vri and *Pdp1* share highly conserved DNA binding domains, suggesting that they bind to the same target sequences (Cyran et al., 2003). *vrille* is >85% homologous to the mammalian E4BP4 protein, and therefore would be expected to bind to a similar consensus sequence (Glossop et al., 2003). Mobility shift assays confirm that *vrille* can bind to the multiple E4BP4 binding sites which are present within the *dClk* promoter (Glossop et al., 2003).

PDP1 and VRI have antagonistic effects on d*Clk* expression. Overexpression of *vri* reduces the level of dCLK, leading to a reduction of *per* and *tim* expression and an elongated period (Blau and Young, 1999). A similar effect is seen in flies heterozygous for the *Pdp1* locus which also have longer free-running periods, and a reduced amplitude of *dClk* mRNA oscillations (Cyran et al., 2003). Thus it appears that PDP1 enhances and VRI represses dCLK expression.

The repressive capacity of VRI has been demonstrated by overexpressing *vri* in cyc^{01} mutants, where the level of *dClk* mRNA normally remains at the peak level at all times. If *vri* expression is induced by heat shock in these mutants, *dClk* mRNA levels fall, indicating that *vri* acts directly to repress *dClk* expression (Glossop et al., 2003).

As PDP1 and VRI compete for the same binding sites within d*Clk*, the ratio of PDP1 to VRI determines the cycle of *dClk* mRNA (Cyran et al., 2003). The difference between the protein cycles of VRI and PDP1 (VRI phase leads PDP1) ensures that dCLK levels cycle. When VRI levels are at their highest, PDP1 levels have not yet peaked. Therefore VRI represses *dClk* expression so at this time the levels of dCLK are at their lowest. As the level of VRI falls, the level of PDP1 is still rising, so the level of dCLK also begins to rise. This means that *vri, Pdp1* and *dClk* form a second feedback loop in the *D.melanogaster* clock (Cyran et al., 2003) as shown in figure 1.6

vri and *Pdp1* are also likely to control the expression of other mRNA's which cycle in phase with *dClk*. There is evidence that VRI regulates the expression of CRY as the *cry* locus contains several E4BP4 binding sites. VRI is able to bind to these sites, and the overexpression of *vri* reduces the abundance of the *cry* transcript *in vivo* (Glossop et al., 2003).

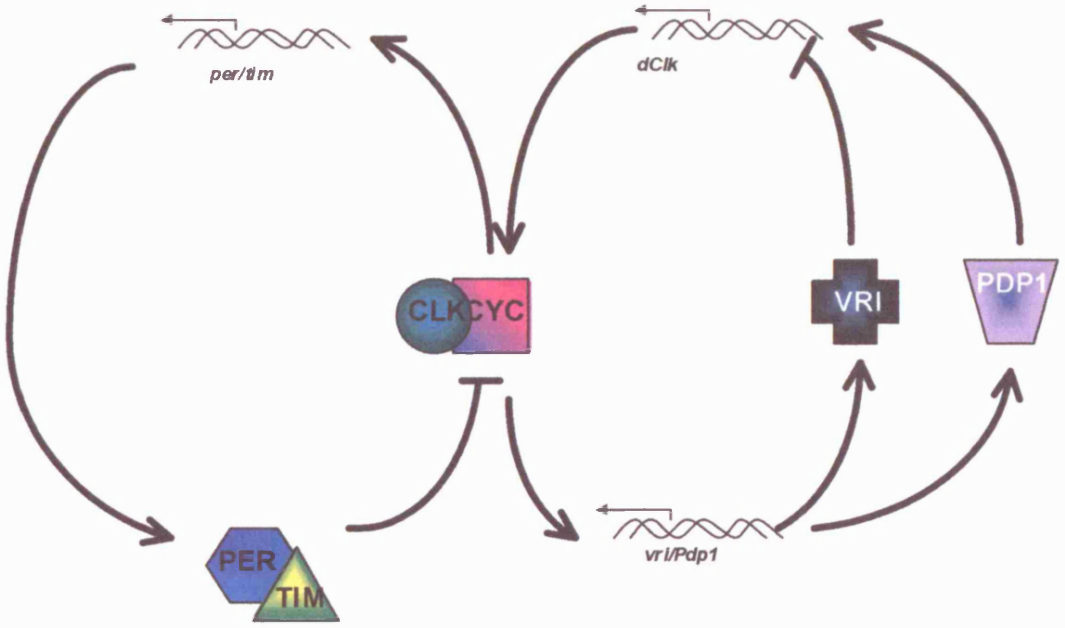


Figure 1.6. Two feedback loops of the *D.melanogaster* circadian clock. Adapted from Cyran et al., 2003. Figure 1.6 shows a model of how the two feedback loops now identified in the *D.melanogaster* clock interact. *per* and *tim* expression is under the control of the CLK/CYC dimer, and in turn, the PER/TIM complex inhibits the action of CLK/CYC on *per* and *tim* transcription. dCLK and CYC also drive the transcription of *vri* and *Pdp1*. VRI protein inhibits and PDP1 enhances *dClk* expression. As VRI and PDP1 compete for the same binding sites within the *dClk* locus, the relative levels of these two proteins determines the cycle of *dClk*. As d*Clk* expression and activity is regulated by the protein products of the mRNA expression it controls, the two feedback loops ensure that the levels of all the RNAs and proteins cycle on a daily basis (Cyran et al., 2003, see text).

1.9 Temperature entrainment of the clock

In order to be in tune with the environment, the circadian clock must respond to changes in temperature. Temperature pulses, like light pulses, induce rapid falls in the levels of PER and TIM proteins. This heat-induced degradation of the proteins seems to be the initial response to elevated temperatures (Sidote et al., 1998). This then alters *per* and *tim* cycling because of the disruption of the feedback loop, and in turn changes the phase of locomotor activity. However, light and heat pulses affect behaviour

in different ways. For instance, a light pulse at night leads to a significant period of reduction in TIM and then PER levels. In contrast, heat pulses induce an immediate reduction in PER and TIM levels, followed by rapid cycling of PER and TIM (Sidote et al., 1998). Additionally, heat pulses at night have no effect on the *per* and *tim* mRNA levels. One possibility is that TIM responds to both light and heat, whereas PER only responds to heat. However, the deletion of a region within the C-domain of PER alters the response of transgenic flies to light pulses, suggesting that there is a direct effect of light on the PERIOD protein as well (Sidote et al., 1998).

Heat pulses delivered to the fly in this way are not an accurate reflection of what goes on in the wild. Instead it is the effect of temperature and photoperiod on the RNA cycles of *per* (and to a lesser extent, *tim*) which provides a truer picture of how the clock adjusts to new climactic and seasonal conditions (Majercak et al., 1999; J.Majercak pers. comm), as discussed in the introduction to Chapter three.

1.9.1 Temperature compensation

A problem for the molecular clock is that it is a series of chemical reactions and protein interactions, and so like any biological system, it should run faster or slower depending on the temperature. Obviously a clock which tells different times in different climates or changes with the seasons is of no use, so the clock has an in-built mechanism to cope with temperature fluctuations so that it always keeps time across the organisms physiological range. This is known as 'temperature compensation', and a well temperature compensated clock will maintain a period close to 24 h across a wide a range of climates (for a review see Hall, 1997).

Pittendrigh first identified temperature compensation as a 'functional prerequisite of a good clock' long before the isolation of the first circadian mutant. By examining the time of eclosion of *D.pseudoobscura* at different temperatures he found that this was unaffected by 10°C drops in temperature (Pittendrigh, 1954; Zimmerman et al., 1968). Thus he demonstrated that the

circadian clock of flies was temperature compensated. Mammalian clocks were later shown also to be temperature compensated (Menaker, 1959; Menaker and Wisner, 1983; Francis and Coleman, 1988). Temperature compensation is therefore also a fundamental property of the clocks of warm-blooded organisms, enabling them to cope with daily fluctuations in body temperature.

A number of mutants of *per* and *tim* have been identified which affect both the period length and temperature compensation but *per* seems to be the key gene. For example, the delay in nuclear entry of per^{L} is temperature sensitive and alters the temperature compensation phenotype of the fly. At higher temperatures the interaction between PER^{L} and TIM is weaker than between TIM and wild-type PER (Gekakis et al., 1995). tim^{SL} restores temperature compensation and the 24 h period of the per^{L} mutation, and also reduces the delay in nuclear accumulation to wild-type periods (Rutila et al., 1996).

It is also possible that the DBT phosphorylation of PER is required for temperature compensation (Bao et al., 2001). When dbt^{s} is combined with per^{s}, the resultant double mutant regains its temperature compensation (Bao et al., 2001). tim^{rit}, a long period mutant displays an increase in period with temperature by increasingly delaying/abolishing the nuclear accumulation of PER. A tim^{+} transgene restores the temperature compensation phenotype, while leaving a long period, suggesting that tim^{+} is also required for temperature compensation (Matsumoto et al., 1999). The rescue of the temperature compensation by one copy of *tim* could be because one copy of *tim* has enough function to take over driving the clock and interact normally with PER, but because there is less *tim* transcribed from only one copy of the gene, the period is longer as it takes longer for TIM to accumulate. The most curious feature of this mutation is that the tim^{rit} phenotype is rescued by the overexpression of *per,* which is probably because although TIM^{rit} is less stable, in excess PER it can translocate into the nucleus (Matsumoto et al., 1999).

Although the mechanism of temperature compensation is not yet well understood, a well temperature compensated clock is clearly important to *D.melanogaster.* Evidence for this comes from a study of the temperature compensation of variants of the Thr-Gly repeat region within the *per* gene. In natural populations of *D.melanogaster*, there is length polymophism within *per* which encodes threonine and glycine repeats (Thr-Gly repeat region). This region is involved in determining the species specificity of song cycles (Wheeler et al., 1991) and the thermostability and temperature compensation of circadian rhythms (Yu et al., 1987; Ewer et al., 1991). Although a *per* gene lacking this region is able to rescue the rhythmicity of per^{01} mutants, the quality of rescue decreases as the temperature is increased (Yu et al., 1987). The role of the Thr-Gly repeat appears to be structural, as in wild populations ~99% of individuals carry a number of repeats within a series of 14-17-20-23. Repeat lengths outside this series e.g. $(Thr\text{-}Gly)_{21}$ are uncommon, suggesting that the $(Thr\text{-}Gly)_3$ repeat motif has a functional role within the protein (Costa et al., 1992).

The two most abundant alleles that encode 17 and 20 Thr-Gly variants, are distributed along a latitudinal cline from North Africa up into Northern Europe (Costa et al. 1992). As there is no longitudinal distribution cline, changes in climate from North to South are probably responsible for this distribution. The $(Thr\text{-}Gly)_{17}$ and $(Thr\text{-}Gly)_{20}$ alleles show differing abilities for temperature compensation, and this may determine their distribution, in that the $(Thr\text{-}Gly)_{20}$ allele is better temperature compensated and is more abundant in Northern Europe. This is presumably because the North of Europe is more temperate, and the fly is likely to encounter a wider range of temperatures. The $(Thr\text{-}Gly)_{17}$ allele is less well temperature compensated, but at 29°C it has a free running period very close to 24 h, and is more abundant in Southern Europe (Sawyer et al., 1997).

1.10 RNA cycling and entrainment

In wild-type flies there is a ~6 h delay between *per/tim* transcription and translation which was for a long time assumed to be vital for the maintenance of daily molecular rhythms (Hardin, 1992). It has subsequently been shown that both the cycling of *per* mRNA and the delay between RNA and protein cycles are not required for rhythmicity, but are required to generate normal behaviour and a 24 h period (Cheng et al., 1998; Suri et al., 2000).

In dbt^{H} and Dbt^{G} long period mutants, there is almost no advance in the phase of the *per* and *tim* RNA cycles ahead of their proteins, but these flies remain rhythmic (Suri et al., 2000). In these *dbt* mutants, PER stability is increased, so that in LD cycles PER and TIM disappearance is delayed. This increases the time during which *per* and *tim* transcription is repressed, so that the levels of RNA rise at the same time as the levels of proteins. In DD these *dbt* mutants have a longer period because there is no longer light induced degradation of TIM and therefore the lag between RNA and protein is restored. This indicates that the delay between RNA and proteins is not required for the robust cycling of *per/tim* RNA, but may be required to generate a 24 h period. It also suggests that the delay before nuclear entry is sufficient to prevent the levels of PER and TIM negatively autoregulating their own transcription and shutting down the clock (Suri et al., 2000).

Rhythmicity does not require a cycle of *per* expression as constitutive transgenic *per* expression within the photoreceptors, does not prevent rhythmic PER expression in either LD or DD (Cheng and Hardin, 1998). This means that the cycling of TIM is sufficient to drive PER rhythms. This is confirmed by examining *per* over-expression which produces high levels of PER, and an early peak in PER levels, but nuclear entry is only slightly advanced, because TIM levels are limiting. Although constitutive PER expression is sufficient for rhythmicity, locomotor activity is weaker than usual, and a stable free-running period is not maintained (Cheng and Hardin, 1998).

A *tim* RNA cycle is also not required to generate rhythms in TIM protein. In DD at 18°C the *tim* RNA cycle quickly breaks down, but the protein continues to cycle (Majercak et al., 1999). In tim^{01} lines, heat shock control of *tim* expression for a 30 minute period each day is enough to give (aberrant) rhythmic behaviour (Suri et al., 1999).

Although flies can be rhythmic in the absence of *per* and *tim* mRNA cycles, these cycles are required to generate normal rhythmic locomotor behaviour. A direct link between the mRNA cycle and locomotor activity was demonstrated by heat-inducing *per* expression. In this case, heat induction shifts the phase of *D.melanogaster* locomotor behaviour (Edery et al., 1994; Frisch et al., 1994). Therefore the regulation of the RNA cycle can be used to alter patterns of behaviour. It has subsequently been suggested that the regulation of *per* RNA levels in response to temperature changes is one way in which the fly is able to alter behaviour to cope with different seasonal conditions (Majercak et al., 1999; discussed in Chapter 3).

The level and cycle of *per* mRNA is controlled by photoperiod and temperature, adjusting the clock to different seasonal conditions. The key light signal controlling *per* RNA cycles is 'lights off', as the level of *per* peaks ~4 h after this event (Qiu and Hardin, 1996). The cycling of *per* is dependent on there being at least 6-8 of darkness every day - without this, the levels of *per* are similar to those seen in per^{01} and tim^{01} flies, an intermediate level between the peak and trough amounts. As a result, it seems that a period of daily darkness is required for normal clock function. In short photoperiods (≤LD 8:16), the *per* peak occurs no later than ZT20, and therefore is no longer four hours after lights off. As TIM is light sensitive, it may be that this is the minimum amount of darkness required to allow TIM to accumulate, stabilise PER, and translocate into the nucleus to run the clock (Qiu and Hardin, 1996).

Upon entering a new photoperiod, the RNA peak entrains to the new regime within one day and locomotor activity within two days, with the evening peak

occurring ~1 h prior to the lights off signal. This is because the mRNA level peaks ~5 h after this so it can only have an effect on locomotor activity on the subsequent day (Qiu and Hardin, 1996). There seems to be a link between the molecular and behavioural peaks as there is a constant phase difference between them even in different light regimes (Qiu and Hardin, 1996). Temperature changes also alter the cycling of both *per* and *tim* RNA. At low temperatures in LD, the position of the peak RNA level of both *per* and *tim* is advanced. Additionally, the overall level of *per* RNA is increased, while that of *tim* is reduced (Majercak et al., 1999). This means that the RNA cycle acts as a memory of the previous day's daylight and temperature conditions.

At the protein level, temperature also modulates the cycle. This is difficult to see in LD, but in DD, although the RNA cycles are advanced for 2-3 days at cold temperatures, the PER/TIM cycle is only advanced during the first day of DD (Majercak et al., 1999). Therefore, in LD cycles, the effect of the cold induced *per* mRNA advance is counterbalanced by the light induced degradation of TIM (TIM is required for PER stability). At higher temperatures, PER accumulates to higher levels, even though the level of *per* RNA is lower, as the TIM level is elevated and therefore more PER is stabilised (Majercak et al., 1999). Temperature and light also regulate the splicing of an intron within the 3' UTR of *per* (Majercak et al., 1999 and reported herein). This regulation of splicing modifies the cycling of *per* RNA in a seasonal manner, and determines the position of the evening activity peak, as discussed at the beginning of Chapter 3.

1.11 Clock outputs

In order to control behaviour, the clock must affect the expression of other genes at the transcriptional and post-transcriptional level. A number of genes involved in the output pathway of the clock have been identified by looking for mutants with normal cycling of PER and TIM and distorted or arrhythmic behaviour.

lark is a vital RNA binding protein, which is required for circadian eclosion but not circadian locomotor behaviour (Newby & Jackson, 1993). Lark seems to

function as a repressor protein which limits the hours during which adult flies can eclose. Null mutants of lark display arrhythmic eclosion, while overexpression delays the timing of this event (Newby & Jackson, 1996). It is therefore likely that *lark* is involved in the post-transcriptional regulation of genes in the output to eclosion pathway of the clock. *lark* RNA does not cycle (Newby & Jackson, 1996) and instead LARK activity is regulated by the clock at the post-transcriptional level (McNeil et al., 1998). The lark protein is under circadian control; a daily cycle in abundance of LARK protein is lost in per^{01} mutants. Therefore the clock controls the levels of LARK which in turn regulates the levels of various downstream RNAs. This then leads to eclosion being gated to a certain time of day (McNeil et al., 1998).

PDF, a homologue of crustacean PDH is a neurotransmitter/hormone which keeps pacemaker neurons and thus activity in phase (Park et al., 2000a). Wild-type PDF is expressed within the four small lateral neurons (s-LN_vs) and the four large lateral neurons (l-LN_vs), which are known to be the location of the pacemaker (see figure 1.2), making PDF a candidate neurotransmitter for the clock (Park et al., 2000a). pdf^{01} mutant flies have an evening peak which is ~1 h early and no morning peak anticipation in LD (Park et al., 2000a). In DD, there is a high incidence of arrhythmicity of hetero- or homozygous pdf^{0} flies (Renn et al., 1999). Misexpression extends the evening peak into the night in LD and, in DD leads to increased activity and complex behavioural patterns arrhythmicity (Helfrich-Forster et al., 2000). The fact that aberrant PDF mutants are not arrhythmic is probably because of the existence of other neurotransmitters. Expression of PDF in neurons outside the central brain has no effect on behavioural rhythmicity (Helfrich-Forster et al., 2000).

pdf RNA does not cycle, so cannot be under circadian regulation (Park et al., 2000a), instead the clock appears to control the cellular localisation of PDF within the brain as the nerve terminals within the brain show day-night cycles of PDF levels. This cycle is dependent on *per* and *tim,* but not on the RNA levels of *pdf* (Park et al., 2000a)

Because flies with ablated PDF-expressing neurons (LN_vs) and pdf^{0} flies still show low levels of rhythmicity in DD, there must be other neurons which

contribute to driving rhythmicity (Renn et al., 1999) such as the *per* and *tim* expressing neurons which do not express PDF (Helfrich-Forster et al., 2000).

Several mutations affecting behaviour have been identified in components of the cAMP-signalling pathway. Mutations in the major catalytic subunit of cAMP-dependent protein kinase 1 (Pka-C1) show normal, entrainable *per* cycling, but abnormal circadian rhythms and DD arrhythmicity. In this case, the loss of PKA, a signalling molecule in the cAMP messenger pathway leads to behavioural arrhythmicity (Majercak et al., 1997). *dCREB2* is the *Drosophila* homologue of CREB/CREM, the **c**AMP **R**esponse **E**lement **B**inding protein. It is known to have a role in many behavioural responses, including drug addiction, stress, memory and mammalian circadian rhythms (Ginty et al., 1993; Stehle et al., 1993; Foulkes et al., 1996). It is possible that dCREB2 regulates *per* expression as there are a number of CREB binding sites within the *per* promoter (Belvin et al., 1999). However, the analysis of *Neurofibromatosis-1 (Nf1)* mutants indicates that dCREB2 is primarily involved in the output of information from the clock (Williams et al., 2001). *Nf1* is involved in cAMP signalling, and all defects caused by mutating this gene are rescued by up-regulating cAMP-dependent signalling levels. Mutants of *Nf1* are almost all arrhythmic in DD, but there is no effect on *per* and *tim* levels, and PER and TIM cycle normally within the LN_vs. *Nf1* must therefore act downstream of the clock to alter behavioural rhythms. It is likely that PDF activates a Ras/MAPK pathway via *Nf1* (Williams et al., 2001).

Finally, *dFMR1*, the homologue of the mammalian RNA binding protein FMR1, the loss of which is the cause of human fragile-X syndrome is also involved in the circadian output. *dfmr1* is required for normal circadian and courtship behaviour, and loss of function mutants are arrhythmic in DD but show periods of hyperactivity. In LD the flies are rhythmic and anticipate lights off, but display occasional abnormal bursts of activity during the day (Dockendorff et al., 2002; Inoue et al., 2002).

1.12 The mammalian clock

The study of the mammalian clock has been aided by its remarkable similarity to the circadian clock of *D.melanogaster.* As the mouse has the best understood mammalian clock, all comparisons made here are between the clock of *Mus musculus* and *D.melanogaster.* Both circadian oscillators are based upon remarkably similar autoregulatory feedback loops involving more or less the same proteins in similar, if not identical roles. These loops utilise the regulation of the nuclear entry of a protein complex, which represses transcription and responds to daily changes in light, to maintain a 24 h period.

The clock of the mouse is located within the suprachiasmatic nucleus (SCN) (Klein et al., 1991; Weaver, 1998), which as well as driving activity rhythms, also synchronises all peripheral clocks. So far eight genes of the mammalian clock have been identified, all of which have fly clock gene homologues. These are the three homologues of *per* (*mPer1-3),* Casein Kinase 1ε (CKIε) which is the homologue of *dbt* (CKIδ also plays a role in the mammalian clock), two homologues of *cry* (*mCry1* and *2*), *Clock (mClk)*, and *Bmal1/Mop3,* the mammalian homologue of *cycle.* There appears to be no role for the homologue of *D.melanogaster tim*[1] (e.g. Field et al., 2000), and the gene that was identified as *mTim* is actually the homologue of *D.melanogaster tim2* that currently has no known circadian function (Benna et al., 2000). Instead the role of *tim* in the *D.melanogaster* clock is carried out by the other clock proteins in the mouse.

There appears to be some overlap of function in mammals of the multiple homologues of the *D.melanogaster* clock genes as it takes longer for arrhythmicity to become manifest in mammalian clock mutants in DD (Vitaterna et al., 1994; Zheng et al., 1999; Bae et al., 2001). It is also more difficult to 'knock out' the clock in mice - so far the only arrhythmic mice

[1] TIM1 homologues have recently been shown to be involved in chromosome cohesion in *C.elegans* (Chan et al., 2003; Golden and Cohen-Fix, 2003). As yet unpublished data has also suggested that mTIM1 may play a circadian role.

identified are either knockouts of both *mCry* genes (van der Horst et al., 1999; Vitaterna et al., 1999), *Bmal1* (Bunger et al., 2000) or *mPer1 and 2* (Bae et al., 2001).

The roles of *CKIε* and *CKIδ* in the mammalian clock are not yet completely clear, although they have been shown to bind and phosphorylate all three mammalian *per* proteins, and are essential for clock function (Lowrey et al., 2000). They may have a similar role to *dbt* and regulate the degradation of the PER complex via the proteasome (Akashi et al., 2002), possibly in a similar manner to the *Slimb* dependent PER degradation in *D.melanogaster* (Ko et al., 2002; Grima et al., 2002).

In the feedback loops of the mammalian clock, *mClk* and *Bmal1* fulfil the same roles as in *D.melanogaster,* driving the transcription of the *mPer* and *mCry* genes. However, their roles are reversed, with *Bmal1* cycling in abundance, and it is the level *Bmal1 rather* than *mClk* which is limiting (Shearman et al., 2000; Tamaru et al., 2000; Yagita et al., 2001; Lee et al., 2001). Despite the absence of a cycle in abundance, the activity of *mCLK* is regulated through altered patterns of phosphorylation of two different CLK isoforms (Lee et al., 2001). The levels of *mCRY* and *mPER* cycle synchronously under the control of m*Clk* as expression is reduced and does not cycle in m*Clk* mutants (Miyamoto and Sancar, 1998; Kume et al., 1999). The other half of the feedback loop involves *mPer2*, *mCry1* and *mCry2,* as when they are at their most abundant, transcription levels are at their lowest (Hastings et al., 1999; Kume et al., 1999).

mPER1 and mPER2 translocate mCRY1 and mCRY2 into the nucleus, and mutants for both of these *per* genes show cytoplasmic localisation of *mCrys* and *vice-versa* (Lee et al., 2001). This forms the driving force behind the mammalian clock. The timed nuclear entry of a mCRY/mPER/CK1ε/CK1δ complex (Lee et al., 2001) inhibits the initiation of *mCry* and *mPer* transcription by the BMAL1/mCLK complex, much the same as the PER-TIM complex of *D.melanogaster* inhibits the action of dCLK/CYC. Within the nucleus, the role adopted by *dPer* is divided between the three mammalian

homologues of *per* and the two of *cry*. In fact *mCry1* and *mCry2* are more important to the feedback loops of the mammalian clock than the *mPer's*, perhaps mirroring their core clock function within the peripheral clocks of *D.melanogaster* (reviewed in Stanewsky, 2002). Although the *mPers* form complexes with *mCRY1* and *2,* the *mCry* protein is sufficient to inhibit the action of the *mCLK/BMAL1* complex (Shearman et al., 2000; Kume et al., 1999) and *mPer2* has only a small inhibitory effect on the BMAL1/mCLK complex. Instead the role of *mPER2* seems to be within the positive leg of the feedback loop where it enhances the expression of *BMAL1* (Shearman et al. 2000; Zheng et al., 1999)

This is not to say that the *mPer's* are not vital for rhythmicity as homozygous mutants of *mPer1* and *mPer2* show disrupted locomotor rhythms, and *mPer1/mPer2* double mutants are arrhythmic (Bae et al., 2001). All three *mPer* genes display circadian phenotypes, the most subtle of which being the slight reduction in free running in mutations of *mPer3* (Shearman et al., 2000). As double mutants of *mPer3* and either *mPer1* or *mPer2* have the same phenotype as the *mPer1* or *mPer2* single mutant, *mPer3* probably has a role outside the core of the clock (Bae et a, 2001; Shearman et al., 2000). There is good evidence that one key role of *mPer1* and *mPer2* may be in light entrainment of the clock, a role taken by CRY and TIM in *D.melanogaster.* Elimination of each *mPer* gene alters the ability to respond to light pulses, but does not prevent entrainment to LD cycles (Albrecht et al., 2001; Bae et al., 2001), which is somewhat analogous to the effect of the cry^b mutation on the behaviour of *D.melanogaster*.

1.12.2 Entrainment by light

Like *D.melanogaster,* light reception by the mammalian circadian clock uses both ocular and non-ocular photoreception, but the structures involved differ from those involved in vision as the clock still responds to light in rodless and coneless mice. This reveals the location of the photoreceptor as being within the inner retina (Lucas et al., 1999; Freedman et al. 1999). Melanopsin is an opsin found in a subset of cells within the retina of mammals known to be

required for circadian entrainment, but so far evidence for the role of melanopsin in entrainment is largely circumstantial (Berson et al., 2002; Gooley et al., 2001; Hannibal et al., 2002; Hattar et al., 2002). Indeed, the latest data suggests that while melanopsin plays a role in the mammalian circadian response to light, it is not essential for entrainment or the response to light pulses (Ruby et al., 2003; Panda et al., 2002). This suggests that within mammals, multiple photoreceptors transmit photic information to the clock[2]. Given that CRY is involved in entrainment in other clocks, the mammalian homologues are also candidate circadian photoreceptors (Selby et al., 2001).

It turns out that as well as being core clock components, *mCry1* and *mCry2* are also required for entrainment, with rodless, coneless mice that are mutant for both *Cry*'s being arrhythmic even in LD cycles (Selby et al.., 2001). This is analogous to the $glass^{60j}$ cry^{b} double mutant of *D.melanogaster* which is also unentrainable (Helfrich-Forster et al., 2001) and indicates that using both the eyes and cryptochrome to entrain the clock is a common mechanism. The precise role of mammalian CRY proteins in the light response of the clock is as yet unknown.

Within *D.melanogaster,* the initial response induced by light pulses or 'lights-on' is down-regulation of CRY and a rapid degradation of TIM, which is then followed several hours later by the degradation of PER, and in this way the clock is reset on a daily basis. Within the mouse, light has no effect on the levels of either mTIM protein or *mCry* transcription indicating that mammals utilise a different light entrainment pathway (Field et al., 2000). One way in which light pulses set the clock is to induce expression of *mPer1* and *mPer2* within the SCN, which then leads to elevated mPER1 and mPER2 protein levels (Field et al., 2000). The response of mPER1 protein and RNA levels to

[2] Recent work generated mice lacking melanopsin and disabled rod and cone phototransduction, but with intact retina (Hattar et al., 2003). These mice could not, amongst other phenotypes, be entrained to LD cycles, wheras this is not the case for rodless coneless mice alone. Therefore the rod-cone system and melanopsin provide all the light input required for entrainment, and melanopsin plays a vital role in this entrainment (Hattar et al., 2003).

light pulses is much greater than that of mPER2 (Field et al., 2000), and the induction of *mPer1* expression allows both phase advances and delays in response to light pulses. How this occurs exactly is unclear, as mice injected with anti-sense *mPer1* RNA do not phase delay in response to a light pulse (Akiyama et al., 1999) but deletions of *mPer1* prevent a phase advance in response to light pulses without affecting the phase delay (Albrecht et al., 2001). *mPer2* may play a greater role in mediating phase delays as this response is lost in mutant mice (Albrecht et al., 2001).

BMAL1 may also play a role in the way in which light regulates the clock as the level within the SCN is significantly reduced in response to light pulses delivered during the night (Tamaru et al., 2000). This is a response which is very similar to that of dTIM in response to light, suggesting that BMAL1 may carry out this role of TIM in mice. Interestingly, *mPer1* and *mPer2* induction occurs concurrently with BMAL1 degradation even though BMAL1 is part of a complex which regulates *mPer* transcription.

1.12.3 Of mice and flies

Differences in the control of clocks between diptera and mammals have arisen to cope with the different demands encountered during evolution from some long-lost clock-bearing ancestor. This has lead to duplications of some clock components within mammals, and the roles of individual genes have become somewhat mixed up in the different systems. Remarkably almost all the core clock genes of *D.melanogaster* have homologues within the mouse which have retained a circadian role, and both systems are based on a negative feedback loop based on BMAL1/CLK induced transcription.

The one exception to this is that mTIM has (as yet) not been attributed a circadian function within mammals despite being a cornerstone of the insect clock. It could be that this is because it has no role in the mammalian clock, but to date the relevant experiments have not been done as mTIM knockouts are lethal in mice (Gotter et al., 2000). It therefore remains to be seen whether TIM is part of the ancestral clock or is a novel gene which has

acquired clock functions only within the flies. In *D.melanogaster,* TIM is required to stabilise PER, and allow nuclear translocation. It is also degraded in response to light, resetting the clock, via an interaction with CRY. In the mammalian clock, these same events are still required for rhythmicity, and therefore several other proteins have equivalent roles to those of TIM in the *D.melanogaster* clock.

mPER2 forms a complex with mCRY1/mCRY2 and the two Casein Kinases, which then translocates into the nucleus. Once within the nucleus, this complex sets up the negative feedback loop by inhibiting BMAL1/CLK induced transcription (reviewed in Stanewsky, 2002). The resetting of the clock by light probably involves the rapid degradation of BMAL1, as this protein is degraded in response to light pulses (Tamaru et al., 2000). This may be the equivalent of the light induced, CRY dependent degradation of TIM seen in *D.melanogaster.*

All the information gleaned from the study of clocks in other species should eventually lead to an understanding of how the human clock works, with all its roles from insomnia to learning. So far only one clock mutant in humans has been identified. This mutation within hPER2 was identified in a large family who suffered from a condition known as familial advanced sleep-phase syndrome (FASPS). The mutation occurs at a phosphorylation site and reduces the ability of casein kinase 1ε to phosphorylate hPER2, and as a result, carriers wake up at 4.30am and fall asleep at 7.30 - the clock is running about 4 h fast. (Toh et al., 2001).

1.13 The clock and non-locomotor behaviour

Within the output pathway of the *Drosophila* clock, there are several genes such as *creb* that have been identified as being involved in memory, drug addiction and stress (Ginty et al., 1993; Stehle et al., 1993; Foulkes et al., 1996). However, the role of the clock, if any, in these complex behaviours is not yet understood. Circadian rhythms have been demonstrated to play an important role in the reproductive cycle of the fly as rhythms in mating activity

are lost if the mating involves per^{01} or tim^{01} females. Different species have different mating rhythms, so this could be a factor in sexual isolation driving speciation (Sakai & Isgida, 2001; Tauber et al., 2003). The loss of the clock in the testes of *D.melanogaster* has been demonstrated to reduce male reproductive fitness, with 40% fewer progeny from each mating (Beaver et al., 2002). More intriguingly, recent work in *D.melanogaster* has shown that the circadian clock may be important in social situations as flies kept together in DD keep their clocks in phase, communicating via the olfactory system (Levine et al., 2002). If arrhythmic flies, or flies which have short or long period mutants ('visitors') are introduced into the population ('hosts'), then the pre-existing synchrony of the host's behaviour is disrupted. If populations are combined, early visitors (6 h advanced phase) have a significant effect on the phase of their late hosts, but not vice-versa. The air circulating around one group of flies can also alter the rhythms of a second set of flies, demonstrating the olfactory nature of this response. As flies are able to communicate timing to other flies, there must be an advantage within a population to having synchronised clocks (Levine et al., 2002).

In animals with more complex social behaviour, the role of the clock becomes even more important, as demonstrated in the honeybee (Toma et al., 2000). Worker bees generally spend the first part of their adult life working in the nest, and the end of their lives as foragers outside the hive. The age at which this change occurs depends on the needs of the hive and is under environmental, genetic and social control. Within the hive, tasks are done arrhythmically, and mutant bees which have an earlier onset of rhythmic behaviour within the hive go on to forage at a younger age. As this development occurs, the level of *per* expression increases, presumably preparing the clock for foraging. Forager honey bees have a highly developed circadian clock, which they use for navigation using the sun, to time visits to flowers to get the most nectar and to communicate this information to other individuals. Thus the timing of the onset of circadian rhythmicity is one factor which allows the bee to move from one phase of life to another (Toma et al., 2000).

1.2 Aims of the project

In order for the clock to set the appropriate pattern of behaviour, it must also respond to seasonal changes in temperature. Majercak et al. (1999) demonstrated the role of the regulation of alternative splicing in the seasonal response of the clock. As it is likely to be important to be able to alter locomotor activity in response to seasonal changes, it would be predicted that the regulation of splicing would be under a tight system of control. Seasonal changes in temperature come with associated changes in photoperiod, so different light regimes might also be expected to set behavioural patterns.

The aim of this project was to see how this alternative splicing event is controlled by temperature and light. This involves examining locomotor activity and splicing levels in different mutant backgrounds at different temperatures and light regimes. Thus the hypothesis is that if the regulation of behaviour is important, splicing should be tightly controlled. Additionally, splicing is repressed at high temperatures, where the day length is likely to be longer, light should also repress splicing. Therefore it should be possible to identify how light information reaches the splicing machinery, and one aspect of how light entrains behaviour.

As well as allowing the fly to adapt to different seasonal conditions, the ability to adjust behaviour to suit different temperatures may also allow *D.melanogaster* to live in a wide range of different climates. Other species closely related to *D.melanogaster* are found in a narrower range of habitats and/or are less able to cope with variable temperatures. It was therefore also decided to see how other species cope with increased temperatures, and whether they regulate the *per* RNA cycle in the same manner as *D.melanogaster.* The hypothesis here is that if there are differences in the ability to adapt behaviour to different seasonal conditions between species, they may correlate with post-transcriptional differences. The first stage of this was to investigate the role that the clock plays regulating the alternative splicing of the *per* RNA transcript, as discussed in Chapter Three.

Chapter two: materials and methods

2.1 Basic molecular techniques

All buffers were prepared and basic molecular techniques (e.g. agarose gel electrophoresis, enzyme digestions etc) performed as in Sambrook et al. (1989) unless otherwise specified. All protocols carried out using kits followed the supplied instructions, unless otherwise stated.

2.2 Rearing *D.melanogaster, D.pseudoobscura, D.virilis*

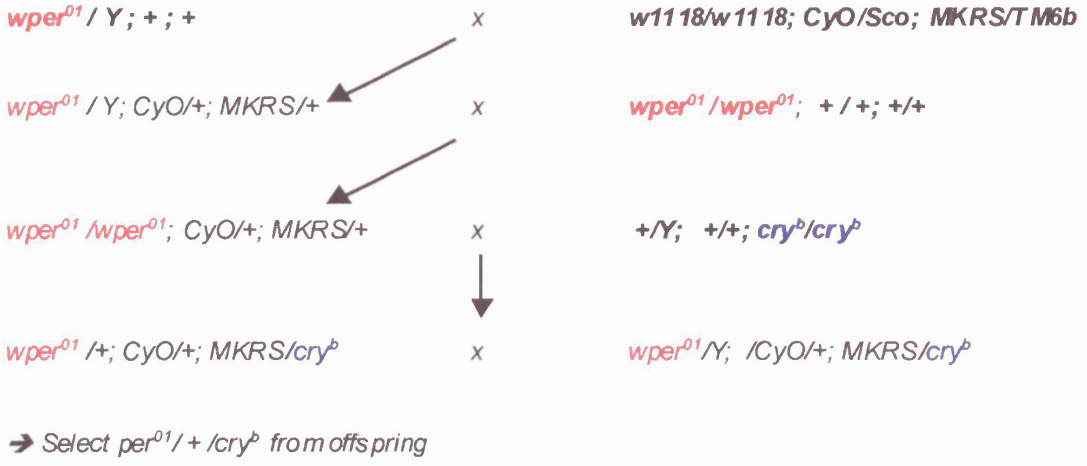
The following strains of flies were used

Canton-S
per^{01}
tim^{01}
cry^{b}
norpA^{P41}
norpA^{P41}; *cry*^{b}
glass^{60j}
glass^{60j} *cry*^{b}
mps3-65c
D.pseudoobscura Ayala
D.virilis

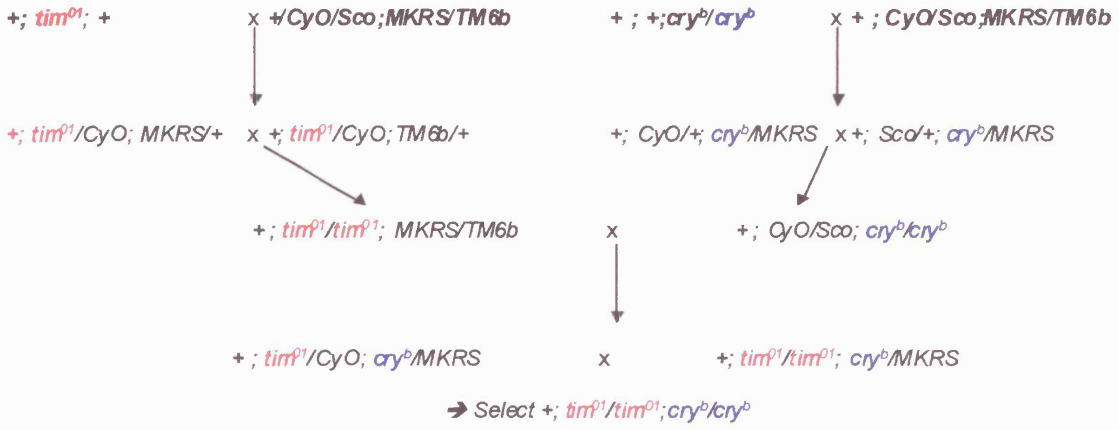
Flies were maintained on sugar/agar food (4.63g sucrose, 4.63g dried brewers yeast, 0.71g agar and 0.2g Nipagin in 100ml of H_20) in 1/3 pint milk bottles or glass vials (10cm x 22cm). Fly stocks were maintained at either 18 or 25°C in temperature controlled rooms.

2.3 Crossing schemes

2.3.1 Cross to make per^{01}; cry^{b}



2.3.2 Cross to make tim^{01}; cry^{b}



(in each cross ♀ or ♂ were used depending on which flies had emerged from the previous cross as there are no genes required on the X-chromosome in this cross)

2.4 Fly collections

For experimental collections, flies were entrained for at least three days to the appropriate LD regime at either 18°C or 29°C. Flies were collected at four-hour intervals and snap-frozen in liquid nitrogen. Heads were removed by vortexing frozen flies, and then filtering out the heads using sieves. Collections of fly heads could then be used for either RNA or protein extraction.

2.5 Locomotor activity

Locomotor activity was recorded by the Drosophix locomotor monitoring system and software (PIXEL Srl., Padova, Italy). Each fly was placed inside a glass tube containing sugar food at one end and a tissue paper bung at the other. The end containing the food was sealed with wax to prevent desiccation. These tubes were then placed inside the monitoring apparatus. Each tube sat between an infrared emitter and detector, with the infrared detector connected to a computer, and each time the fly broke this infrared beam, one 'unit' of locomotor activity was recorded. The number of times this beam was broken within a 30-minute window was recorded as the locomotor activity for that time bin. Forty-eight 30-minute bins of activity data were then collected every day, and recorded in an Excel spreadsheet.

Flies were placed inside incubators at either 18 or 29°C, with a LD 12:12 light cycle. Flies were then allowed to entrain to this LD and temperatures regime for three days. Once the flies had entrained to the desired light regime, the activity was monitored. Locomotor activity was recorded in LD for three days, and then the lights were turned off (or kept on continuously) to record the free-running activity in DD (or light). Before analysis of data, any dead flies were removed.

The period of free-running flies was determined by spectral analysis using the CLEAN algorithm (Roberts et al., 1987; Kyriacou and Hall, 1989). The

output from the program generally produces a spectrogram with peaks representing the waves that best fit the locomotor activity profile. The highest peak in the spectrogram was taken as the period, and if there were a peak ~48 h, a peak ~24 h, and one ~9 h, the relevant period would be the peak closest to 24 h. In order to calculate the 95% and 99% confidence intervals, the data for each fly was randomised 100 times and CLEAN analysis repeated on each fly. The 95^{th} and 99^{th} highest values generated at each frequency represented the 95% and 99% confidence intervals based on the Monte Carlo simulation of random data. A peak was considered significant if it was above the 99% confidence interval (Hennessey, 1999).

In order to compare the behavioural profiles of flies, activity data was 'wrapped' as described by Hennessey (1999). In LD, this means successive days activity are superimposed on top of each other, to present an average activity pattern over several days, so bins 1, 49, 97 etc are averaged together, and so on. All LD activity figures presented herein are the averages of the wrapped data of three days LD activity for many flies.

In DD, wrapping is slightly more complex, as individual flies have periods that differ slightly from 24 h. The activity bins were redistributed depending on the free running spectral period of each fly. The number of bins for each flies 'day' were re-distributed into 48 bins. Thus, if a fly had a period of 25 h, each day would consist of 50 30-minute time bins. In order to plot this on a 24 h scale, the 50 bins needed to be converted to 48. Each bin is then assigned a value, so the first 30 minute bin, rather than occurring at time 1 occurred at time 48/50 (0.96), bin two at 2 x 48/50 (1.92) and so on. Regression between actual time points then determined the activity at times 1,2 and so on, producing 48 activity values.

Comparisons of activity between different genotypes were carried out using STATISTICA (Statsoft). In order to determine how different temperatures affect different genotypes, locomotor activity was compared across three different regions. The time bins were divided into the morning activity peak

(11-15), during the day (20-29) and the evening activity peak (30-38), as shown in figure 2.1.

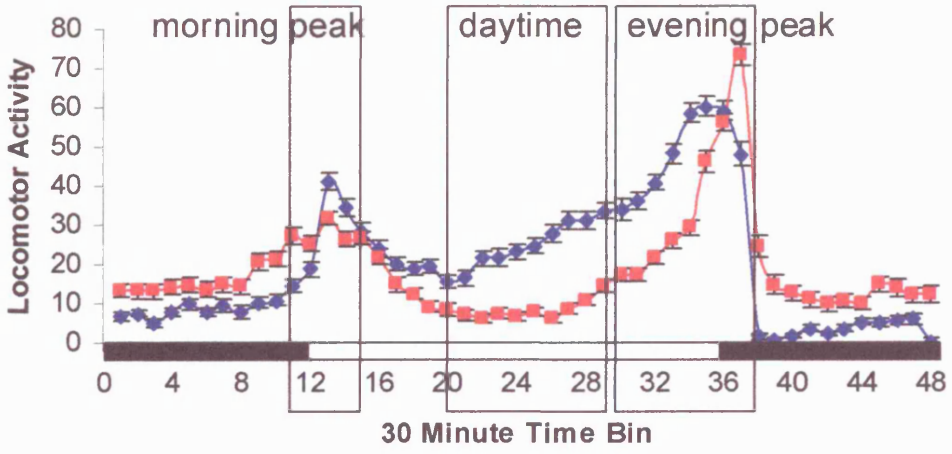


Figure 2.1: Division of locomotor activity profiles for analysis Morning activity peak (11-15); daytime activity (20-30), evening activity peak (30-38)

2.6 DNA and RNA extraction

DNA was extracted using the single fly preparation method (Gloor et al., 1993).

RNA was extracted using Tri-reagent (Sigma, St Louis, MO) and RNA levels quantified using a spectrophotometer.

2.7 3' RACE

3' RACE was carried out using a 3' RACE kit (Invitrogen, Karlsruhe, Germany). PCR was then carried using the Expand High Fidelity PCR kit (Roche) using the primers specified in Table 2.1. PCR products were resolved on 1% agarose-TAE gels, and relevant bands gel extracted using the QIAquick gel extraction kit (QIAGEN, Valencia, CA). PCR products were cloned into the TOPO TA cloning vector (Invitrogen), and sequenced in house by PNACL, using the big dye terminator reaction. Sequences were

then assembled using the GCG package (Accelrys Inc., Burlington, MA) or Autoassembler (Applied biosystems, Foster City, CA).

2.8 polyA selection

polyadenylated RNA was selected using the QIAGEN Oligotex kit (QIAGEN).

2.9 One-Step RT-PCR

In each cycle of a PCR reaction, each product is replicated once. This means that over a certain range, the number of copies of a product will double with each PCR cycle. The levels of the products therefore amplify exponentially until factors such as the levels of dNTPs and primers become limiting. At this point, additional cycles no longer double the number of copies of the product, and the PCR reaction is said to have plateaued. This makes it possible to use PCR to quantify the number of copies of a product which were originally present in a sample.

One step RT-PCR was used to determine the levels of spliced and unspliced *per* RNA using a single set of primers. In order to determine the absolute levels of *per* RNA present in any reaction, a fragment of *per* was co-amplified with a fragment of *rp49*, a constitutively expressed housekeeping gene. This was done in a multiplex RT-PCR reaction, using two different sets of primers, one for each gene. In this way, the level of *per* RNA in each sample could be quantified relative to the level of *rp49*. Both types of quantitative PCR were set up in the same way.

In order for an RT-PCR reaction to accurately quantify levels of RNA, the amplification of all products must be within the exponential phase of amplification (i.e. in the range where the levels of all products are doubling each cycle). If one product reaches the plateau phase of amplification before the other, then relative quantification will be inaccurate. To determine that amplification of the RNA products for each set of primers used for

quantification were in the exponential amplification phase the following procedure was used.

A master mix for 6-10 reactions was set up, containing the PCR reagents, the primers of interest, and RNA. This was done using the QIAGEN One Step RT-PCR kit (QIAGEN). 200-250 ng of RNA was added per reaction into the master mix. The master mix was aliquoted into 25 µl reactions, which were then put onto the PCR machine simultaneously (50°C, 30 min; 95°C, 15 min; 20-30 Cycles of: 95°C, 30s; 55-65°C, 30s; 72°C, 1 min.). After at least 20 cycles, one reaction was removed from the PCR block at the end of the 72°C extension step of each cycle. To this 5µl of loading buffer containing EDTA was added to stop the PCR reaction and the sample was put on ice. In this way, a series of PCR reactions were collected, differing only by the number of PCR cycles. These were then run on 2% agarose gels and photographed. The levels of each product were then quantified using the gelplot2 macro in Scion Image (Scioncorp, Frederick, MA). A typical reaction is shown in figure 2.1

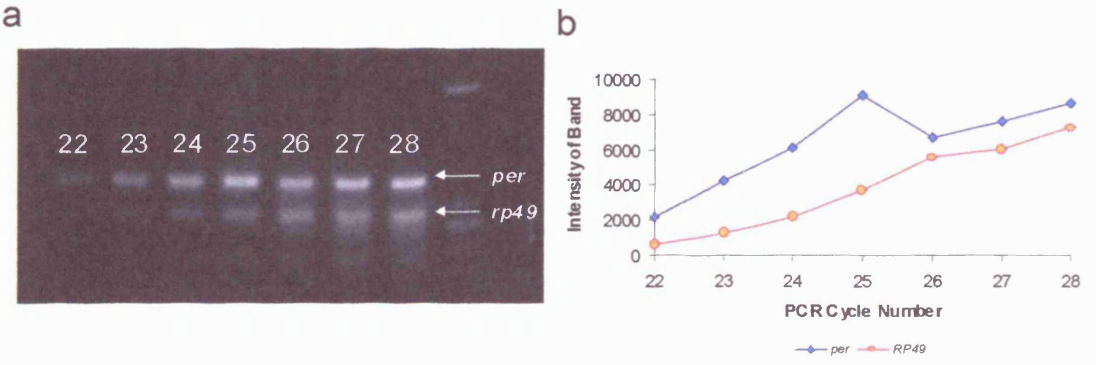


Figure 2.1: Setting up quantitative RT-PCR. a) typical gel of multiplex RT-PCR reaction. b). Quantified levels of *per* and *rp49* mRNA

In the example shown in figure 2.1, the amplification of both PCR products remained in the exponential phase from cycle 22-25 (figure 2.2b). From cycle 26 onwards the amplification of *per* has plateaued, while the amplification of *rp49* continues to be exponential. This allows the identification of the range over which the amplification of *per* and *rp49* from

200 ng of RNA will be exponential, in this case 22-25 cycles. All subsequent reactions in this case would then be carried out around 23 cycles to ensure than amplification of both products remains exponential, even if there are small variations in RNA concentrations and levels of each PCR product.

An identical process was carried out to show that the primers which span the 3' UTR *per* intron amplify both spliced and unspliced *per* RNA with equal efficiency. In this case, only one set of primers was required to amplify both products. As a result, PCR amplification remained linear over a much wider range. The number of PCR cycles used for each primer pair, determined in this way, is shown in table 2.1.

All RT-PCR reactions were carried out in 25 µl volumes using the One-Step RT-PCR kit (QIAGEN). PCR products were run on 2% agarose gels, and photographed. The level of each PCR product was then determined by image analysis using Scion Image software (Scion Corp.). The primer combinations annealing temperatures and number of PCR cycles for each reaction are shown in table 2.1

	Species	Gene	Forward Primer	Reverse Primer	Annealing Temp.	PCR Cycles
1	*D.melanogaster*	*per*[3]	DMPERF1	DMPERR1	65°C[4]	29
2	*D.melanogaster*	*per*[5]	DMPERF2	DMPERR2	60°C	22-23
3	*D.melanogaster*	*rp49*	DMrp49F1	DMrp49R1	60°C	22-23
4	*D.pseudoobscura*	*per*	DPPERF1	DPPERR1	60°C	28
5	*D.pseudoobscura*	*rp49*	DPrp49F1	DPrp49R1	60°C	28

Table 2.1 Primer combinations for one step RT-PCR.

2.10 Real-Time PCR[6]

In the analysis of *D.virilis per* RNA (Chapter 7), the different *per* transcripts differed not by the presence or absence of an intron, but by length. This means that RT-PCR cannot be used to quantify the levels of *per* transcript in

[3] primer combination used to quantify the relative amounts of spliced and unspliced *per* RNA
[4] This is the only reaction which required the addition of 'Q-solution' for accurate quantification
[5] primer combination used to quantify the total amount of *per* RNA relative to *rp49*

D.virilis as the primers which match the 'short' product will also amplify the 'long' product. In order to overcome this problem, real-time PCR was employed.

The Real-Time PCR system (Roche, Basel, Switzerland) used for the experiments reported here works as follows. As the PCR product is amplified, SYBR green is incorporated into the dsDNA. This then produces a fluorescent signal that is detected by the Real-Time PCR machine. This means that the level of the product can be monitored at each cycle. It is therefore very easy to see whether the PCR reaction is still in the exponential amplification phase. To quantify the amount of each product, samples are run alongside a series of standards. The 'crossing point' for the samples and standards is then calculated. This is number of cycles at which a line drawn from the exponential phase of the amplification curve crosses the X-axis. By comparing the crossing points of samples with those of the standards, the amount of product present in each sample can be calculated.

For each experiment, RNA was extracted, quantified and treated with DNAseI (Promega, Madison, WI) to ensure that there was no DNA contamination, and then phenol-chloroform extracted to remove the DNAse. cDNA synthesis was carried out using Superscript II (Invitrogen) using a saturating amount of RNA (usually 5 µg/Unit Superscript).

Primers were designed to amplify the 'short' and 'long' *D.virilis per* products, and to *rp49*. As the 'short' primers also amplify the same fragment from 'long' products, the amount of short products present in a sample is calculated by subtracting the amount of products from the 'long' PCR from those from the 'short' PCR. To control for variations in the total amount of cDNA in each sample, the levels of 'short' and 'long' product were then divided by the level of *rp49*.

[6] Real-time PCR is sometimes also referred to as 'RT-PCR' . Here RT-PCR refers to 'Reverse transcriptase PCR' while Real-Time PCR is referred to by its full title.

For each set of primers shown in Table 2.2, the PCR reaction was optimised. As any dsDNA will incorporate SYBR green it is essential to ensure that only one PCR product is produced by each set of primers. Therefore each experiment was run with a range of $MgCl_2$ concentrations, and under different PCR conditions. The final concentration of $MgCl_2$ used for each reaction is contained in the notes in table 2.2.

The 'melting curves' were also determined for each PCR product. The fluorescence at the end of a run is measured as the temperature is increased. The point at which fluorescence falls is the melting temperature of the PCR product. The long PCR product had two melting temperatures, so fluorescence measurements were made at 84°C so that only the level of the higher temperature melting product would be measured.

The Real-Time PCR cycles for each set of primers were as follows: DV*rp49* (95°C, 10 minutes; 55 cycles of 96°C, 2 s; 65°C→55°C, -0.8°C/cycle, 5 s; 72°C, 15 s); DVSHORT (95°C, 10 minutes; 55 cycles of 96°C, 2 s; 70°C→58°C, -0.8°C/cycle, 5 s; 72°C, 15 s,); DVLONG (95°C, 10 minutes; 55 cycles of 96°C, 2 s; 75°C→63°C, -0.8°C/cycle, 5 s; 75°C, 15 s, 84°C, 0s).

2.11 Protein Extractions and Western Blotting

For each time point ~30 heads were homogenised in 2 volumes BW extraction buffer (0.1 M KCl, 0.01 M HEPES pH 70.5, 5% Glycerol, 0.1% Triton-X 100, 10 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 µg/ml Aprotinin, 5 µg/ml Leupeptin, 5 µg/ml Pepstatin) on ice. This was then spun down twice on a microcentrifuge and the supernatant collected.

The amount of protein was determined using the Bradford Protein Assay (Biorad, Hercules, CA), recording the absorbance at 595nm. Protein samples were then diluted to the lowest OD and ½ volume of 3x loading buffer added (188 mM Tris-Cl pH 6.8, 6% v/v SDS, 30% v/v Glycerol, 15% v/v β-mercaptoethanol, 0.03% v/v bromophenol blue. 15 µl of each protein

sample was then loaded onto a 6% SDS-page gel in gel running buffer (2.5 mM Tris, 0.25 M Glycine, 0.1% v/v SDS). Gels were run for ~2 h at 20 mA. Proteins were then blotted onto nitrocellulose () O/N at 50 mA in transfer buffer (20 mM Tris, 0.15 M Glycine, 20% Methanol). All SDS PAGE electrophoresis and subsequent western blotting was carried out using the Biorad Mini-Protean system.

Antibody detection on Western Blots

Nitrocellulose membranes were blocked for 1 h at room temperature in TBST (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% w/v Tween 20) containing 5% dried milk powder. Following this they were incubated in a 1/15,000 dilution of the primary antibody in TBST + 5% milk. The membrane was then washed 3 times for 5 minutes in TBST, and incubated in a 1/10,0000 dilution of the secondary antibody for 1 h at 4°C. The primary PER antibody was supplied by Jeff Hall (Dembinska et al., 1997), and had been raised in rabbit. Secondary antibody was an anti-rabbit-HRP conjugate (Jackson Immunoresearch, West Grove, PA). This was then washed 3 times in TBST before immunodetection. The membrane was then incubated for one minute in 0.1 M Tris-Cl, pH 8.5, 6.25 µM Luminol (Sigma), 6.38 µM p-Coumaric acid (Sigma), 2.7 mM H_2O_2), which creates a luminescent signal. This was then detected on autoradiographic film.

2.12 Primer sequences

Primer	Sequence 5'-3'	Notes
DMPERF1	AAG ACG GAG CCG GGC TCC AG	Quantification of spliced and unspliced *per* in *D.melanogaster*
DMPERR1[7]	AAT TGG ATC CGT GGC GTT GGC TTT CG	
DMPERF2	GGT AAT GAA GAA GGG TCA GAC	Quantification of total level of *per* in *D.melanogaster*
DMPERR2	AGA CGG TCA ACT GCT TCT CG	
DM*rp49*F	GTT AGT CCG TGC GCC GTT CG	Quantification of total level of *rp49* in *D.melanogaster*
DM*rp49*R	CCT GTA TGC TGG GCG GAT GG	
DPPERF1	GCA CGT TGA GCA GCA AGA TAA TGG A	Quantification of spliced and unspliced *per;* also used to quantify total level of *per* in *D.pseudoobscura*
DPPERR1	CAT CGC AGT GTT TAT TCT AAA GGA C	
DP*rp49*F1	CCG AGT AAC ATG TTG GCT C	Quantification of total level of *rp49* in *D.pseudoobscura*
DP*rp49*R1	GCT GGA ATT TGA TAG TTG AGA CC	
DM5'TG[8]	AAC TAT AAC GAG AAC CTG CT	Determination of Thr-Gly repeat length
DM3'TG[8]	TTC TCC ATC TCG TCG TTG TG	
DP3'RACEF1	CAA CTC ACC CAT CGC CTC CG	3' RACE of *D.pseudoobscura per* 3'UTR. Primers used in order F1, F2
DP3'RACEF2	As DPPERF1	
DV'RACEF1	CTG TCC GAT TTG CCC AGT ACA TCG	3' RACE of *D.virilis per* 3'UTR
DV'RACEF2	TGG CAA CAG CGA TGA CAT GGA TGG C	
DVSHORTF	CGA AAG TTC ACA AGC TCA AGC	Real-Time PCR of *D.virilis per* short product – 3.5 ng/µl $MgCl_2$
DVSHORTR	AGC GTT TGG TTT GGG TGT C	
DVLONGF	GGA TAG CAT TCC AAA GGA CG	Real-Time PCR of *D.virilis per* long product - 3.0 ng/µl $MgCl_2$
DVLONGR	GGA TAT GAA AAT TTG TCG CTT G	
DV*rp49*F	TAT CTG ATG CCC AAC ATC GG	Real-Time PCR of *D.virilis rp49* - 3.5 ng/µl $MgCl_2$
DV*rp49*R	CAA TCT CCT TGC GTT TCT TCG	

Table 2.2 PCR Primer sequences

[7] Adapted from Majercak et al., 1999
[8] From Piexoto 2000

Chapter three: *D.melanogaster period* mRNA and the response to seasonal light and temperature changes: the role of the clock

3.1 Introduction

D.melanogaster locomotor activity in LD cycles is bimodal, with a morning peak owing primarily to the startle effect of lights on, and an evening activity peak anticipating lights off. There is good evidence that the morning activity peak is only partially under control of the clock as it is diminished, but not always lost in arrhythmic Clk^{Jrk} mutants (Allada et al., 1998). This effect is probably due to the regulation of genes within the phototransduction pathway by *Clk* (Kim et al., 2002). However, as most clock gene mutants do not affect the morning peak, studies have concentrated on the analysis of the effect on the evening peak position. There is also a theory that the two peaks are under the control of separate oscillators, with the LN_Vs representing the oscillator which controls the evening peak (see Helfrich-Forster, 2000).

The pattern of locomotor activity is dependent on the season, with higher levels of activity during the day and an earlier evening peak at lower temperatures. Temperature has no effect on the position of the morning activity peak in LD cycles, so seasonal effects are likely to be mainly manifested in evening peak position (Majercak et al., 1999). As the temperature rises, the fly in effect has a 'siesta' to avoid the midday sun and, as the photoperiod is reduced, shows progressively more nocturnal behaviour at high temperatures (Qiu and Hardin, 1996).

The evening activity peak of pdf^{01} mutants occurs ~1 h earlier than wild-type. As this is probably a mutation in the clock output pathway, and is not one of the core clock components (Renn et al., 1999) *pdf* is unlikely to be involved in

the response to temperature changes. Instead, the primary determinant of evening peak position is the cycle of PER protein. The long period mutants dbt^H and Dbt^G show altered entrainment to LD cycles. These mutants entrain to 24 h LD cycles with no anticipation of the morning or evening activity peak, but the evening peak instead extends several hours into the night. Comparisons with the yeast homologue of *dbt,* HRR25, indicate that mutants of both these genes have severely reduced kinase activity (Suri et al. 2000). This is likely to increase PER stability resulting in the delay in evening peak position. The anticipation of dawn and dusk is restored in combination with the per^s mutation, confirming that these long period *dbt* mutants increase PER stability (Rothenfluh et al., 2000). Additionally, *slimbΔF* overexpression produces an evening peak delayed into the night. This is again most likely owing to the increased stability of PER (Ko et al., 2002).

As the position of the evening activity peak is dependent on the cycle of PER protein, changes to the *per* RNA cycle will alter the position of evening activity. Evening activity is regulated by seasonal changes to this cycle, with light and temperature controlling the abundance of *per* at the RNA level, fine-tuning behaviour to changing environmental conditions (Majercak et al., 1999), At lower temperatures, the cycles of both *per* and *tim* transcripts are advanced. This is not, however, the primary determinant of evening peak position. Temperature seems to primarily set the clock by altering the levels of *per* because levels of *per* but not *tim* mRNA respond very rapidly to shifts in temperature (Majercak et al., 1999). The regulation of mRNA need not only involve changes in abundance. Within the head of *D.melanogaster,* two alternatively spliced isoforms of the *per* transcript are present differing only by the presence (unspliced) or absence (spliced) of an 89bp intron within the 3' UTR. As the ratio of spliced to unspliced *per* RNA varies in different tissues, the alternative splicing of this intron would be predicted to have a functional role (Cheng et al., 1998).

Within the head, both spliced and unspliced RNAs cycle in abundance, with more spliced RNA present at all times (Cheng et al., 1998). Transgenes that

produce only spliced, only unspliced or both spliced and unspliced *per* RNA all rescue rhythmicity in per^{01} flies. Interestingly, flies which can only produce the unspliced transcript have longer periods due to the delay in the rise of PER levels as the presence of the intron delays the production of a mature mRNA (Cheng et al., 1998).

The difference in ability of the transcripts to rescue the clock indicates that the two isoforms have functional differences. In many cases the *cis*-acting elements that regulate mRNA stability are found within the 3' UTR of genes (Majercak et al., 1999). The regulation of splicing of this intron by light and temperature turns out to alter the timing of the daily upswing in PER protein levels, and sets the evening activity peak position. In 12:12 LD cycles, there is 2-3 times as much of the spliced isoform present at 18°C than at 29°C. During the day, there is a slight reduction in splicing levels, and splicing levels respond rapidly in response to temperature changes (Majercak et al., 1999)

The role of this alternative splicing event in determining the activity pattern is demonstrated by examining the transgenic lines that cannot splice out this intron. Transgenic flies producing only unspliced (with inactivated splice sites) or only spliced *per* transcript have a delayed evening peak in comparison to wild-type flies at low temperatures. At high temperatures, the period is also lengthened in these lines. There is no difference in the evening peak phenotype between these transgenic flies, indicating that it is the act of splicing, rather than the presence or absence of the intron which determines the position of the evening activity peak (Majercak et al., 1999).

Therefore, the act of splicing of the *per* 3' UTR intron is responsible for the advance of the evening activity peak seen on cold days. This can be related directly to an effect on PER levels: flies which cannot splice out this intron have delayed PER accumulation and disappearance and, at cold temperatures, the cycle of *per* RNA is dampened. Majercak et al. concluded that the splicing of the 3' UTR intron is elevated at low temperatures and this stimulates the formation of the 3' end of the *per* RNA transcript, which advances the onset of PER accumulation, as shown in figure 3.1).

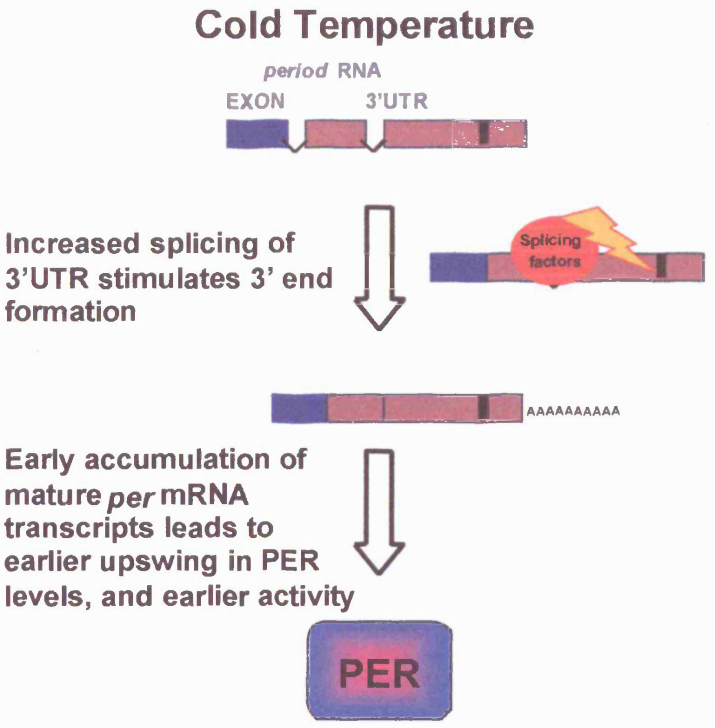


Figure 3.1: At cold temperatures the splicing of the 3' UTR intron of per is stimulated, producing an earlier upswing in PER levels resulting in an earlier phase of activity. Adapted from Majercak et al., 1999.

Because the accumulation of PER is also dependent on TIM, and TIM is degraded in the presence of light, PER accumulation is dependent on the photoperiod. In shorter photoperiods, TIM accumulation is also advanced, leading to earlier PER upswing, and corresponding locomotor activity peaks, but in long photoperiods TIM accumulation is delayed, and as a result activity occurs later despite the low temperature advance in PER. The role of splicing is therefore likely to advance the point at which the minimum level of PER has accumulated to dimerise with TIM, and in this way the clock adjusts behaviour to the seasons.

3.2 Aims and objectives

As the splicing of the 3' UTR intron of *per* determines the position of the evening activity peak, it provides an opportunity to relate a molecular process

(splicing of an intron) directly to a behavioural phenotype (evening locomotor activity peak position). As the adaptation to different seasonal conditions requires the measurement of photoperiod and temperature, both of which alter the levels of *per* and *tim* RNA, it would not be surprising if the clock also controlled the *per* splicing level.

No role was identified for *per* in the rapid response of splicing levels to altered temperatures. However, per^{01} flies show a slight increase in splicing levels during the day in comparison to wild-type flies (Majercak et al., 1999). To determine whether the clock plays a more prominent role in the regulation of splicing, the levels of splicing in per^{01} and tim^{01} clock mutants around the clock were compared to those of a (wild-type) Canton-S strain at both 18°C and 29°C. Changes in temperature are also usually associated with changes in photoperiod, and therefore the levels of *per* RNA splicing were compared in a reduced photoperiod, and DD, in the Canton-S and per^{01} strains to see what role light plays in regulating splicing.

per^{01} is a null mutant, with a premature stop codon preventing the production of the PER protein (Yu et al., 1987; Baylies et al., 1987). In these flies, a full length *per* transcript is still transcribed so the regulation of *per* splicing can be assayed, although it can have no effect on behaviour patterns as no protein is produced. In the tim^{01} line, PER protein is produced, but the lack of TIM to stabilise PER means that the level of PER is very low, and without TIM PER cannot enter the nucleus. This means that altering the splicing of *per* mRNA in tim^{01} mutants is unlikely to have any effect on the clock or locomotor activity.

Finally, altering the splicing of the *period* gene is thought to alter the timing of the accumulation of PER, and as such, might be predicted to alter the free-running period of the clock by speeding up or slowing down the PER protein cycle. The *period* gene is also involved in temperature compensation (see introduction), and therefore it is possible that this is also linked to the splicing levels. To determine whether the temperature dependent regulation of

splicing impinges on the period and/or temperature compensation phenotype, the period length, temperature compensation, locomotor activity and splicing phenotypes of 4 natural *per* variants were analysed. The splicing levels, periods and degrees of temperature compensation can then be compared to see if there are any significant correlations. This also makes it possible to see whether different genetic backgrounds affect the *per* splicing levels.

The lines used in these experiments, except for Canton-S, had been collected from the wild for the previous work of Sawyer et al (1997), and characterised in terms of Thr-Gly repeat length. These four lines include representatives of the two most abundant Thr-Gly repeat lengths (17 and 20), as well as a $(Thr\text{-}Gly)_{23}$ and $(Thr\text{-}Gly)_{21}$. Thr-Gly repeat lengths were confirmed by PCR, as described in materials and methods, and the Canton-S strain also carries 23 Thr-Gly repeats.

3.3 Methods

mRNA was prepared from fly heads collected at 4 h intervals from Canton-S, per^{01} and tim^{01} flies. Canton-S flies were selected as they were previously used in the work of Majercak et al. (1999). Flies were collected while entrained to LD 12:12 or LD 6:18 cycles, or during the first day of DD after prior entrainment to LD 12:12. To determine the relative amounts of spliced and unspliced *per* RNA transcripts, One-Step RT-PCR was then performed using the primers described in Chapter 2. Locomotor activity was recorded as described in Chapter 2. Resultant data was analysed by ANOVA using Statistica (StatSoft)

3.4 Results

3.4.1 Locomotor activity and splicing in Canton-S flies

Initially it was important to establish whether the same behavioural and splicing phenotypes previously reported were also present in the Canton-S line used here. Locomotor activity profiles of Canton-S flies in LD 12:12 at 18

and 29°C were compared to see whether there was an earlier evening peak at 18 than at 29°C, as shown in figure 3.2.

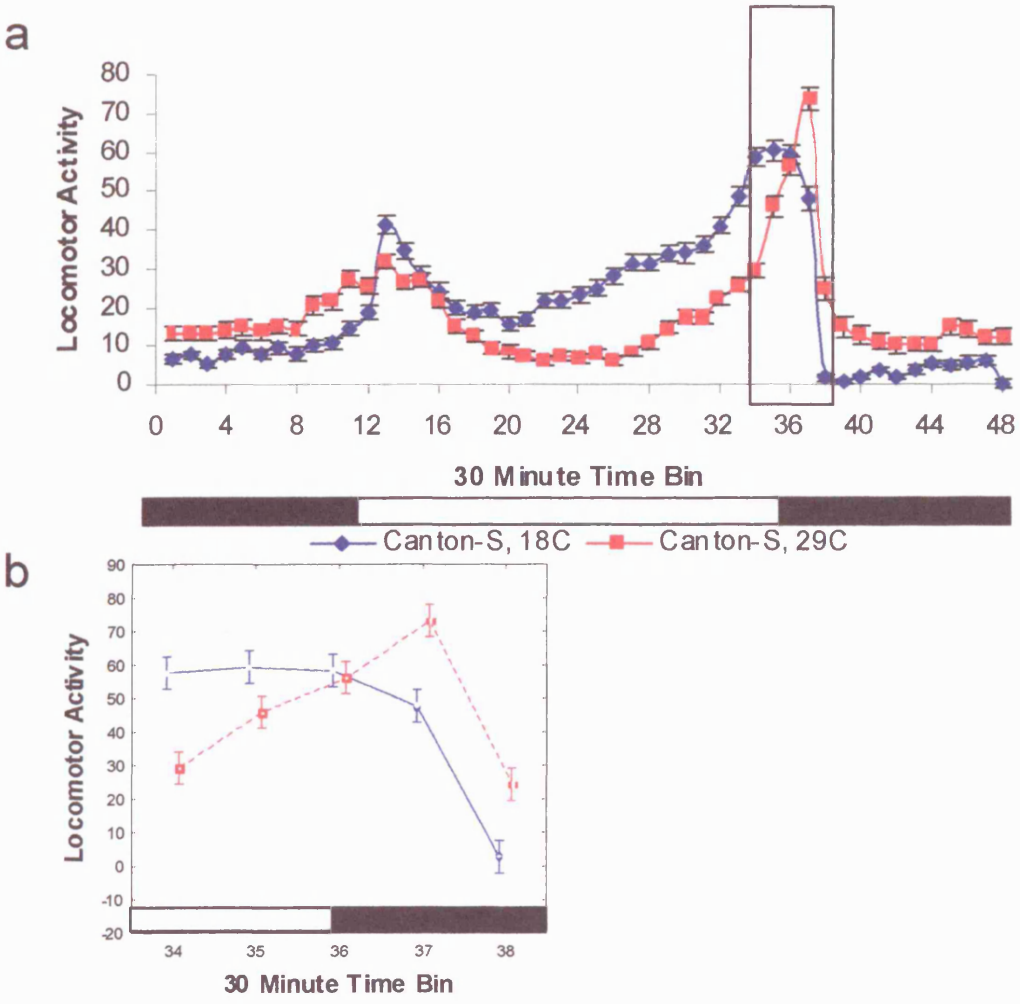


Figure 3.2: Locomotor activity in Canton-S in LD 12:12

a). 18°C (blue) and 29°C (red). Error bars represent SEM. b). Error bars represent 95% confidence interval.

ANOVA of the locomotor activity profiles of Canton-S flies reveals a smaller, broader morning activity peak at 18 than 29°C (Appendix (A) 3.2.2, temp x time, F=8.8, p<<<0.001), although the peak of locomotor activity occurs in time bin 13 at both temperatures. This is the first time bin after 'lights on', reflecting the role of the 'startle effect' in generating this peak. There is an earlier upswing in locomotor activity at 18 than 29°C (A 3.2.3, temp x time, F=4.4, p<<<0.001) (figure 3.2a). A comparison of time bins 30-38 (figure 3.2b, A 3.2.4) reveals highly significant effects of temperature (F=58.6, p<<<0.001), time (F=99.7, p<<<0.001) and time x temperature (F=36.6, p<<<0.001). This confirms that the evening peak of this Canton-S strain is earlier in phase at 18 compared to 29°C, as previously published (Majercak et al., 1999).

To confirm that the evening peak position is related to the level of spliced *per* RNA present, the levels of splicing in Canton-S flies at 18 and 29°C in LD 12:12 were also measured as shown in figure 3.3.

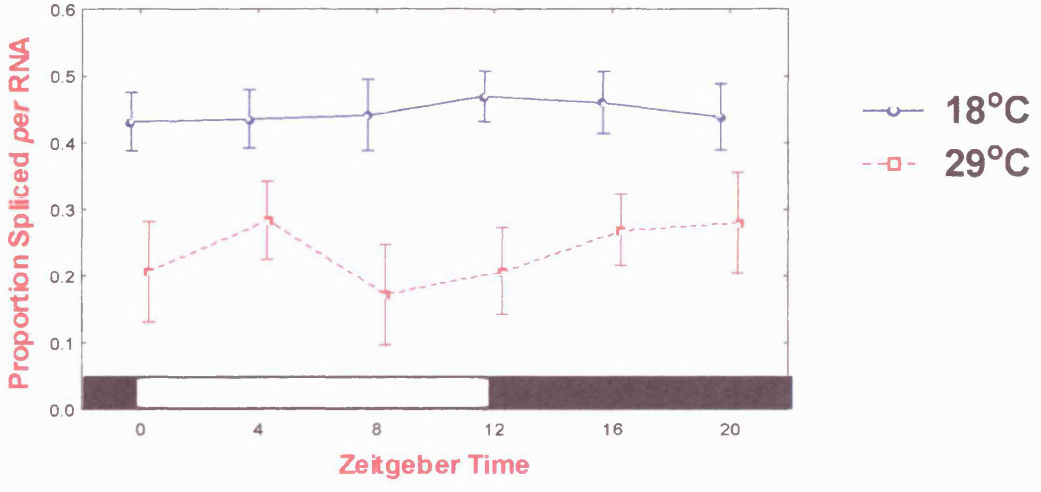


Figure 3.3: Splicing in Canton-S in LD 12:12.

splicing levels in Canton-S at 18°C (blue) and 29°C (red) around the clock. The values shown represent the proportion of *per* which is spliced at any given time point, independent of the underlying *per* RNA cycle.

As can be seen from figure 3.3, the splicing level is higher at 18 than 29°C (A 3.3, F=156.5, p<<<0.001), but there is no significant change in the level of splicing around the clock (F=1.3, p=0.27). At 18°C ~45% of *per* RNA transcripts are spliced, whereas only ~25% of *per* transcripts are spliced at the higher temperature. This data is consistent with the results of Majercak et al. (1999), and therefore provides a control set of data to which all mutations and altered conditions can be compared.

3.4.2 Locomotor activity and splicing in per^{01} and tim^{01} clock mutants

The locomotor activity of per^{01} and tim^{01} flies at 18 and 29°C was compared to that of the Canton-S wild-type strain, as shown in figure 3.4.

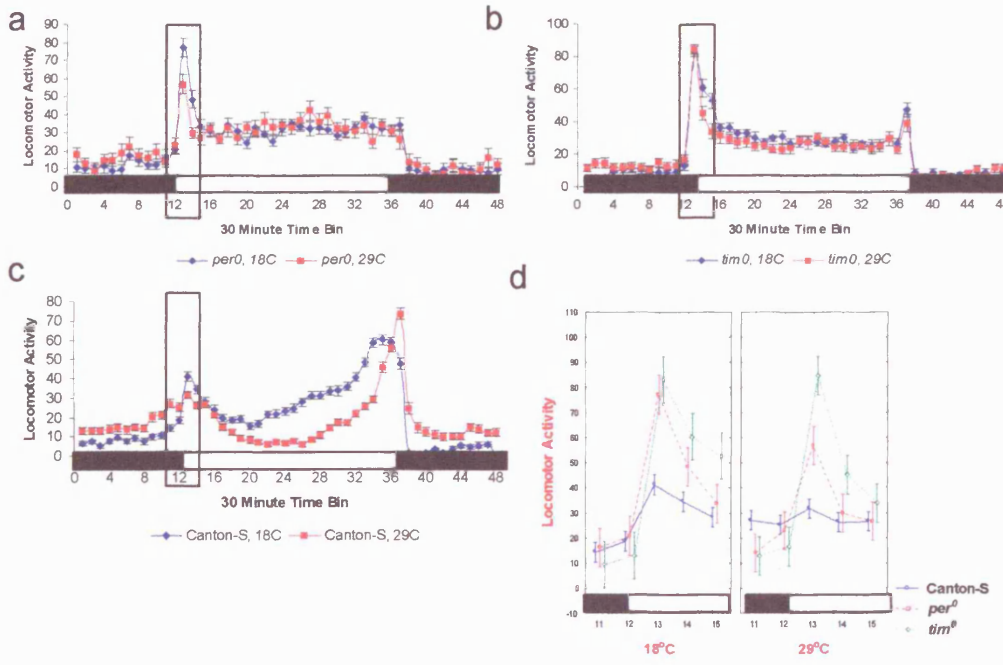


Figure 3.4: Locomotor activity at 18 and 29°C in LD 12:12. a). per^{01}, b). tim^{01}, c). Canton-S, 18°C (blue) and 29°C (red), error bars represent SEM. d). expansion of morning boxes (bins 12-14) a-c, error bars represent 95% confidence interval.

Figure 3.4 illustrates that in per^{01} and tim^{01} mutants, there is a morning activity peak occurring immediately after 'lights-on', at the same time as in

Canton-S flies (time bin 13, compare figures 3.4 a-c), confirming that the clock has no role in determining the positioning of this peak. In per^{01} flies there is no evening activity peak, while in tim^{01} the evening activity peak does not anticipate lights off (figure 3.4 a-b) as in Canton-S (figure 3.4c). While there are clearly differences in the activity peaks at different temperatures in Canton-S flies (panel c), there are no differences in the overall patterns of activity in per^{01} (A 3.4.1; F=1.2, n.s.) and tim^{01} (A 3.4.2;, F=2.1, n.s.) mutants. The only temperature difference seen in either per^{01} or tim^{01} concern the size of the morning activity peak.

Statistical comparison of all 3 morning activity peaks (figure 3.4d) reveals significant major effects and interactions (A 3.4.4). However, in all genotypes at both temperatures the morning peak level of activity occurs in time bin 13, the first after lights on (genotype x temp. x time, F=1.2, n.s.). This reflects the fact that it is mainly attributable to lights on

Comparisons within genotypes of the level of morning activity reveals that there is a significant effect of temperature in Canton-S (A 3.4.5; F=5.1 $p<<0.05$) and per^{01} lines (A 3.4.6; F=11.8, $p<<0.05$) with a reduced morning peak at high temperatures. This is not the case in tim^{01} (A 3.4.7; F=1.3, n.s.), indicating that there is no effect of temperature on the morning activity peak in this mutant.

At 18°C, the morning activity is higher in per^{01} and tim^{01} than in Canton-S (A 3.4.9; F=46, $p<<<0.001$), and figure 3.4d. This is also the case at 29°C, where the level of morning activity in per^{01} (A 3.4.11; F=11.6 $p<0.05$) and tim^{01} (A 3.4.12; F=72.4 $p<<<0.001$) are elevated above those of Canton-S. At the higher temperature, the morning peak of tim^{01} is also higher than per^{01} (A 3.4.10; F=14.9, $p<0.001$).

Most of the temperature dependent differences in locomotor behaviour are lost in per^{01} and tim^{01} mutants, with only a slight reduction in the

size of the morning activity peak seen in *per*^{01} mutants in response to elevated temperatures.

The splicing levels at 18 and 29°C in both *per*^{01} and *tim*^{01} lines were compared to those of the Canton-S strain, as shown in figure 3.5

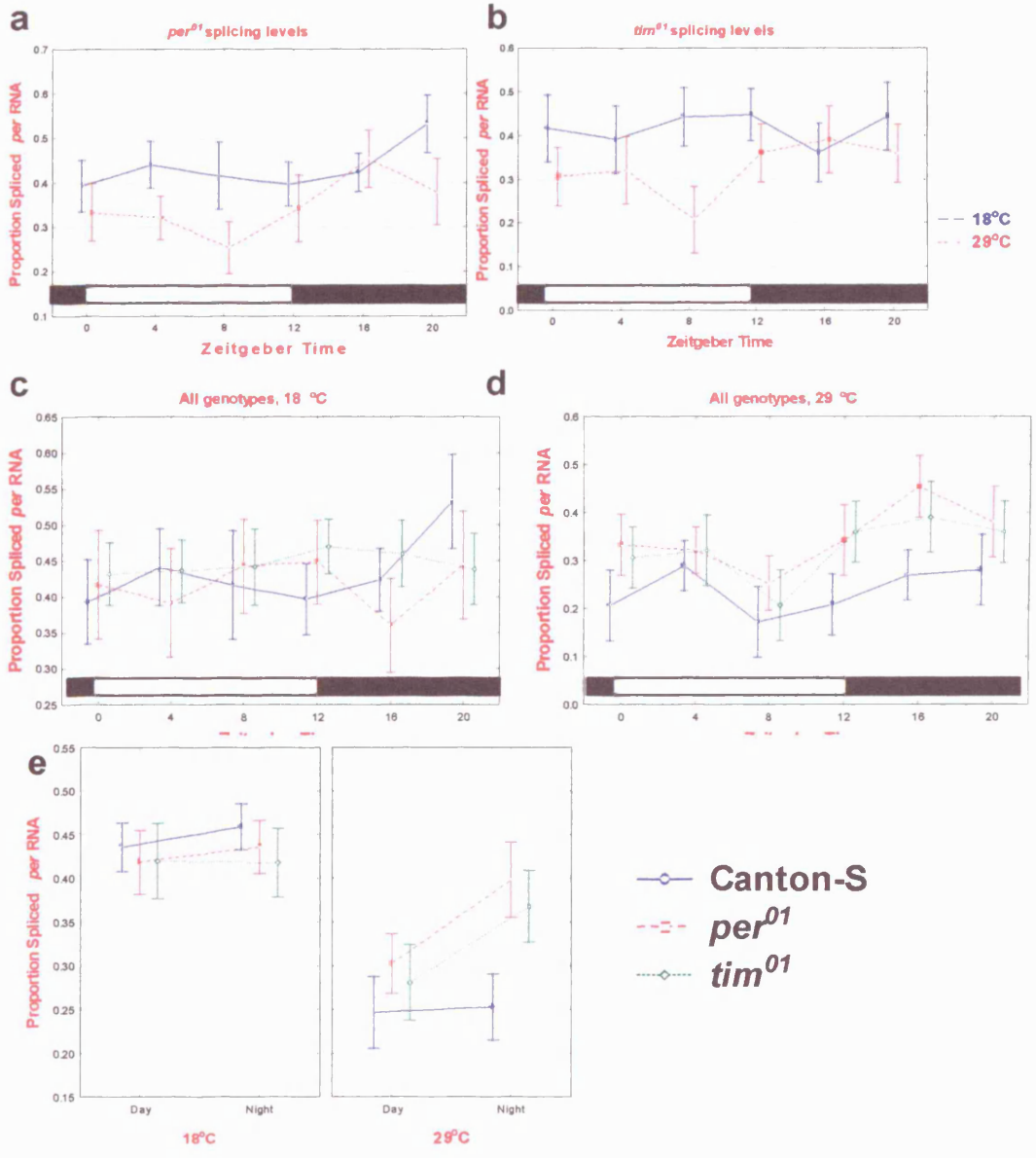


Figure 3.5: Splicing in *per*^{01} and *tim*^{01} compared to Canton-S at 18 and 29°C. a). *per*^{01} and b). *tim*^{01} at 18°C (blue) and 29°C (red). c) and d). *per*^{01} (red), *tim*^{01} (green) and Canton-S (blue) at 18°C and 29°C e). shows the difference in splicing levels between the day and night. All error bars represent 95% confidence intervals.

In per^{01} (A 3.5.2; F=23.8, p<<0.001) and tim^{01} (A 3.5.3; F=21.3, p<<0.001), there is significantly more spliced *per* mRNA at 18 than at 29°C, as shown in figures 3.5a and 3.5b. At 18°C the level of splicing in per^{01}, tim^{01} and Canton-S flies is identical, as shown in 3.5c (A 3.5.4; F=1.5, n.s). At 29°C, the levels of splicing in per^{01} and tim^{01} flies are identical (A 3.5.6; F=1.2, n.s.) and differ from those of Canton-S (A 3.5.5; F=18.9, p<<0.05). In the mutant lines there is a cycle in splicing levels at 29°C (A 3.5.6; F=5.7, p<<0.05), with an elevated level of splicing at night. As can be seen from figure 3.5e, the level of splicing at 29°C during the day in the two mutant lines is similar to that of Canton-S flies, while the level of splicing at night is elevated. Although the level of splicing during the day in per^{01} and tim^{01} flies appears to be above that of Canton-S at 29°C, this difference is not significant. Therefore it seems that the clock is only involved in the regulation of splicing at night at elevated temperatures.

The clock (or PER/TIM) is only involved in the regulation of splicing at higher temperatures, in the absence of light.

3.4.3 The effect of photoperiod on splicing

In the wild, seasonal changes in temperature will usually be accompanied by changes in photoperiod. Therefore the effect of altering the photoperiod on the splicing level of Canton-S and per^{01} was examined by measuring the *per* mRNA splicing level of wild-type and per^{01} mutants in a LD 6:18 photoperiod, as shown in figure 3.6.

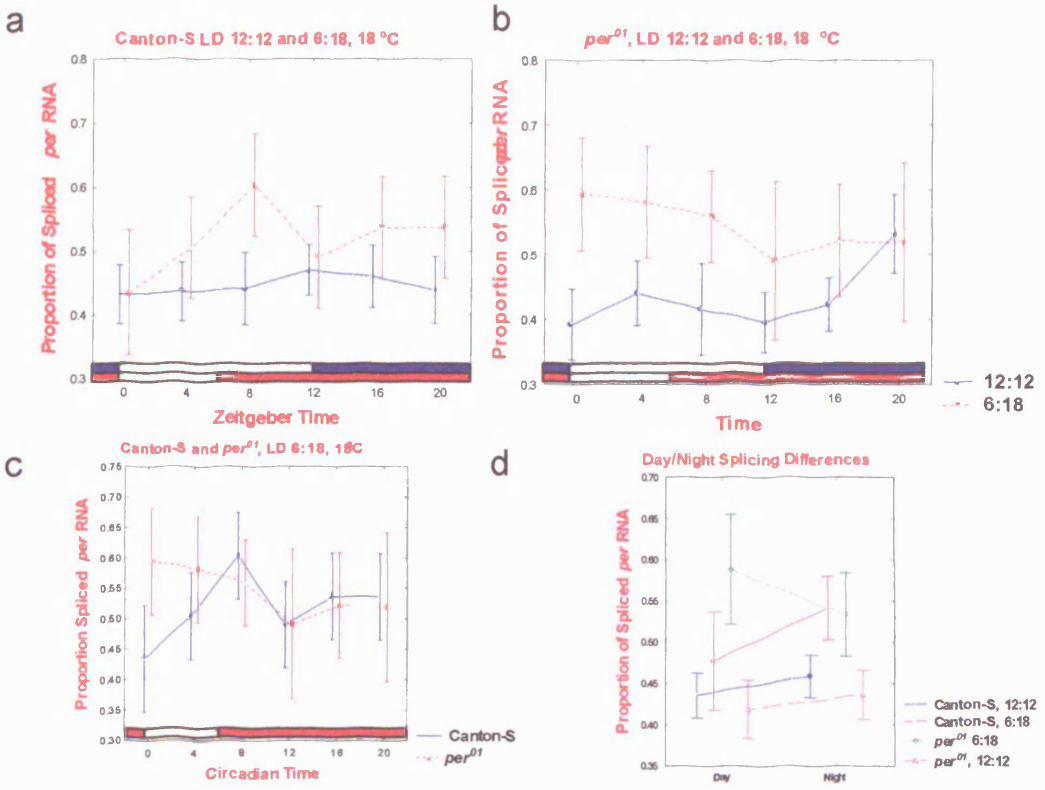


Figure 3.6: Splicing in different photoperiods a). splicing in Canton-S and b). splicing in *per*^{01} in LD 12:12 (blue) and 6:18 (red). c). splicing levels of Caton-S (blue) and *per*^{01} (red) in LD 6:18. d). splicing levels during the day and night in Canton-S (blue/red) and *per*^{01} (green/purple) in LD 12:12 and 6:18. Error bars represent 95% confidence intervals.

In both Canton-S (A 3.6.2; F=12.7, p<0.001, figure 3.6a) and *per*^{01} (A 3.6.3; F=24.3, p<<0.001, figure 3.6b), there is a significant effect of reduced photoperiod on the splicing level with an elevated level of splicing in LD 6:18 compared to LD 12:12. There is no significant difference in splicing levels in LD 6:18 between Canton-S and *per*^{01} flies (A 3.6.1; figure 3.6c), the same situation as found in LD 12:12 at 18°C (see figure 3.5c, above), indicating that the clock may not be required to regulate splicing at low temperatures even in reduced photoperiods. In Canton-S flies, the most obvious difference in splicing between LD 12:12 and 6:18 is observed at ZT 8. To examine this difference further each time point was classified as either 'Day' (ZT 0-8 of LD 12:12 and ZT 0-4 of 6:18) or 'Night' (ZT 12-20 of 12:12 or 8-20 of 6:18) (figure 3.6d). In *per*^{01} flies there is no significant difference between the day and night levels of splicing. As the levels of *per*^{01} splicing are indistinguishable from Canton-S in LD 6:18, it may be that there is also an

elevated level of splicing at night in this genotype which cannot be recovered from this data. In any case, in both per^{01} and Canton-S flies, the level of splicing of *per* mRNA is elevated in response to reduced photoperiods.

As this is the same result as obtained by another group, the examination of the effect of altered photoperiod on the splicing levels of different mutants was not pursued any further (J. Majercak, pers. com; see discussion, p 75).

3.4.4 Splicing during the first day of DD in Canton-S and per^{01}

The level of splicing in DD at different temperatures in Canton-S and per^{01} is shown in figure 3.7.

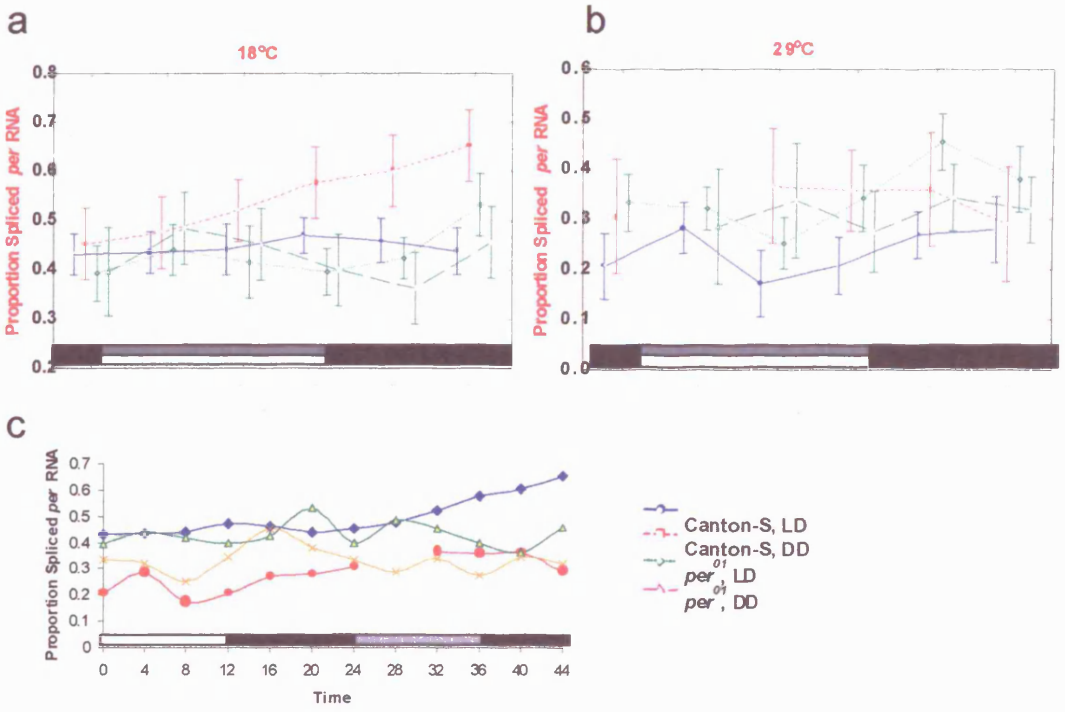


Figure 3.7: *per* mRNA splicing during the first day of DD in Canton-S and per^{01}. a). splicing at 18°C in LD (Canton-S, (blue), per^{01} (green)) and DD (Canton-S, (red), per^{01} (purple)) b). splicing at 29°C in LD and DD c). splicing during the transition from LD to DD. Error bars, 95% confidence intervals.

In Canton-S, there is a significant difference in splicing levels between LD 12:12 and the first day of DD at both 18°C (A 3.7.1; F=28.7 p<<0.001, figure 3.7a) and 29°C (A 3.7.2; F=27.6, p<<0.001, figure 3.7b). At 18°C, there is a significant effect of time (A 3.7.1; F=3.4, p<0.05) in Canton-S but not per^{01}. Therefore, the rise in splicing levels during the first day of DD at 18°C in Canton-S is significant, and there is no similar effect in per^{01} (compare figures 3.7a and b).

At 18°C, there is a significant difference between per^{01} and Canton-S flies in DD (A 3.7.5; F=52.3, p<<<0.001) but not in LD. Therefore, in Canton-S flies, with a functional clock, during the first day of DD, the level of splicing rises, but this does not happen in per^{01} flies. This indicates that the clock is required to initiate a rapid change in splicing levels in response to altered photoperiods; as per^{01} flies show elevated levels of splicing after entrainment to LD 6:18 (fig. 3.6), flies without clocks must still be able to respond to altered photoperiods.

In the absence of LD cycles, the level maximum level of splicing is dependent on the temperature, which sets a level of ~60% at 18°C, and ~40% at 29°C. Rapid adaptation to new photoperiods requires a functional clock.

3.4 Analysis of natural *per* variants

3.4.1 Free-running periods of natural *per* variants

In order to determine which lines are well temperature compensated, and which are not, the free running periods of each of these five strains were measured at different temperatures, as shown in figure 3.8.

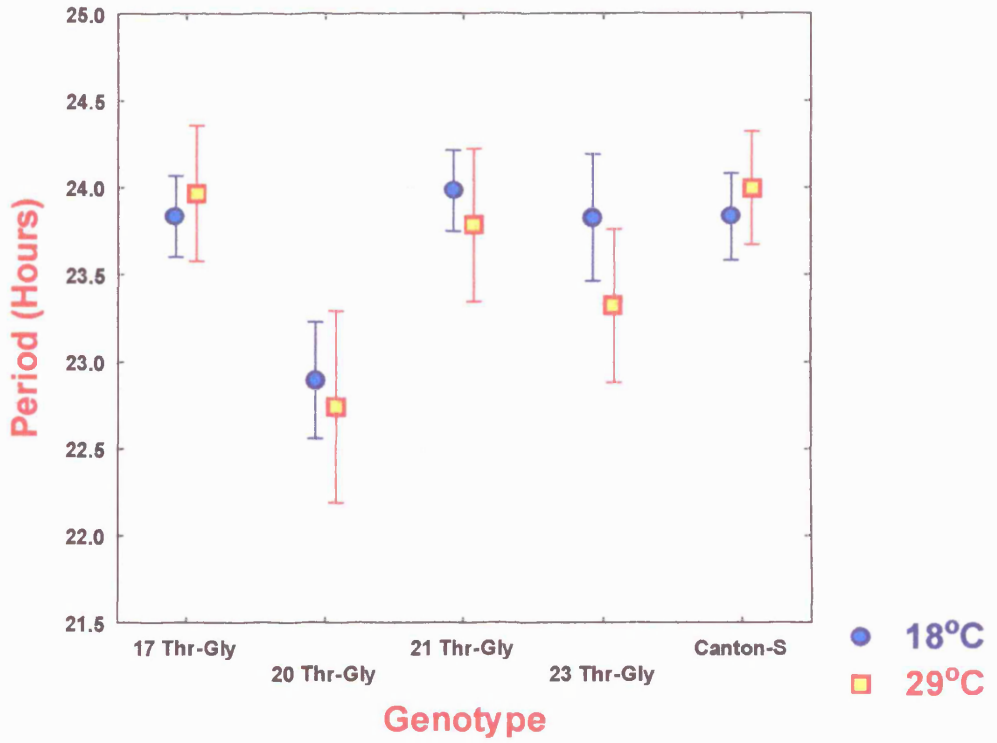


Figure 3.8: Free-running periods of Thr-Gly variants 18°C (blue) and 29°C (red). Error Bars represent 95% confidence intervals

Although the difference in period lengths at different temperatures is not significant in any individual line (A 3.8.2 - 6), it appears from figure 7.1 that of these variants, $(Thr\text{-}Gly)_{17}$, $(Thr\text{-}Gly)_{20}$, $(Thr\text{-}Gly)_{21}$ and Canton-S strains are better temperature compensated than $(Thr\text{-}Gly)_{23}$. Therefore in both this study and previously published work the $(Thr\text{-}Gly)_{23}$ variant is the least well temperature compensated (Sawyer et al, 1997). Sawyer et al analysed a large number of lines carrying each Thr-Gly variant and therefore were able to pick up differences in period lengths which are not apparent here as this is a much smaller sample size and the background effect will be much greater.

3.4.2. Evening peak activity in Thr-Gly variants

To determine whether there is any difference in the evening peak position between the different lines, the locomotor activity was assayed for all five variants at 18 and 29°C, as shown in figure 3.9.

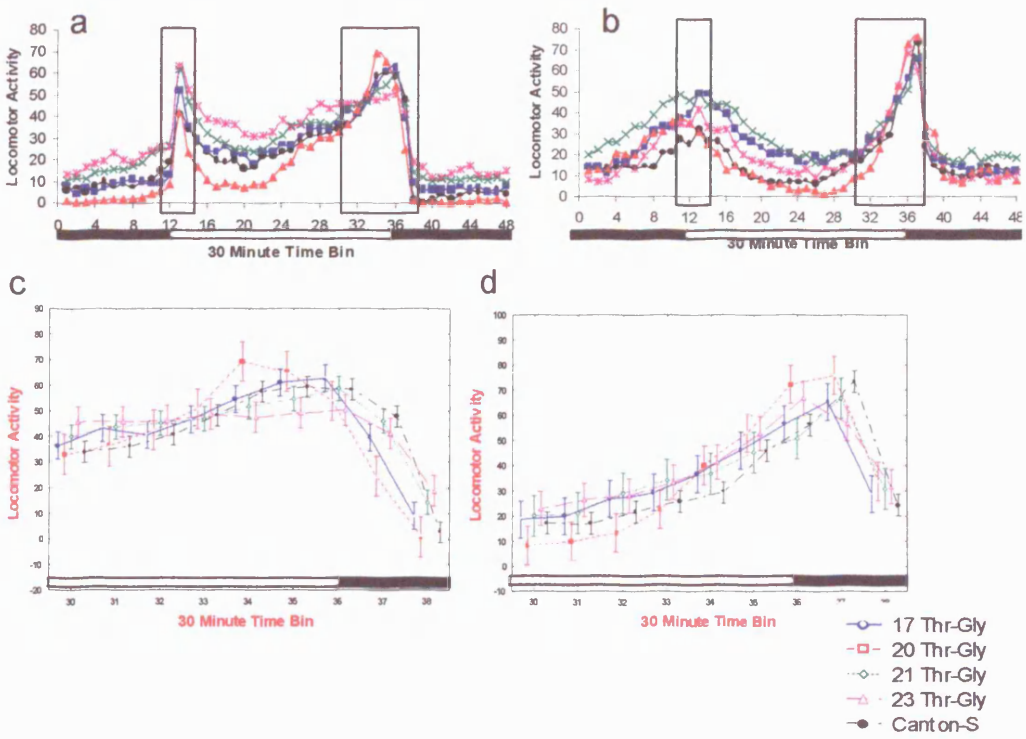


Figure 3.9: Locomotor activity in Thr-Gly variants a) $(Thr\text{-}Gly)_{17}$ (blue), $(Thr\text{-}Gly)_{20}$ (red), $(Thr\text{-}Gly)_{21}$ (green), $(Thr\text{-}Gly)_{23}$ (purple) and Canton-S (black), at 18°C. b) at 29°C. Error bars represent SEM. c). evening activity at 18°C. d) evening activity at 29°C. Error bars represent 95% confidence intervals

In each individual line, the evening activity peak occurs earlier at 18 than at 29°C (there is always a significant interaction between temperature and time; A 3.9.1 - 3.9.4). From figure 3.9c it can be seen that at 18°C, the evening peaks of the $(Thr\text{-}Gly)_{17}$ and $(Thr\text{-}Gly)_{21}$variants, and Canton-S occur at the same time (A 3.9.5; temp x time F=1.4 n.s.), and are significantly different from $(Thr\text{-}Gly)_{20}$ (A 3.9.6; F=3.4, p<<<0.001) and $(Thr\text{-}Gly)_{23}$ (A 3.9.7; F=1.8, p<<<0.001). Additionally, $(Thr\text{-}Gly)_{20}$ peaks earlier than $(Thr\text{-}Gly)_{23}$ (A 3.9.7; F=8.1, p<<<0.001).

With $(Thr\text{-}Gly)_{23}$ there is not a well defined evening activity peak at 18°C; instead activity simply seems to fall immediately after lights off. This lack of a pronounced evening activity peak, rather than an earlier phase of activity

makes *(Thr-Gly)$_{23}$* behaviour different from other variants. Therefore only the phase of the evening peak of *(Thr-Gly)$_{20}$* is advanced at 18°C.

As can be seen from figure 3.9 d at 29°C *(Thr-Gly)$_{20}$* and $_{23}$ evening activity peaks earlier than the other three variants (A 3.9.9; genotype x time, F=2.9, p<<<0.001). There is a significant difference between the evening activity peaks of *(Thr-Gly)$_{20}$* and $_{23}$ as the peak level of activity lasts longer in *(Thr-Gly)$_{20}$* (A 3.9.10; time x genotype, F=5.2, p<<0.001).

So, at 18°C the evening peak of *(Thr-Gly)$_{20}$* is advanced, and at 29°C this is also the case for *(Thr-Gly)$_{23}$*.

If splicing levels of *per* determine the position of the evening activity peak then there should be more spliced *per* mRNA in these two variants than *(Thr-Gly)$_{17, 21}$* and Canton-S. The splicing levels in the Thr-Gly variants are shown in figure 3.10.

3.4.3. *per* splicing levels in Thr-Gly variants

As shown in figures 3.10 a - d, there is significantly more spliced *per* mRNA at 18°C than 29°C in all variants (A 3.10.1 - 3.10.4; 17 Thr-Gly, F=52.2, p<<<0.01; 20 Thr-Gly, F=33.1, p<<0.001; 21 Thr-Gly, F=49.1, p<<<0.001; 23 Thr-Gly, F=16.8, p<0.05). In *(Thr-Gly)$_{20}$*, there is a significant cycle in splicing levels at 18 but not 29°C (figure 3.10b; A 3.10.2; temperature x time, F=3.9, p<0.05). Thus *(Thr-Gly)$_{20}$* is the only 'wild-type' where the level of splicing cycles around the clock.

Figure 3.10 f shows the average splicing levels for each different line. There is significantly more spliced *per* transcript in Canton-S than in any other line at 18°C (A 3.10.7; F=4.2, p<0.05). At 18°C, the levels of splicing are identical in the 17, 20, 21 and 23 Thr-Gly variants (A 3.10.6; F=1.0, n.s.).

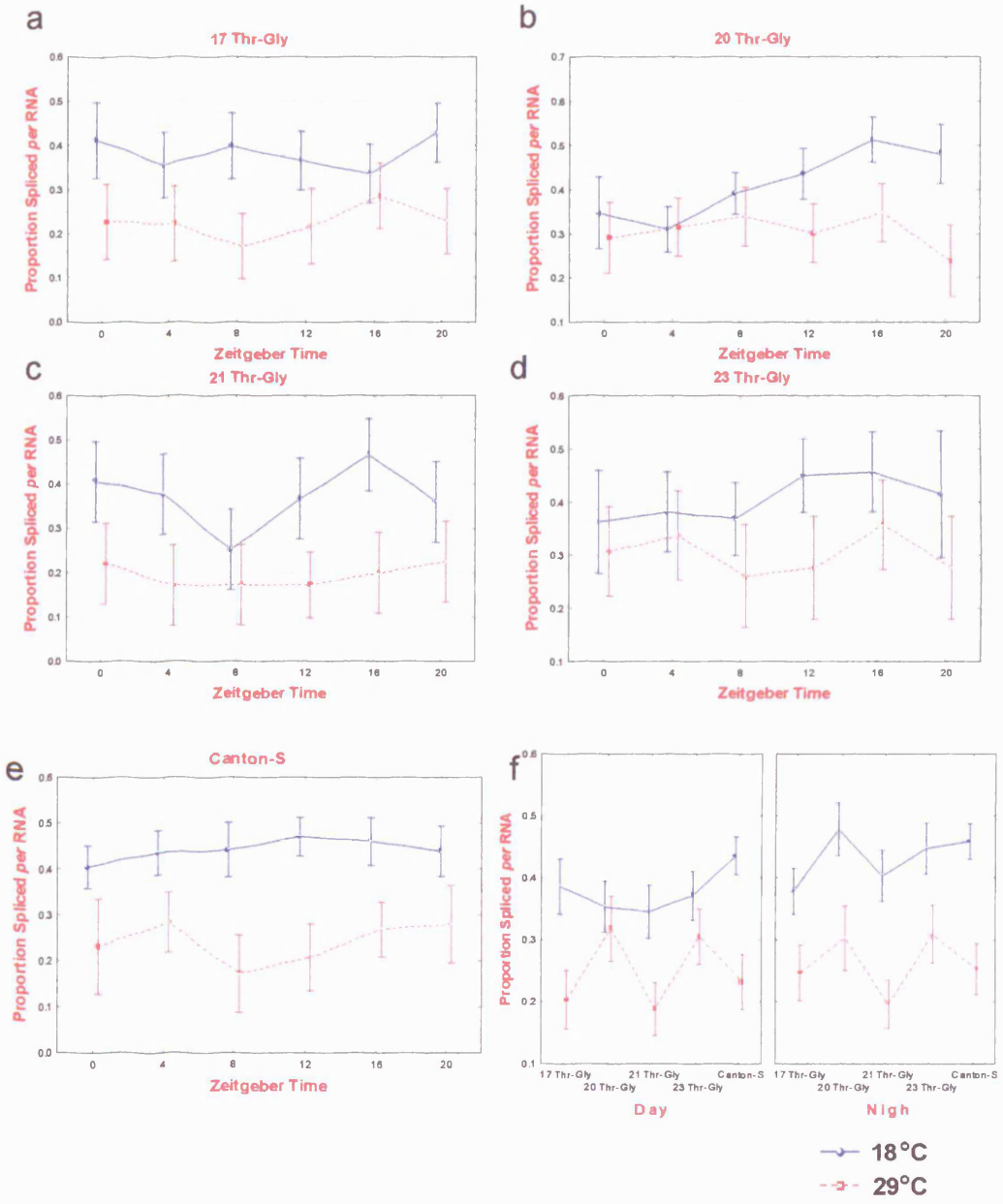


Figure 3.10 Splicing levels in Thr-Gly variants. a) $(Thr\text{-}Gly)_{17}$, b) $(Thr\text{-}Gly)_{20}$, c). $(Thr\text{-}Gly)_{21}$, d). $(Thr\text{-}Gly)_{23}$ and e). Canton-S at 18°C (blue) and 29°C (red). f) average level of splicing in each during the day and night at 18°C and 29°C. Error bars represent 95% confidence intervals.

Further differences in splicing levels emerge if comparisons made only for the night (ZT12 -20). There is no significant difference in the splicing levels at night between 17, 21, 23 *(Thr-Gly)*$_{17,21}$ and $_{23}$ and Canton-S at 18°C (A 3.10.9; F=2.7, n.s.); if the splicing levels of *(Thr-Gly)*$_{20}$ are included, then there is a difference between the different genotypes (A 3.10.10; F=3.2, $p<0.05$).

Therefore at 18°C at night the highest level of splicing is seen in *(Thr-Gly)*$_{20}$, which also displayed the earliest evening activity peak at this temperature (above).

At 29°C the levels of splicing of *(Thr-Gly)*$_{17}$ and Canton-S are not statistically different (A 3.10.11; F=1.2, n.s.), as are the levels in *(Thr-Gly)*$_{20}$ and *(Thr-Gly)*$_{23}$ (A 3.10.12; F=0.02, n.s.), which are higher than in the other lines (A 3.10.12 - 17; *(Thr-Gly)*$_{17}$ and *(Thr-Gly)*$_{20}$, F=33, $p<<0.05$; *(Thr-Gly)*$_{17}$ and *(Thr-Gly)*$_{23}$, F=15.4, $p<0.05$; Canton-S and *(Thr-Gly)*$_{20}$, F=21.6, $p<0.05$; Canton-S and *(Thr-Gly)*$_{23}$, F=10.3, $p<0.05$).

At 29°C, the highest levels of splicing are seen in *(Thr-Gly)*$_{20}$ and *(Thr-Gly)*$_{23}$ which again correlates with the positions of the evening activity peaks

T-tests were used to determine whether the splicing levels were related to the free-running period or the temperature compensation of each variant. As can be seen from appendix table 3.1, there were no significant correlations between the splicing level and either the period or the difference in free-running period at different temperatures. Therefore there is probably no relationship between the splicing of the *per* 3' UTR intron in these lines and temperature compensation.

The strains used in these experiments all have different genetic backgrounds, so by comparing the splicing levels in these natural variants it is possible to determine whether the observations reported here are

independent of genetic background. At 18°C, there was no effect of the per^{01} mutant in comparison to Canton-S, which shows the highest level of splicing at this temperature of all the 'wild-type' strains analysed. The lowest level of splicing at 18°C is seen in $(Thr\text{-}Gly)_{21}$, and ANOVA analysis shows that even if this variant is compared to per^{01}, there is no significant difference (A 3.10.21, F=1.5, n.s.). This confirms that per^{01} has no effect on splicing at low temperatures.

At 29°C, the level of splicing in the per^{01} mutant is higher than that of Canton-S during the night. The highest level of splicing at 29°C is seen in $(Thr\text{-}Gly)_{20}$, but even in comparison to this variant, the per^{01} mutation still has a significant effect on splicing levels only at night (A 3.10.22 - 24, F=7.5, $p<0.05$). This means that the effect on the splicing levels of the various mutations studied is unlikely to be caused by an effect of genetic background, particularly as the data for several natural variants is so similar, despite their differing genetic origins. Although splicing levels cycle at 18°C in $(Thr\text{-}Gly)_{20}$, no such cycle was identified on any other genetic background, and therefore this does not appear to be typical of *D.melanogaster*.

In wild-type strains the splicing level correlates with the position of the evening activity peak. Although there is enough variation in the splicing levels to produce differences in evening peak position in different natural variants, these differences are smaller than those seen between 'wild-type' and the clock mutants.

3.5 Discussion

3.5.1 Locomotor activity and splicing

The analysis of the locomotor behaviour and splicing of Canton-S flies entrained to 12:12 LD at 18 and 29°C confirms previously published observations (Majercak et al., 1999). There is a later evening activity peak at high temperatures, which corresponds to a lower level of splicing of the intron

within the 3' UTR of the *per* transcript at 29°C (figures 3.2 and 3.3). This is confirmed by the analysis of a number of natural *per* variants (figures 3.9-3.10). The splicing levels and evening peak positions of the Canton-S strain therefore provide a 'wild-type' set of data for comparison to mutant strains. In addition, it was found that although the level of *per* mRNA has a distinct cycle (e.g. So and Rosbash, 1997; Majercak et al., 1999), the proportion of this *per* RNA transcript which has been spliced remains constant around the clock. This means that at all times ~45% of *per* transcripts at 18°C, and ~25% of *per* transcripts at 29°C are spliced in Canton-S flies.

Without a functional clock, as in per^{01} and tim^{01}, there is no anticipation of dawn and dusk, so the locomotor activity profiles of per^{01} and tim^{01} flies have no anticipatory activity peaks. In these lines there is a very clear morning activity peak immediately after lights on, followed by a constant level of activity until lights off (see figure 3.4). As the morning peak is present in these mutants, and occurs at the same time as in Canton-S, it does not require a functional clock. The morning activity peak in per^{01} and tim^{01} is much larger than in wild-type strains, so one role of the clock may be to reduce the 'startle effect' of lights on, presumably because the timing of dawn can be anticipated. Without a clock, the startle effect cannot be attenuated and the morning peak activity is much greater. The role of the clock and visual system in determining the morning peak is discussed further in chapters 4 and 5.

The abolition of the clock removes most of the locomotor activity responses to altered temperatures. The only temperature difference remaining in either clock mutant is the higher level of morning peak activity at 18 than at 29°C in per^{01} flies, a difference also seen in Canton-S. As this difference is lost in tim^{01} flies, it raises the possibility that PER and TIM have different roles in the temperature control of morning activity. Apart from the difference in the morning peak size in tim^{01} mutants, and a small, non-anticipatory evening peak in tim^{01}, the activity levels in per^{01} and tim^{01} flies are identical at both 18 and 29°C.

The small evening activity peak that occurs in tim^{01} flies is dependent on the signal of 'lights off' and shows no temperature dependence or anticipation of the event. As there is no similar peak in per^{01} flies, *per* may be more important than *tim* in producing the evening activity peak, although how PER is able to affect activity without the stabilising effect of TIM is unclear. In per^{01} mutants, PER protein is not translated, while in tim^{01} mutants there is no TIM protein to stabilise PER, so PER protein is present only at very low levels.

At 18°C, the level of splicing is identical in per^{01}, tim^{01} and Canton-S in a 12:12 photoperiod (figure 3.5), and per^{01} and Canton-S in a 6:18 photoperiod (figure 3.6) indicating that the clock is not required to regulate splicing at low temperatures. At 29°C, the levels of splicing in per^{01} and tim^{01} flies are identical, and differ from those of Canton-S flies (figure 3.5). During the day at 29°C, splicing is repressed to the same extent as in Canton-S flies. This suggests that during the day at high temperatures, the presence of light ensures that splicing is repressed.

However, in per^{01} and tim^{01} mutants, the level of splicing at night is significantly higher than during the day at 29°C, and higher than the level of splicing in Canton-S at this temperature. The level of splicing at night at 29°C is the same as at 18°C in per^{01}, tim^{01} and Canton-S lines, indicating that the repression of night-time splicing is lost in these mutants at 29°C. Therefore the clock is required to repress splicing at elevated temperatures at night.

The 45% level of splicing seen at 18°C in LD 12:12 appears to be a 'default' level of splicing for this photoperiod, as the same level is observed in clock mutants at 29°C at night, when the apparent repression of splicing is lifted (figure 3.5e). It is also the level seen in per^{01} flies at 18°C and 29°C in the first day of DD, suggesting that this is the level at which splicing has been set by temperature and the previous LD 12:12 photoperiod (figure 3.7). With no

functional clock the level of splicing rises at night at high temperatures, so therefore the clock must repress splicing at high temperatures. This is in contrast to the published model (Majercak et al., 1999), where it was suggested that splicing is enhanced at low temperatures. This fits with the idea that the regulation of behaviour is more important at elevated temperatures, when it is important to avoid the heat of mid-day.

3.5.2 Splicing in altered photoperiods

There is more spliced *per* mRNA at 18°C in LD 6:18 than LD 12:12, mainly due to an elevated level of splicing in the early night (figure 3.6). This elevated level of splicing in a reduced photoperiod may be essential to the correct functioning of the clock. During the day, the repression of splicing by the PER/TIM complex is lifted by the light-induced degradation of TIM. This produces a rise in *tim* and *per* mRNA levels, eventually leading to an upswing in PER and TIM protein levels (Stanewsky, 2002). In LD 12:12 the level of *per* transcription does not begin to rise until several hours after the degradation of TIM, when the levels of PER fall (Rothenfluh et al., 2000). This means that in LD 6:18, there is a much shorter window in which *per* mRNA transcripts are able to accumulate, and therefore more will need to be spliced to maintain the same levels of PER and the timing of the PER protein upswing in PER.

The first day of DD is equivalent to the first day of a very short photoperiod. During this time, the level of splicing rises constantly at 18°C in the wild-type strain, but not in per^{01} mutants. As per^{01} flies show the same level of splicing as Canton-S in LD 6:18 after entrainment, the splicing machinery must be able to respond to reduced photoperiods in the absence of a clock; the clock must therefore speed up the adjustment to a new photoperiod. However, in nature, the change between photoperiods is very gradual. Therefore the role of the clock in eliciting a rapid response to altered photoperiods is more likely a consequence of the role of the clock in 'fine-tuning' splicing and behaviour. This may be undetectable under this protocol perhaps because there is no daily variation in light intensities or temperature.

The comparison of splicing levels in DD at 18 and 29°C suggests that there is a limit as to how high the splicing level can rise which is temperature dependent. At 29°C, the level of splicing is only slightly elevated from the LD 12:12 levels (~25% in LD and ~30-35% in DD, whereas at 18°C, the level of splicing rises constantly throughout the first day of DD (45% in LD and 60% after a day in DD). This suggests that at high temperatures there is a mechanism which acts to set the maximum level of spliced *per* which can be reached. At 29°C, the level of splicing is very similar in both per^{01} and Canton-S strains, suggesting that this limiting mechanism may be clock independent. In order to confirm this the level of splicing needs to be checked after several days of DD at both 18 and 29°C.

Recent work from J.Majercak (pers. com.) confirms many of the findings in reduced photoperiods reported here, including the increasing levels of splicing. In DD mutants for any of the clock genes showed lower levels of splicing than wild-type, indicating that the clock is required to regulate splicing in the absence of LD cues (compare DD splicing of per^{01} and Canton-S, figure 3.7 above). Light pulses during DD repress splicing, but not below the level of splicing seen during the day in LD cycles, confirming the role of light in the repression of splicing.

Regulation of *tim* RNA levels by light and temperature was also identified, and, like the regulation of *per* splicing, there is a different effect on *tim* levels at different temperatures (J. Majercak, pers. com). At low temperatures, a light pulse during dark leads to a rise in *tim* but not *per* levels. So, in cold conditions light controls the splicing of the 3' UTR intron and *tim* RNA levels, and as a result there are elevated levels of *per* and *tim* present on cold days, generating the appropriate behaviour despite the reduced daylight hours. At higher temperatures, the regulation of splicing comes under the control of the clock, and light no longer alters *tim* expression. Therefore both *per* and *tim* RNA levels are under different systems of control at different temperatures (J. Majercak, pers. com.).

Although both sets of work show that light and temperature control the splicing of the *per* 3' UTR intron, there are some differences. For instance, a daily cycle in splicing levels in LD 12:12 cycles was previously reported (Majercak et al., 1999), although this only became apparent in reduced photoperiods in the work reported here[9]. The cycle observed is very shallow (Majercak et al., 1999) and could therefore be accounted for by differences in experimental conditions - light intensities in incubators, RNA amplification protocols and strain differences. The same is probably true of the oscillations observed in DD (J. Majercak, pers. com.), although it may be that if the analysis of DD cycles is extended beyond the first day of DD the splicing level begins to fall. Interestingly, the clock was found to be required to regulate the response of splicing to reduced photoperiods, and as a result contradicts the results reported here. As only the per^{01} line has been analysed, and it shows altered splicing in DD, it could be that further analysis of clock mutant lines would reveal a requirement for the clock in the photoperiodic regulation of splicing.

Conversely no role was found for the clock in the temperature regulation of splicing (Majercak et al. 1999; J. Majercak, pers. com.). As an identical effect of both the per^{01} and tim^{01} mutant was observed here, and the results are consistent, it seems likely that this was not picked up by the alternative protocol.

3.5.3 Splicing and temperature compensation

This work has identified no definite links between the regulation of splicing and temperature compensation, and the regulation of splicing seems to have little if any effect on the free running period of the clock. Therefore temperature compensation and the regulation of *per* alternative splicing are two separate mechanisms by which the circadian clock copes with environmental changes in temperature.

[9] Although cycling of splicing was observed in the $(Thr\text{-}Gly)_{20}$ variant, the strain used in the work of Majercak et al. was Canton-S.

Of the natural *per* variants studied, *(Thr-Gly)$_{20}$* stands out as it has the highest splicing levels at both 18 and 29°C, and correspondingly early evening locomotor activity compared to the other lines. Having early evening activity is likely to be disadvantageous at high temperatures as activity will occur during the hottest part of the day. This means that *(Thr-Gly)$_{20}$* appears to have an evening locomotor activity peak more suited to living in cooler climates. *(Thr-Gly)$_{20}$* is also the best temperature compensated of these *per* variants, and this is thought to explain the predominance of this variant in northern Europe (Sawyer et al, 1997). Therefore both the splicing and temperature compensation phenotypes of this line fit with its geographical distribution, and it may be interesting in the future to see if earlier evening activity is also found in other *(Thr-Gly)$_{20}$* lines, and even whether there is a cline in splicing levels as well as temperature compensation across Europe.

3.5.4 Summary

The regulation of splicing generates altered patterns of behaviour because it alters the timing of the daily upswing in PER protein levels, leading to altered evening peak positioning. The splicing machinery needs to be under a tight system of control which can adapt to changes in light and temperature so that activity can be fitted to different climates and respond to seasonal variations in photoperiods and temperatures (Majercak et al., 1999). Although experiments in the laboratory are only a crude approximation of the gradual changes in light and temperature seen on a daily basis in the wild, they provide a picture of how the fly fine tunes behaviour through the control of a single splicing event.

The regulation of behaviour by alternative *per* transcript splicing is controlled by different input pathways at high and low temperatures, as if the high temperature response is only switched on at a certain point. At low temperatures, the primary determinant of the level of splicing appears to be the photoperiod. Under LD 12:12 the level of splicing is constant around the clock, with ~45% of all *per* transcripts being spliced. If the photoperiod is

then shortened to LD 6:18, the level of splicing rises, and there is slightly more spliced RNA present at night than during the day, peaking after lights off at around ZT8. At low temperatures, the clock is not required for the setting of the splicing level by photoperiod as at 18°C the level of splicing in per^{01} mutants is identical to that seen in the wild-type Canton-S strain in both LD 12:12 and LD 6:18.

At 29°C, the repression of splicing is light dependent during the day and clock dependent at night. In per^{01} and tim^{01} at 29°C ~30% of transcripts are spliced during the day, and ~40% at night, compared to ~25% of transcripts day and night in Canton-S. This means that the clock is much more important in controlling splicing at night, with the level in clock mutant flies at night at 29°C identical to that seen in either wild-type or clock mutant flies at 18°C. This suggests that in the absence of a clock, all temperature dependent repression of splicing is lost at night.

The regulation of splicing, and therefore of behaviour, is under tighter control at high temperatures, when the clock is recruited. It is thought to be more important to regulate behaviour at high temperatures so as to avoid desiccation in the mid-day heat. On a cold autumnal day, the temperature does not get particularly high, so there is no need to be active at any particular time of the day, as long as it is still light. Therefore only the photoperiod matters when setting the splicing level. In contrast, at high temperatures, it is vital to avoid mid-day activity, and therefore splicing is tightly regulated by the clock to ensure that most activity is concentrated into the evening activity peak around dusk.

However, to ensure that the pattern of activity fits as well as possible to daily temperature changes, some control of splicing levels is clock independent, as demonstrated by the repression of splicing by light during the day in clock mutant flies even at high temperatures.

The regulation of splicing at high temperatures by the clock only at night is probably owing to the nature of the cycling of PER and TIM. During the day at high temperatures PER and TIM levels are low and cytoplasmic rather

than nuclear, so are unlikely to regulate splicing. At night the levels of PER and TIM are at their highest, and able to enter the nucleus, so these clock components are available to set the splicing level as required. As the difference between 'day' and 'night' under laboratory conditions is the presence or absence of light, and light represses splicing, as shown by the elevated levels of splicing in LD 6:18 and DD, this probably involves a light input. Therefore during the day at high temperatures, light represses splicing, and at night the clock carries on this repression. The elimination of the light input to the splicing machinery should abolish daytime repression at high temperatures, and leave splicing levels similar to those seen in DD.

Chapter four: splicing and behaviour in cry^b mutants

4.1 Introduction

Examination of wild-type and clock mutant flies under different light regimes revealed that light has a prominent role in setting the *per* splicing level. The entrainment of behaviour to LD cycles uses both ocular and non-ocular photoreception pathways (e.g. Helfrich-Forster, 2001), so it is likely that the same pathways set the *per* splicing level. The most obvious candidate gene for this is the circadian photoreceptor cryptochrome, and its role in the regulation of splicing can be deduced by looking at flies carrying the cry^b mutation (Stanewsky et al., 1998).

cry^b is a missense mutation of a highly conserved residue within the C-terminal of the protein, which disrupts a flavin binding region and produces little or no expression of the mutant CRY protein (Stanewsky et al., 1998). Although cry^b is not a null mutation, it causes arrhythmic *per* and *tim* expression in whole head extracts, but unlike cyc^0 and Clk^{Jrk} the overall RNA levels are unaffected. This seems to be because the light dependent degradation and phosphorylation of TIM is blocked in cry^b mutants, preventing the correct regulation of RNA levels through the negative feedback loop, so PER and TIM levels remain high at all times in LD cycles, but only within the eyes (Stanewsky et al., 1998). The cry^b mutation has no effect on locomotor activity in DD, indicating that CRY is involved in the transmission of light information to the clock, and in the absence of light, it has no effect on PER/TIM cycling (Stanewsky et al., 1998)

Despite the lack of light-induced TIM degradation and PER/TIM cycling in the eyes, cry^b flies can be entrained to light:dark cycles and remain rhythmic in DD, so the clock is still functional. Entrainment to LD cycles occurs because the clock is still sensitive to the light input from the visual system even in cry^b

mutants, because within ventral lateral neurons (LN_vs), PER and TIM levels cycle and are able to generate rhythmic locomotor activity. Thus CRY is not a core clock component (Stanewsky et al., 1998). However, normal entrainment of locomotor activity requires CRY expression in the central clock because CRY overexpression produces an earlier phase of activity without affecting the free running period (Ishikawa et al., 1999) making it an ideal candidate for regulating evening locomotor activity peak position. cry^b mutants take ~3 days longer to entrain to a new light regime and the mutation primarily affects evening peak entrainment confirming yet again that the evening peak, more than the morning peak, is under circadian control (Emery et al., 2000b).

Could it then be that this evening peak effect is due to CRY altering the splicing of *per* mRNA in a temperature dependent manner? Analysis of the cry^b mutation has provided evidence that CRY interacts with PER and TIM and alters the levels of RNA, and some aspects of these effects are temperature dependent (Ceriani et al., 1999; Rosato et al., 2001). The cry^b mutation alters both the *per* and *tim* expression levels probably because the abolition of the light dependent degradation of TIM causes the breakdown of the feedback loop in the eye. The role of CRY in the negative feedback loop seems to be to stop the PER/TIM complex from being able to inhibit its own transcription in the presence of light as *in vivo,* the CRY^b mutant protein prevents this inhibition in S2 cells (Ceriani et al., 1999).

The regulation of PER levels by CRY could therefore be an indirect effect due to the degradation of TIM. Coimmunopreciptiation assays using S2 cells and yeast 2-hybrid assays initially suggested that this was the case, as CRY could only be shown to bind TIM, which would explain why TIM levels fall fastest in response to light (Ceriani et al., 1999). Subsequently, an interaction with PER was observed at both the molecular and phenotypic levels (Rosato et al., 1999).

There is good evidence supporting an association between the PER/TIM complex and CRY that is vital for the light regulation of PER/TIM levels. CRY

subcellular localisation is dependent both on light and the PER/TIM complex. Normally CRY is predominantly nuclear, but in the presence of light and PER/TIM, it becomes localised to speckles within the nucleus (Ceriani et al., 1999). The interaction between CRY and TIM in yeast two hybrid assays or S2 cells is also light dependent, irrespective of whether TIM is monomeric, or dimerised with PER, and is lost with the CRY^b mutation. It seems that CRY^b cannot respond correctly to light owing to the mutation in the flavin binding site and, as a result, is destabilised (Ceriani et al., 1999). In wild-type flies, the light activation of CRY causes it to undergo light-induced degradation. In cry^b, proteasomic degradation of TIM does not occur, so it is likely that CRY targets TIM for degradation before CRY itself is degraded (Lin et al. 2001). TIM reacts to light in a similar way, being ubiquinated before proteasome degradation (Naidoo et al., 1999). These results explain why CRY affects the PER/TIM complex in a light dependent manner (Ceriani et al., 1999).

Further evidence that CRY may be involved in the temperature dependent regulation of splicing is that the interaction between the PER/TIM complex and CRY is temperature sensitive and potentially mediated by the PER protein (Rosato et al., 2001). The behavioural phenotype of the per^s cry^b double mutant reveals a genetic interaction between *per* and *cry* (Stanewsky 1998; Rosato et al., 2001). At 25°C in LD, this double mutant displays predominantly 24 h rhythms, but there is also a 19 h rhythmic component in ~40% of flies, a property of per^s non-entrainment (Rosato et al., 2001). This phenotype is temperature sensitive, and at higher temperatures, this 19 h period becomes the main rhythmic component in ~80% of flies. These flies are perfectly capable of light detection as there is still a morning peak, but there is a temperature dependent defect in the transmission of this information to the clock (Rosato et al., 2001).

The PER/CRY protein interaction, like the phenotype of per^s; cry^b, is temperature dependent, decreasing at higher temperatures. The C terminus region of CRY is required for mediating the light response and the per^s mutation lies within the region of PER which interacts with the CRY C

terminus, perhaps explaining the temperature sensitivity of the interaction (Rosato et al., 2001).

Although the CRY/PER interaction can be temperature sensitive (Rosato et. al 2001), the primary role of CRY is in light rather than temperature dependent regulation of the clock as the cry^b mutation has no effect on the entrainment of PER/TIM cycles to temperature fluctuations (Stanewsky et al., 1998). In fact, cry^b does abolish some temperature differences in the activity profile, as reported below. CRY therefore is a good candidate for the light input pathway to the splicing mechanism, but there are indications that other factors are involved. cry^b flies are rhythmic in LL, and therefore 'blind' to constant light, yet can be entrained to LD (Emery et al., 2000a), so *cry* cannot be the only light sensitive component detecting dawn/dusk changes.

4.1.2 Peripheral Clocks

It is also worth noting that in peripheral tissues, CRY is a core clock component as the cry^b mutation abolishes the cycling of PER and TIM at both the protein and RNA level (Stanewsky et al., 1998). When whole head extracts are analysed, the majority of protein or RNA comes from the eyes. CRY has a different role in the LN_vs (central clock) to that within other tissues, such as the eyes or antennae (peripheral clocks).

Most *Drosophila* tissues have autonomous clocks in the periphery that are similar in phase to the central clock located in the LN_vs (Plautz et al., 1997). These clocks use many of the same genes as the central clock - with cycling *per* and *tim* levels, and TIM degradation in response to light (e.g. Giebultowicz & Hege 1997; Giebultowicz et al. 2000; Krishnan et al., 2001). The central pacemaker cannot regulate the clocks in many of these tissues as they have no neural connection to the visual system or the brain, yet light still entrains their clocks.

These independent clocks govern processes that run in different phases, such as oviposition, sperm release, metabolism, excretion and ecdysis (for review, see Giebultowicz, 2001). One reason for having more than one light

input into the clock may be that the same genes are used in running clocks in peripheral tissues. Peripheral tissues may not have a direct neural connection to the visual system, and as a result they must either utilise their own photoreceptor, most likely CRY, if they cannot be driven by the central clock.

These peripheral clocks have some novel properties. A loss-of-function mutation of a core clock component should produce an arrhythmic clock. CRY is not a core clock component within the LN_vs, as cry^b flies can still be entrained to light and are rhythmic in DD. However, within the antenna, cry^b behaves as a mutation of a core clock gene - the cycling of *per* and *tim* RNA is lost in ~75% of flies, and the overall levels of *per* and *tim* are elevated (Krishnan, 2001).

In the antennae of the fly, the olfactory response varies in a circadian manner, and the cry^b mutation severely reduces this response in LD and abolishes it in DD. The cry^b mutation also prevents the antennal clock entraining to temperature cycles (Krishnan, 2001). The same is true in the malpigian tubules (MT), where cry^b flies also show no TIM cycling in DD (Ivanchenko et al., 2001), indicating that CRY is a core component of these peripheral oscillators (Krishnan, 2001; Ivanchenko et al., 2001). CRY probably also acts as a photoreceptor in the periphery, as in cry^b the *per-tim* cycle entrains more strongly to LD than temperature cycles. This also indicates that CRY may have a role in the temperature input pathway, at least in peripheral clocks (Krishnan, 2001)

Although it is a core clock component within peripheral clocks, CRY also has many of the same functions as seen within the LN_vs clock. In both the brain and peripheral clocks, CRY degrades TIM in response to short light pulses. Even within the MTs, there is nuclear localisation of PER and TIM late at night in cry^b flies, unlike the eyes (Stanewsky et al., 1998; Ivanchenko et al., 2001). It thus seems that all peripheral clocks are not the same. The cycling of *per* and *tim* can be seen to be reduced by the cry^b mutation within the heads, bodies, antennae and forelegs, but not the wings of the fly,

suggesting that CRY has a central clock function within these tissues. In the other tissues, there are differences in the peaks of *per* and *tim* expression, suggesting that the different clocks differ slightly from each other in function (Levine et al., 2002).

4.2 Aims and objectives

The regulation of the splicing of the intron within the *per* mRNA transcript is partially controlled by light, and partially through the action of PER and/or TIM (Chapter 3). As CRY is the circadian photoreceptor, and known to interact with PER and TIM (Emery et al., 2000a; Rosato et al., 2001; Ceriani et al., 2000) the behaviour and levels of splicing were analysed in cry^b mutants to see if CRY is also part of the splicing regulatory machinery. To test the role of PER and CRY, per^{01}; cry^b double mutants were produced, and the locomotor activity and splicing phenotype of this line was also analysed. To see what role CRY has in mediating the temperature response of the clock, all experiments were carried out at 18 and 29°C.

4.3 Methods

Flies were collected and analysed as described in Chapters 2 and 3. Data from per^{01}, tim^{01} and Canton-S is taken from Chapter 3. per^{01}; cry^b were generated as described in Chapter 2.

4.4 Results

4.4.1 cry^b Locomotor Activity

The locomotor activity at 18 and 29°C of cry^b and a comparison with Canton-S is shown in figure 4.1

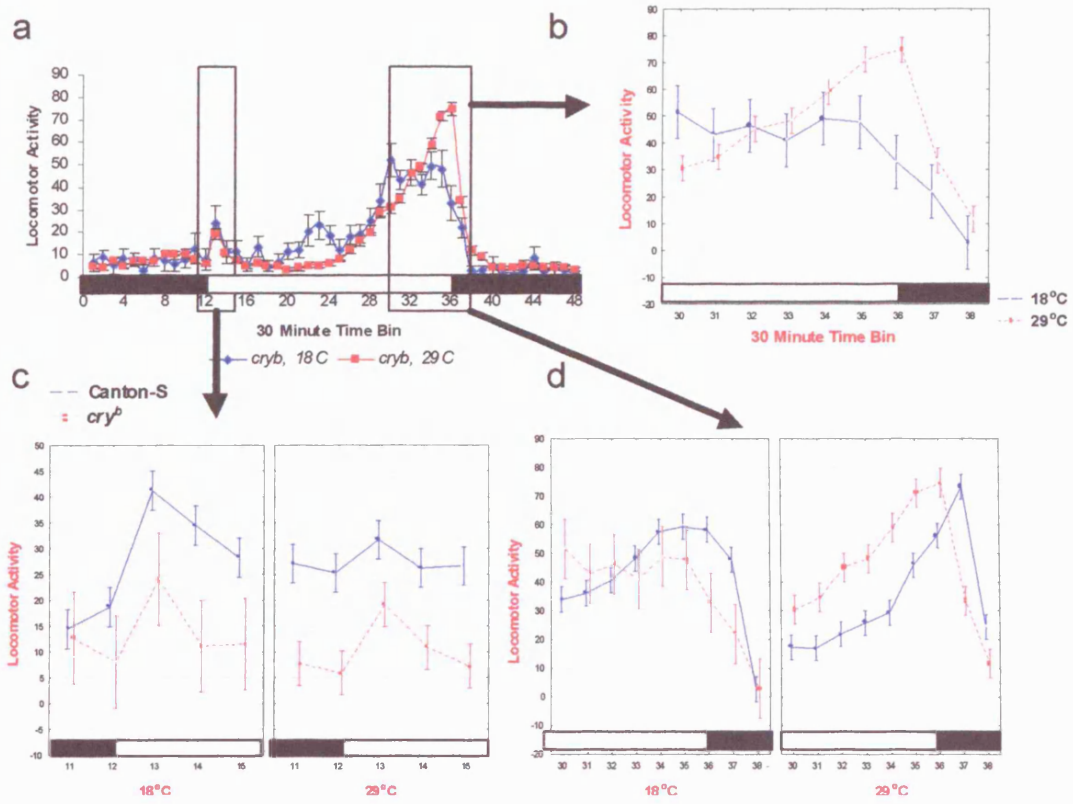


Figure 4.1: Locomotor activity in *cry*^{b}: a). at 18°C (blue) and 29°C (red). Error bars represent SEM. b). expanded evening activity peaks. c). Canton-S (blue) and *cry*^{b} (red) morning peaks at 18°C and 29°C d). Canton-S (blue) and *cry*^{b} (red) evening peaks at 18°C and 29°C. Error bars represent 95% confidence intervals

Although *cry*^{b} flies are rhythmic, the distribution of locomotor activity differs in a number of ways from the wild-type profile. As the *cry*^{b} mutation reduces the effect of temperature changes on locomotor activity, indicating that CRY is involved in temperature regulation of behaviour. In Canton-S flies, both the morning and evening peaks change with temperature (see Chapter 3, fig. 3.2). In *cry*^{b} flies the morning peak is identical at both 18 and 29°C (figure 4.1a,c,e, A 4.1.2; F=2.6, n.s.), but there is a significant delay in evening peak position at high temperatures (4.1b, A 4.1.3; temp. x time, F=10.5, p<<<0.001). In Canton-S, the evening activity peak also begins to rise ~2 h earlier at 18 than at 29°C, generating more activity during the day at 18 and at night at 29°C. This phase difference is lost in *cry*^{b} mutants (compare 4.1a with 3.2a; A 4.1.4 time x temp, F=1.4, n.s.).

The morning activity peak, a result of the startle effect of lights on, is smaller than the equivalent peak of Canton-S at both 18 and 29°C (A 4.1.2; F=34.1, p<<<0.001; F=201.3, p<<<0.001), but the peak position remains constant (time x genotype, 4.1e). The evening activity peak is advanced in comparison to that of Canton-S at both 18 and 29°C (4.1d; A 4.1.6 - 4.1.7; genotype x time F=6.0, p<<<0.001; F=48.4, p<<<0.001 respectively).

The difference in morning peak levels is completely lost in *cry*^{b} mutants, but the evening activity peak still occurs earlier at lower temperatures. This peak is earlier at 18 and 29°C than the corresponding peak in Canton-S.

4.4.2 *cry*^{b} Splicing levels at 18 and 29°C

As the evening activity peak occurs at different times at 18 and 29°C in *cry*^{b} flies, a difference in splicing levels at 18 and 29°C is expected. As the evening peaks at both temperatures are earlier than those of Canton-S flies, the levels of splicing would also be predicted to be generally higher than those of Canton-S. The splicing levels of *cry*^{b} flies and comparisons with Canton-S, *per*^{01} and *tim*^{01} are shown in figure 4.2.

In *cry*^{b} there is significantly more spliced *per* mRNA at 18 than 29°C (A 4.2.1; F=47.4, p<<<0.001). At 29°C, the level of splicing cycles around the clock, so that there is more spliced RNA at night (temp x time, F=3.2, p<0.05), figure 4.2a. The level of splicing at 18°C is significantly different from that of *per*^{01}, *tim*^{01} and Canton-S strains (A 4.2.3; F=4.7, p<0.05), all of which have identical levels of splicing at this temperature (figure 3.5). This is due to an elevated level of splicing at ZT 16 in *cry*^{b} flies (if this is repeated excluding ZT 16, the difference between the genotypes is not significant; F=1.231, p=0.302). This elevated level of splicing at night must therefore account for the earlier phase of activity seen in these flies in comparison to Canton-S

assuming that the level of splicing is responsible for determining evening peak position (figure 4.1).

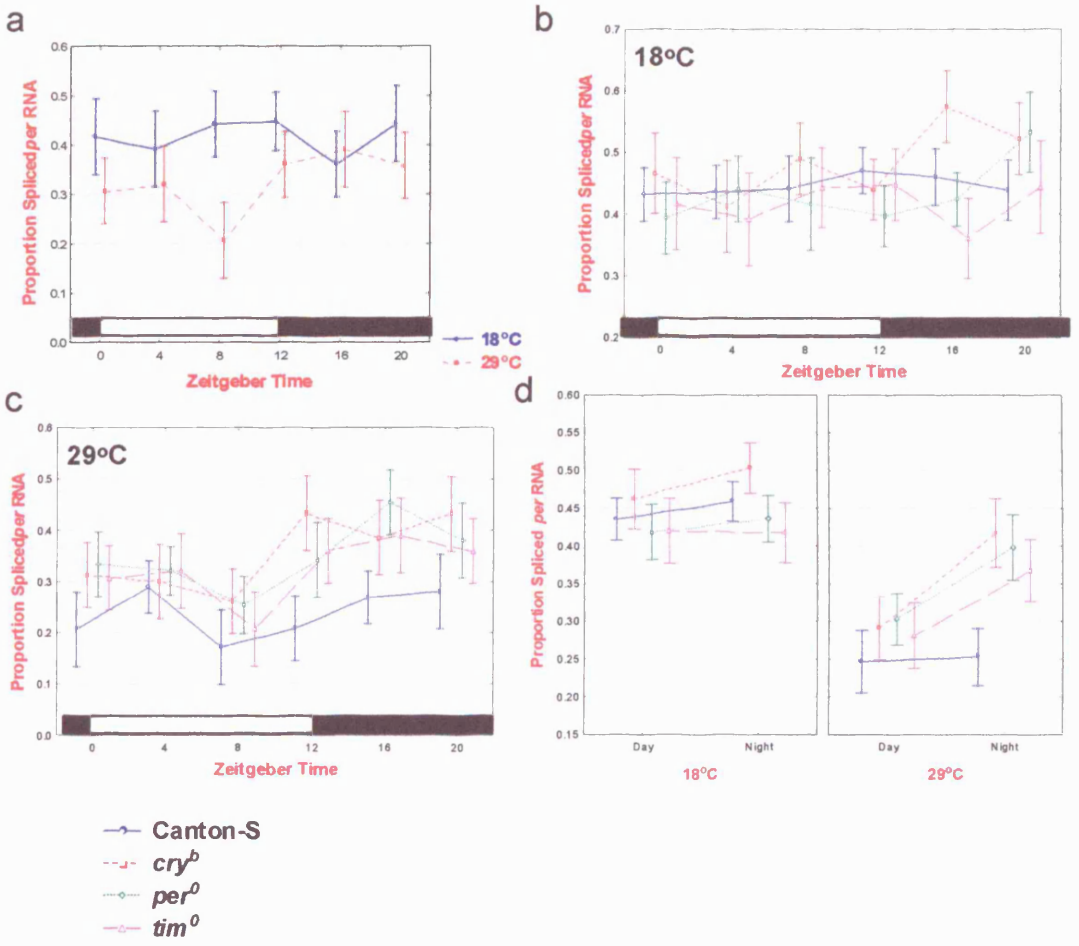


Figure 4.2: Splicing levels in *cry*^{b} flies. a). 18°C (blue) and 29°C (red). b). splicing levels in *cry*^{b} (red), Canton-S (blue) *per*^{01} (green) and *tim*^{01} (purple) at 18°C. c). splicing levels in *cry*^{b} , Canton-S, *per*^{01} and *tim*^{01} at 29°C. d). splicing levels in these lines during the day (ZT 0-8) and night (ZT 12-20) in *cry*^{b} , Canton-S, *per*^{01} and *tim*^{01}

At 29°C, the level of splicing in *cry*^{b} flies is identical to that of *per*^{01} and *tim*^{01} lines, with repression of splicing during the day but not at night (4.2c and d; A 4.2.5; F=0.67, n.s.); this is significantly different from that of Canton-S flies (F=16.171, p<<<0.05). The level of splicing is elevated only at night in comparison to Canton-S flies (4.2d) so any differences in evening peak

position in cry^b flies compared to Canton-S must be a result of elevated levels of splicing during the night at both 18 and 29°C.

The splicing levels of cry^b are elevated at both 18 and 29°C compared to wild-type, which correlates with the earlier evening activity peaks. *At* 29°C cry^b, per^{01} and tim^{01} all eliminate the repression of splicing at night, suggesting that all three proteins act to repress splicing at elevated temperatures at night.

4.4.3 per^{01}; cry^b double mutants

At 29°C, per^{01}, tim^{01} and cry^b have the same splicing phenotype, with partial repression of splicing during the day, and a complete loss of the repression of splicing at night. In order to determine whether PER, TIM and CRY act together or independently to regulate splicing of the *per* intron, double mutant per^{01} cry^b flies were bred (see Chapter 2), and their locomotor activity and splicing phenotypes examined under 12:12 LD conditions as shown in figures 4.3 –4.4.

per^{01} flies have a pronounced morning peak and no anticipation of lights off (figure 3.3), whereas cry^b single mutants have a reduced morning peak, presumably because they are partially blind to the light, and a fairly normal, if broad, evening activity peak (figure 4.1). Thus cry^b almost 'knocks out' the morning peak, while per^{01} eliminates the evening peak. The double mutant would therefore be expected to have a much reduced morning peak, due to the cry^b mutation, and no anticipatory evening peak as the per^{01} mutation means that there is no functional clock.

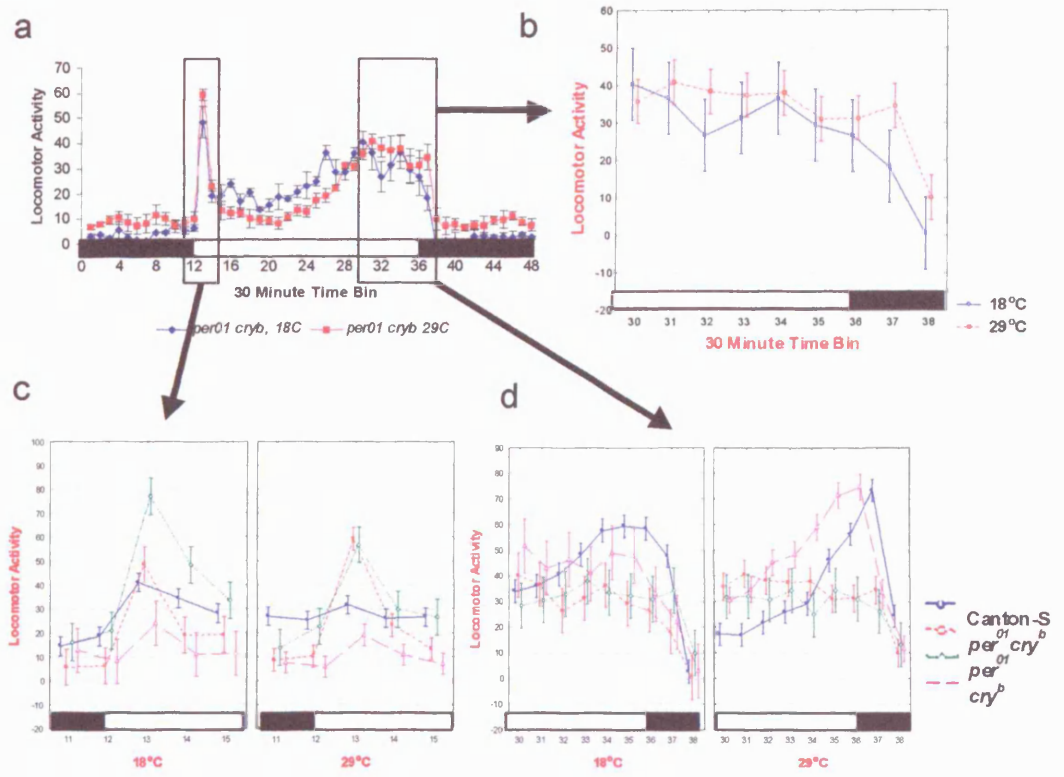


Figure 4.3: Locomotor activity in *per*^{01}; *cry*^{b}

a). 18°C (blue) and 29°C (red). Error bars SEM b). expanded evening peaks at 18°C (blue) and 29°C (red). c). morning activity peaks of Canton-S (blue), *per*^{01}; *cry*^{b} (red), *per*^{01} (green) and *cry*^{b} (purple) at 18°C and 29°C. d). evening activity peaks of Canton-S, *per*^{01}; *cry*^{b}, *per*^{01} and *cry*^{b} at 18°C and 29°C. Error bars 95% confidence interval

Remarkably, the locomotor activity of the *per*^{01}; *cry*^{b} double mutant does not match the prediction, with a morning peak more similar to wild-type than *cry*^{b} flies (figure 4.3 d), and an anticipatory evening activity peak similar to that of the *cry*^{b} mutant at 18°C rather than the *per*^{01} strain (figure 4.3e). Under constant conditions rhythmicity does not persist beyond the first day of DD, as confirmed by spectral and autocorrelation analysis (see Appendix 4.5), indicating that there is no functional clock, presumably owing to the *per*^{01} mutation, yet the fly appears to anticipate 'lights off' in LD cycles. One unique feature of the *cry*^{b} mutation is rhythmicity in LL, which would normally cause the degradation of TIM and produce arrhythmicity (Emery et al., 2000a). Both autocorrelation and spectral analysis of *per*^{01}; *cry*^{b} activity in LL revealed no rhythmicity, again indicating the lack of a functional clock.

There is no difference in the per^{01}; cry^{b} morning peak activity at different temperatures (A 4.3.2; F=2.24, n.s.), a phenotype it shares with cry^{b} but not per^{01} (compare figures 3.4 and 4.1). The level of activity around this peak is higher than the much-reduced level seen in cry^{b} strains (figure 4.3c). At 18°C, the level of activity differs both from per^{01} (A 4.3.5; F=61.7, p<<<0.001) and cry^{b} (A 4.3.10; F=4.7, p<0.05), producing a peak of intermediate size (figure 4.3c). At 29°C, the peak is the same size as in the arrhythmic per^{01} strain (F=3.11, n.s.).

The evening peak is very similar at 18 and 29°C in per^{01}; cry^{b} flies (4.3b), and the peak occurs at the same time at each temperature (A 4.3.3 temp. x time, F=1.16, n.s.). The evening activity peak resembles that of cry^{b} rather than per^{01} as there is an upswing in activity anticipating lights off, as confirmed by ANOVA analysis comparing per^{01} and per^{01}; cry^{b} (A 4.3.7 - 4.3.8 time x genotype; 18°C, (F=2.1, p<0.05; 29°C F=24.709, p<0.001). At 18°C, activity peaks at the same time in both per^{01}; cry^{b} and cry^{b} flies (A 4.3.12; genotype x time (F=0.64, n.s.) (figure 4.3d). At 29°C, the activity peak of per^{01}; cry^{b} is reduced in size compared to that of cry^{b}, and peaks earlier (A 4.3.13; genotype x time, F=24.7, p<0.05) (figure 4.3d).

Thus the locomotor activity of the per^{01}; cry^{b} strain is a composite of the two mutant phenotypes, with per^{01} determining the morning peak, and cry^{b} the shape of the evening peak. The cry^{b} mutation also eliminates the difference in morning peak size seen in per^{01} mutants, but there is an earlier upswing in activity levels at 18°C, which is not seen in cry^{b} mutants. The evening activity peak does not move at different temperatures, and at both temperatures most resembles that of cry^{b} mutants at 18°C.

The simplest explanation for the rhythmic profile of per^{01}; cry^{b} double mutants would be that in the absence of PER and CRY, another clock component is able to anticipate lights off. The obvious candidate for this would be TIM, as TIM is known to interact with both CRY and PER. To confirm whether this is the case, tim^{01}; cry^{b} double mutants were generated and their behaviour in LD 12:12 assayed, as shown in figure 4.4.

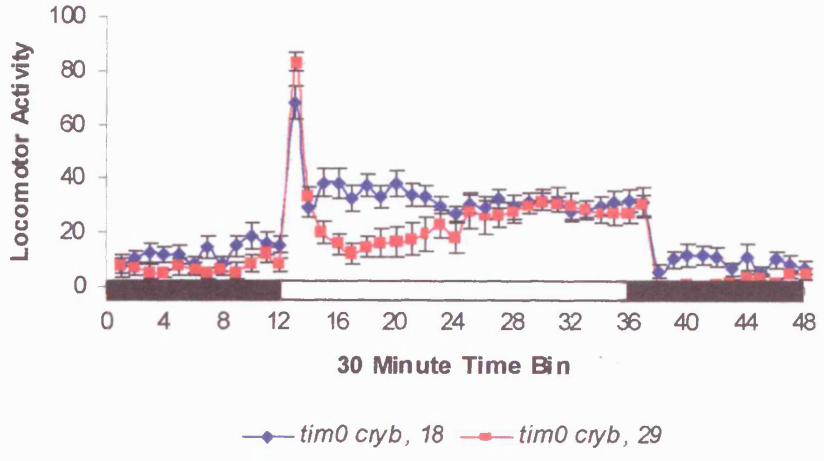


Figure 4.4. Locomotor activity of tim^{01}; cry^{b} in LD 12:12 18°C (blue) and 29°C (red)

As can be seen from figure 4.4, tim^{01}; cry^{b} flies do not anticipate lights off. As anticipation of lights off is seen in per^{01}; cry^{b}, this seems to confirm that in the absence of PER and CRY, the light dependent degradation of TIM is sufficient for this. Like per^{01}; cry^{b}, tim^{01}; cry^{b} are arrhythmic in DD.

per^{01}; cry^{b} are able to anticipate lights off, despite the lack of a functional clock under constant conditions. This seems to be attributable to the light-dependent degradation of TIM

The effect of the per^{01}; cry^{b} double mutant on splicing levels can then reveal whether the temperature response is controlled only by the clock and *cry*, and whether they work together or independently. The splicing levels at different temperatures are shown in figure 4.5.

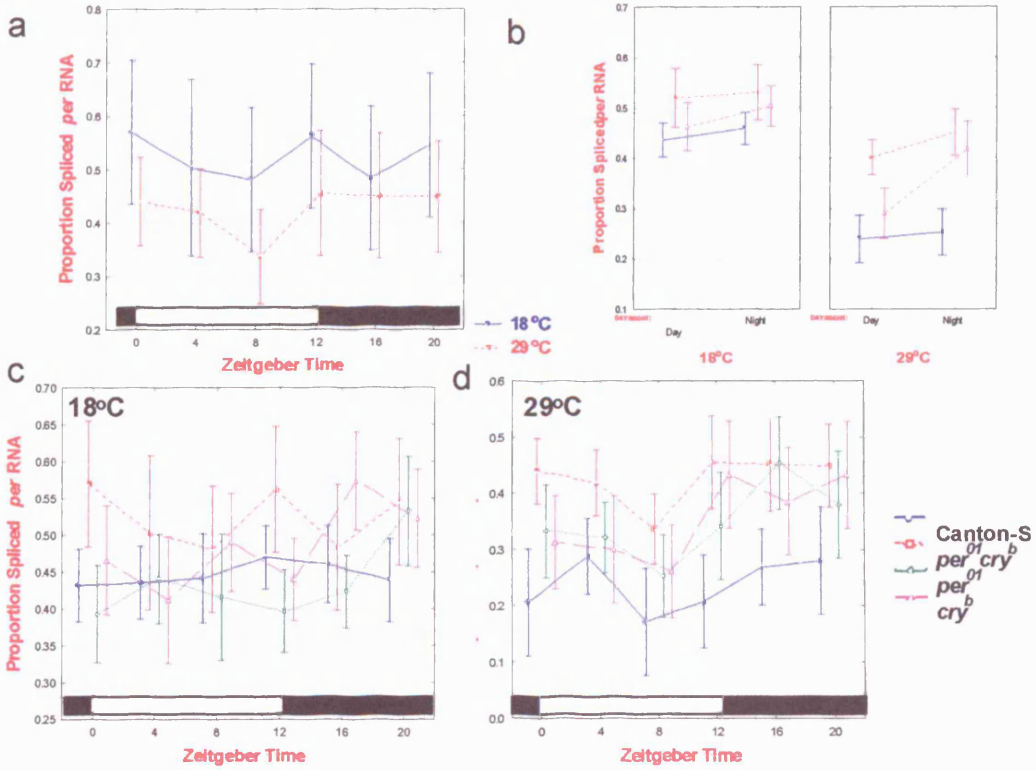


Figure 4.5: Splicing in *per*^{01}; *cry*^{b}. a). 18°C (blue) and 29°C (red). b). splicing levels during the day and night in *per*^{01}; *cry*^{b} (red), *cry*^{b} (purple) and Canton-S (blue). c). splicing levels in *per*^{01}; *cry*^{b} (red), *cry*^{b} (purple), *per*^{01} (green) and Canton-S (blue) at 18°C. d). splicing levels in *per*^{01}; *cry*^{b}, *cry*^{b}, per01 and Canton-S at 29°C. Error bars: 95% confidence intervals.

In *per*^{01}; *cry*^{b} there is more spliced *per* mRNA at 18 than at 29°C (A 4.5.2; f=8.1, p<0.05) (figure 4.5a). These levels are also high in comparison to the other genotypes analysed (figures 4.5 c and d), with ~60% of transcripts spliced at both temperatures, compared to 45% at 18°C and 25% at 29°C in Canton-S. At 18°C, the level of splicing in the *per*^{01}; *cry*^{b} double mutant is elevated in comparison to Canton-S (A 4.5.3; F=10.44, p<0.05) but not to *cry*^{b} (A 4.5.4; F=2.11, n.s., and 4.5c). At 29°C, the level of splicing is elevated in comparison to all other lines (A 4.5.4; F=6.9, p<0.01) and figure 4.5d. At 29°C, the level of splicing of the double mutant does not rise at night (A 4.5.2; no time effect; F= 0.8565, n.s.) unlike the *cry*^{b} (and *per*^{01}) single mutant (see figures 3.5 and 4.2).

Therefore per^{01}; cry^{b} has lost the repression of splicing during the day at high temperatures, so that the level of splicing at 29°C is identical during the day and night.

The elimination of a day/night difference at 29°C in the per^{01}; cry^{b} mutant indicates that during the day the clock (PER/TIM) and CRY contribute independently towards setting the splicing level so only by eliminating both factors is the temperature dependent repression of splicing lost.

4.4 Discussion

All the work reported here is analysis of whole head extracts, and as such any effect is not necessarily the same as that observed within the LN_vs central clock. However, much of the current knowledge of the *D.melanogaster* clock has come from the analysis of whole head extracts, including demonstrating the role of *per* splicing in determining locomotor behaviour (Majercak et al., 1999). It therefore seems likely that the level of splicing observed in these experiments is directly related to the splicing level responsible for determining evening activity. In any case, the mechanics of the temperature and light dependent regulation of splicing levels within the central and peripheral clocks are likely to be similar as they utilise identical clock proteins. In both central and peripheral clocks CRY acts as an input to the clock via a light dependent interaction with the PER/TIM complex (Ceriani et al., 1999).

4.4.1 cry^{b} locomotor activity

CRY is involved in determining the size of the morning activity peak and positioning of the evening peak, and the temperature regulation of both. In wild-type (Canton-S) lines, higher temperatures cause a reduction in the size of the morning activity peak, a delay in the rise in evening peak activity by ~2 hours and a shift in evening activity so that it peaks after lights off (see figure 3.2). The cry^{b} mutation affects all three of these responses to elevated

temperature. The temperature differences in morning peak height and the timing of evening activity upswing are both lost in this line (figure 4.1). Some temperature responses remain, however, and evening activity still peaks earlier at 18 than 29°C in these flies (figure 4.1). Therefore as well as being the circadian photoreceptor, *cry* may also be a component of the temperature response of the clock.

The morning activity peak is primarily a result of the startle response to lights on (Allada et al., 1998), and there is no difference in the timing of this peak in any of the strains examined here at either temperature. The clock seems to have a role in reducing the size of this response as the size of the startle effect is increased in both per^{01} and tim^{01} flies (figure 3.4). This could be because the clock allows the organism to 'expect' dawn at a certain time. CRY also seems to have a role in modulating the startle response in a temperature dependent manner. In wild-type flies, the size of the morning peak is decreased at higher temperatures. This difference is maintained in per^{01} mutants, but lost in cry^{b} and tim^{01} mutants (figures 4.1 and 3.4). Therefore the repression of the startle effect is dependent on CRY and TIM, and possibly on the interaction between these two proteins.

Morning peak activity in cry^{b} flies is low in comparison to the other lines, indicating that the elimination of this photoreceptor makes the clock less sensitive to dawn. CRY may be involved in reducing the startle response at high temperatures, because at high temperatures the light intensity is likely to be higher, meaning that it may be necessary to elicit a lesser response to the same intensity of light as the temperature rises.

As can be seen in figure 4.1, in the cry^{b} mutant the evening activity peak is earlier at 18 than 29°C, and at both temperatures, the peak occurs earlier than in Canton-S flies. At 18°C, the evening activity peak is also broader than the corresponding wild-type peak, and activity reaches a lower level earlier in the evening while at 29°C, the peak rises and falls about 30 mins in advance of the wild-type peak. The difference in evening peak position both

at different temperatures and between the Canton-S and cry^b strains can be accounted for by differences in the splicing levels of the *per* mRNA.

4.4.2 Splicing in cry^b mutants

In the cry^b mutant there is more spliced *per* mRNA at 18 than 29°C, accounting for the earlier evening activity peak. Both evening peaks occur earlier than the corresponding peaks in the wild-type line, and again this correlates with the observed splicing levels.

At 18°C there is significantly more spliced *per* RNA at night in cry^b (ZT16, ~55%) than in Canton-S, per^{01} and tim^{01} strains (~45%). This is presumably responsible for the earlier evening activity peak seen in cry^b flies when compared to Canton-S at 18°C. If this is the case, it indicates that the level of spliced *per* present in the early night is the primary determinant of evening peak position. This is when PER levels will be rising as TIM is present for PER stabilisation, so changes in splicing levels are likely to have the biggest effect around dusk or the early evening.

This also reveals a role for CRY in the regulation of splicing at 18°C; this must be independent of its clock function as per^{01} and tim^{01} have no effect on splicing levels at 18°C. Other factors, presumably other photoreceptors, must be involved in the regulation of splicing at 18°C as the levels of splicing in cry^b mutants are lower than in Canton-S flies in reduced photoperiods or DD (compare figure 4.2 to 3.6 and 3.7).

At 29°C, *cry* shares a splicing phenotype with per^{01} and tim^{01} with splicing repressed during only during the day (30%) (figure 4.2). At night there is no repression of splicing at 29°C in cry^b mutants, with the level of splicing similar to that seen during the day and night at 18°C in Canton-S flies (45%). The elevated level of splicing at night can account for the earlier evening peak seen in cry^b flies at 29°C in comparison to Canton-S. This again indicates the importance of the night-time splicing level.

Thus the level of splicing in cry^b flies is elevated at night at both 18 and 29°C in comparison to Canton-S, resulting in an earlier evening activity peak at both temperatures. A difference in splicing levels between the temperatures is still observed at night, despite this loss of repression, due to the elevated night time splicing at 18°C in the cry^b line, and accounts for the earlier phase of activity at 18°C in cry^b mutants.

4.4.3 per^{01}; cry^b Locomotor Activity

The per^{01}; cry^b double mutant was produced to help determine the relative roles of PER (the clock) and CRY in regulating splicing level of *per* mRNA. The analysis of locomotor activity threw up a very odd result - this combination of two mutant strains produced a pattern of LD locomotor activity that is in many ways more similar to wild-type than either individual mutant. cry^b flies have a reduced morning peak, and an anticipatory evening activity peak, while the converse is true of per^{01} mutants. Therefore the double mutant would be expected have a reduced morning peak, and no anticipation of lights off. Surprisingly, in LD cycles, per^{01}; cry^b have an elevated morning activity peak, and clearly anticipate lights off (figure 4.3). per^{01}; cry^b flies do not have a functional clock, as they are arrhythmic in constant light and darkness.

How the fly can anticipate lights off without a clock is not clear. One possibility is that the remaining clock components are sufficient to drive rhythmicity in LD in the absence of both *per* and *cry,* but not when either is present. As both PER and CRY interact with TIM, the simplest explanation would be that LD driven TIM cycles generate rhythmicity in the absence of PER and CRY. This seems to be confirmed by the lack of an anticipatory evening activity peak in tim^{01}; cry^b flies (figure 4.4). Therefore it seems that the cycling of TIM levels and nuclear entry in per^{01}; cry^b flies is what produces the anticipatory evening activity peak. This would suggest that TIM is able to enter the nucleus of the pacemaker cells alone, which goes against

conventional clock wisdom. As a result this needs to be confirmed by using a technique such as immunocytochemistry to see whether TIM is present in the nuclei of the LNs in per^{01}; cry^{b} heads.

In per^{01} TIM is not detectable in the nucleus (Hunter-Ensor et al., 1996), and the overall level of TIM is much reduced (Zeng et al., 1996). The simplest explanation of per^{01}; cry^{b} anticipation is that in per^{01} mutants, although TIM is present, in the presence of wild-type CRY, TIM is all degraded in the presence of light, and therefore it is unable to enter the nucleus, so the clock cannot function. If per^{01} and cry^{b} are combined, the level of TIM remains low, but as the activity of CRY is much reduced if not completely abolished by the cry^{b} mutation, TIM is no longer degraded. Therefore these low levels of TIM are able to drive the anticipation of lights off. Another possibility is that CRY somehow prevents TIM entering the nucleus on its own.

In order to confirm that the anticipation of 'lights off' seen in per^{01} cry^{b} is under some sort of clock control, it needs to be shown that it takes time for this double mutant to entrain to new LD cycles. In order to do this, the behaviour of per^{01}; cry^{b} needs to be assayed in altered photoperiods, and after entrainment to non 24 h LD cycles.

Although per^{01}; cry^{b} flies anticipate dusk, the locomotor activity profile in LD has lost many aspects of 'normal' locomotor activity, particularly in terms of temperature regulation. The morning peak is intermediate in size between that of per^{01} and cry^{b}, and is the same size at both temperatures. cry^{b} also shows no temperature differences at this peak, but the peak is smaller at high temperatures in per^{01} mutants. This seems to confirm that functional *cry* is essential to get the temperature-induced repression of the morning peak and/or startle effect (see above).

Unlike the cry^{b} single mutant, in the double mutant without the functional clock, there is no difference in the timing of the evening activity peak at different temperatures. This confirms that the timing of the position of the evening activity peak at different temperatures is dependent on the

expression of PER, and the modification of the *per* expression profile through altered splicing leads to altered behaviour at high temperatures, as suggested by Majercak et al. (1999). The evening activity peak resembles that of cry^b at 18°C at both temperatures, indicating that the temperature regulation of the evening activity peak requires the presence of the PERIOD protein. This fits with the model of evening peak position determination dependent on the splicing of the intron within the 3' UTR of *per* (Majercak et al., 1999).

In any strain carrying the per^{01} mutation, the splicing level can have no effect on locomotor behaviour or the clock as although *per* is transcribed it is never translated into a mature protein. This means that the behaviour of lines such as per^{01}; cry^b is independent of the splicing of *per* and of PER protein. Analysis of cry^b suggested that in this strain the splicing level is related to the position of the evening activity peak (above), yet the evening peak at 18°C also occurs at the same time in per^{01}; cry^b flies (see figure 4.3). This suggests that the position of the evening peak in cry^b mutants at 18°C may be independent of the splicing level of *per* mRNA. This 18°C peak could therefore be a 'default' evening peak position, then, when PER is present, it is able to modulate the position of the peak in response to elevated temperatures. It seems that the position of the peak is modified at high temperatures as the peak position at 18°C is the same with and without PER (cry^b and per^{01}; cry^b) but at 29°C the peak of activity is delayed in cry^b.

The idea of a default clock at 18°C that is modified at high temperatures is borne out by examination of the splicing levels. The splicing levels in the double mutant at both 18 and 29°C are comparable to that of the cry^b single mutant at 18°C, or at night at 29°C. Therefore in the double mutant, the repression of splicing has been lost, leaving a 'default' level of splicing. These are the default levels for a 12:12 photoperiod as the splicing levels increased in reduced photoperiods and DD (figures 3.5 and 3.6).

So, in per^{01}; cry^{b} flies both the locomotor activity and splicing levels are very similar at both 18 and 29°C, and most closely resemble the behaviour/splicing associated with the lower temperature. It therefore seems that the default level of splicing is seen at the lower temperature, and is then modified in response to elevated temperatures. This fits with the assumption that it is more important to regulate activity when it is hot to ensure the avoidance of the mid-day sun. This means that splicing is repressed at high temperatures rather than being enhanced at low ones.

While there is no difference in the position of the evening activity peak, temperature still has some effects on the behaviour of per^{01}; cry^{b} double mutants (see figure 4.3). In wild-type flies the level of evening activity begins to rise ~2 h earlier at 18 than at 29°C, but in the per^{01}; cry^{b} double mutant, this phase difference is reduced to ~1 h. This response must be independent of the splicing of *per* as this transcript is not translated in the per^{01}; cry^{b} mutant. Interestingly, this difference is completely lost in cry^{b} flies, but it cannot be under the control of *cry* alone or else it could not be regained in the double mutant.

It can be seen that the timing of the activity upswing is very similar (at 18°C) in both wild-type and double mutant flies, whereas it is delayed in the cry^{b} single mutants. Therefore it seems that the clock and *cry* have antagonistic effects on the timing of the upswing in activity, with CRY working to advance and the clock to delay it at low temperatures. The effect of the *cry* advance must be greater than the clock dependent delay in order to generate the 1 h difference seen in the double mutant. Thus, CRY adds a ~2 h advance and the clock a ~1 h delay.

As there are few temperature differences in behaviour in the per^{01}; cry^{b} double mutant, it could be that eliminating both PER and CRY has eliminated most of the temperature input into the clock. Certainly, CRY appears to be key to the temperature regulation of the morning peak, and in the timing of the upswing of evening peak activity. However, the evening activity peak is controlled by the level and timing of accumulation of PER protein, which is

not a factor in per^{01}; cry^{b} flies. It is therefore not possible to say whether the temperature response has been lost simply by looking at the locomotor activity profile.

4.4.4 per^{01}; cry^{b} Splicing

Although the per^{01}, tim^{01} and cry^{b} mutants all have the same splicing phenotype at 29°C, the analysis of individual mutants does not reveal whether they are in the same splicing regulation pathway. The analysis of the *per* transcript splicing levels in the per^{01}; cry^{b} double mutant is therefore useful in determining how the clock and the light input mediated via CRY interact to determine the splicing level in a temperature dependent manner.

At 18°C the level of splicing in per^{01}; cry^{b} (~55%) is significantly higher than Canton-S and per^{01} (~45%) but not cry^{b} (~55% at ZT 16) (figure 4.4). The elevated level of splicing seen in the per^{01}; cry^{b} mutant at 18°C is probably a result of the cry^{b} mutation, and does not indicate that *per* plays a role in regulating splicing at this temperature. This is probably related to the role of *cry* in photoreception, but as level of splicing in cry^{b} is not as high as that seen in DD, there must be additional light inputs to the splicing machinery (figure 3.7). Although much reduced in comparison to Canton-S or cry^{b}, there is still significantly more spliced *per* mRNA in per^{01} cry^{b} at 18 than at 29°C (see figure 4.4b). PER/TIM and CRY therefore probably play some role in mediating the temperature response, but even in the absence of PER and CRY, the splicing machinery is still able to detect changes in temperature.

At 29°C, PER, TIM and CRY are all involved in the regulation of splicing and per^{01}, tim^{01} and cry^{b} mutants have identical levels of splicing. During the day, splicing is repressed, and this repression is lifted during the night, so that the level of splicing is identical to that seen normally at 18°C. Therefore each of these proteins is required for the repression of splicing at night at elevated temperatures. During the day at 29°C, splicing is repressed in all these lines, but in the per^{01}; cry^{b} double mutant this repression of splicing is lost.

Therefore at high temperatures, during the day (in the presence of light(?)) the repression of splicing requires the presence of either the clock or CRY, while at night, repression of splicing requires both the clock and CRY.

4.4.5 Conclusions

The examination of the levels of splicing in per^{01}, tim^{01}, cry^{b} and per^{01}; cry^{b} flies demonstrates how the alteration of behaviour at higher temperatures is effected. At 18°C the default pattern of activity is primarily under the control of the photoperiod via the regulation of splicing, perhaps explaining why only *cry* has a (small) effect on splicing levels at this temperature. At higher temperatures, both the clock and CRY modify the splicing level in response to light and temperature, as shown in figure 4.5.

In comparison to wild-type flies, in per^{01}, tim^{01} and cry^{b} flies, at high temperatures there is repression of splicing during the day, but not at night. The repression during the day is likely to be driven by light, but this input does not come directly though CRY, and only when *per* and *cry* are eliminated is the repression of splicing at high temperatures lost. This means that at high temperatures, light sets the splicing level during the day. As the elimination of either PER or CRY does not prevent daytime repression, but the loss of both eliminates repression, this is dependent on the presence of one or other of these proteins.

The level of splicing seen during the day, as repressed by light, then sets the level of splicing at night. The repression of splicing at night is dependent on the clock, as the elimination of PER, TIM or CRY causes a complete loss of repression. During the night PER, TIM and CRY are known to associate (Stanewsky et al., 1998; Emery et al. 1998; Ceriani et al.; Rosato et al., 2001) so it is not unexpected that the elimination of any one of the three proteins abolishes all repression of splicing at night at high temperatures. This is also the time when the levels of these proteins are at their highest, and therefore most likely to be able to repress splicing.

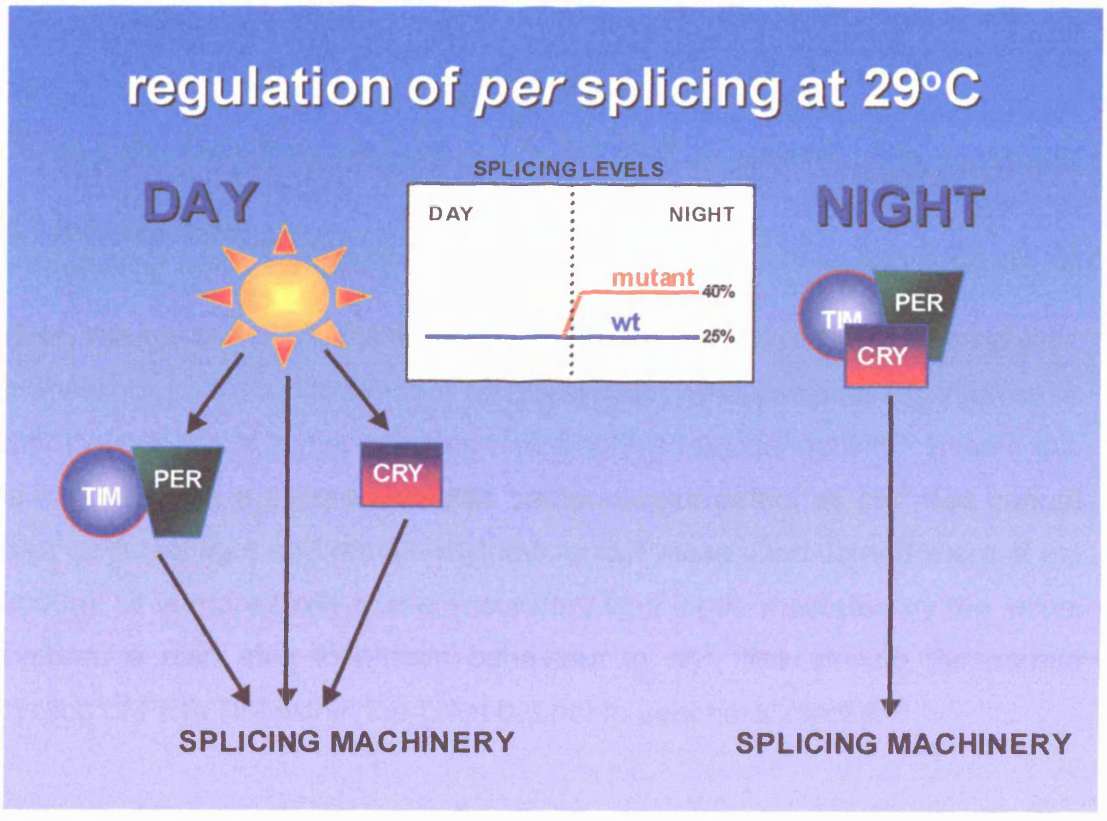


Figure 4.5: The regulation of splicing at high temperatures is dependent on the clock and CRY

Chapter five: *period* splicing and activity in visually blind flies

5.1 Introduction

Even though cry^b is a mutation of the only dedicated circadian photoreceptor, it does not prevent entrainment to LD cycles. One possible explanation is that the cry^b is not a null mutation and displays residual activity. Even if this is the case, it is a severe circadian photoreceptor defect as cry^b flies cannot 'see' constant light and remain rhythmic under these conditions (Emery et al., 2000a). It is more likely that a secondary light input, mediated by the visual system, is also able to entrain behaviour in cry^b flies, driving the normal cycling of PER/TIM within the LN_Vs but not in peripheral clocks.

Mutants of the visual photoreception pathway such as *norpA*, *disco* and *glass* (Dushay et al., 1989; Helfrich-Forster et al., 2000) have long been known to affect entrainment of activity to LD cycles especially at low light intensities. *disco* mutants have normal PER/TIM cycles within the eye, but can display arrhythmic behaviour if the *disco* lesion removes the LN_Vs (Dushay et al., 1989). The phenotypes of *norpA* and *glass* mutants are of more interest as they alter the light input to the clock. *norpA* encodes a phospholipase-C and null mutations of *norpA* blocks phototransduction from the compound eyes and the ocelli (Hu et al., 1978; Pearn et al., 1996). The loss of light sensitivity means that it takes longer for *norpA* mutants to entrain to new LD cycles (Emery et al., 2000b; Wheeler et al., 1993), and they also have altered patterns of behaviour, including a period ~1 h shorter than normal (Dushay et al., 1990).

In an attempt to eliminate all light inputs to the clock, the *norpA* mutation was combined with cry^b. The $norpA^{P41}$; cry^b double mutant, which is almost completely blind, can still be entrained to LD regimes, albeit with a reduced ability to adapt to new LD cycles particularly at low light intensities or under

blue light. In *norpA*^{P41}; *cry*^{b} mutant flies the only known active photoreceptors are the putative extraretinal eyes of the Hofbauer-Buchner (H-B) eyelet (Hofbauer and Buchner 1989).

The rhythmicity of behaviour, like that of *cry*^{b} flies, seems to be generated by PER and TIM cycling within the LN_vs (Stanewsky et al., 1998; Emery et al., 2000b). Thus, the H-B eyelet alone seems to be sufficient for entrainment to LD cycles. The axon terminals of the eyelet overlap with putative dendrites of the s-LN_v indicating that it is not impossible for these to allow entrainment. The role of the H-B eyelet in the entrainment of circadian rhythms seems to depend on the other photoreceptor structures present. In *eyeless* mutants, the eyelet mediates phase delays, but in *cry*^{b} it does not (Helfrich-Forster et al., 2002).

The *glass* (*gl*) gene encodes a transcription factor required for the development of opsins within the external photoreceptor cells (Moses et al., 1989). In the *gl*^{60j} mutant, all ocelli and retinal photoreceptor cells including those in the H-B eyelets, are removed so the only photoreceptor available to entrain locomotor activity is CRY (Moses et al., 1989; Helfrich-Forster et al., 2001). *gl* mutations affects entrainment to new LD cycles with the primary effect on the morning peak which takes up to six days to entrain to a new phase and is less compact and poorly entrained than in a wild-type background. The evening activity peak of *gl* mutants is able to adapt to phase shifts relatively quickly while the converse is true of the *cry*^{b} mutant, where the evening peak takes time to entrain to new light regimes (Stanewsky et al., 1998). This indicates that the visual system is part of a separate entrainment pathway from *cry,* with the entrainment of the morning peak primarily under visual control and the evening peak controlled by *cry* photoreception and/or the clock (Helfrich-Forster et al., 2001).

The *gl*^{60j} *cry*^{b} double mutant lacks all the photoreceptors which can be used to entrain behaviour, and cannot be entrained to LD cycles. Even so, the 'blind' *gl*^{60j} *cry*^{b} mutants behaviour contains a small rise in activity after lights off. However, the PER/TIM cycles of individual flies are not synchronised,

indicating that there is no functional clock, and this peak was therefore attributed to 'masking' (Helfrich-Forster, 2001).

Therefore the visual system has a direct effect on the entrainment to LD cycles, presumably owing to altered cycling of PER and TIM. In cry^b flies, PER/TIM cycling continues in the LNs with reduced amplitude, and is lost from DN2 neurons. In $norpA^{P41}$; cry^b flies, PER/TIM cycling is additionally lost from the l-LN and the LN_D indicating that the eyes contribute to PER/TIM cycling in certain neurons (see figure 1.2). Although PER and TIM levels cycle in individual gl^{60j} cry^b flies, without either light input the protein cycles are not synchronised to the LD cycle, indicating that the PER/TIM cycle is unregulated (Helfrich-Forster et al.., 2001). A role for the eyes in setting PER/TIM cycles is also indicated by mutants of *transient receptor potential (trp)*, part of the visual transduction cascade. *trp* mutants show an altered phase of activity in DD and light pulses produce less of a change in TIM levels and the corresponding phase shift in activity is reduced (Yang et al., 1998).

5.2 Aims

The examination of splicing levels in *per*, *tim* and *cry* mutants indicate that the clock is only required for the regulation of splicing at high temperatures and for rapid adaptation to new photoperiods at 18°C. The repression of splicing is dependent on light at both 18 and 29°C, and is not eliminated in the cry^b mutant (Chapters 3 and 4). In DD, there is no light input to the splicing machinery, and the resultant splicing levels are higher than those of cry^b mutants. Therefore, a light input to the splicing machinery of the clock independent of CRY must allow the adaptation of splicing levels to different photoperiods and the repression of splicing by light. It is likely that this light input is the same as that driving PER/TIM cycles within the LN_vs of cry^b flies, namely the visual system.

Although mutations of the visual system seem primarily to affect the entrainment of the morning activity peak, the extended time taken for visual mutants to entrain their locomotor activity to LD cycles suggests that an input from the visual system is partially responsible for determining the distribution of locomotor activity (e.g. Helfrich-Forster et al., 2001; Stanewsky et al., 1998). It is likely that any mutation which affects the entrainment and positioning of the evening activity peak will have altered *per* splicing levels as this has been demonstrated to be the primary determinant of evening peak position (Majercak et al., 1999). In order to uncover the role of the visual system in regulating splicing and locomotor activity, $norpA^{P41}$, $norpA^{P41}$ cry^{b}, gl^{60j}, and gl^{60j} cry^{b} flies were all assayed, with each line showing increasing levels of visual impairment.

5.3 Methods

Fly Strains

$norpA^{P41}$ (Emery et al., 2000b)
$norpA^{P41}$; cry^{b} (Stanewsky et al., 1998)
gl^{60j} (Lindsley and Zimm, 1992)
gl^{60j} cry^{b} (Helfrich-Forster et al., 2001)

Canton-S, per^{01} and tim^{01} data is taken from Chapter 3
cry^{b} data is taken from Chapter 4. Genotypes were analysed as in Chapter 3.

5.4 Results

5.4.1 Locomotor activity in $norpA^{P41}$ and $norpA^{P41}$; cry^{b}

The analysis of locomotor activity of $norpA^{P41}$ and $norpA^{P41}$; cry^{b} flies is shown in figures 5.1 ($norpA^{P41}$) and 5.2 ($norpA^{P41}$; cry^{b}).

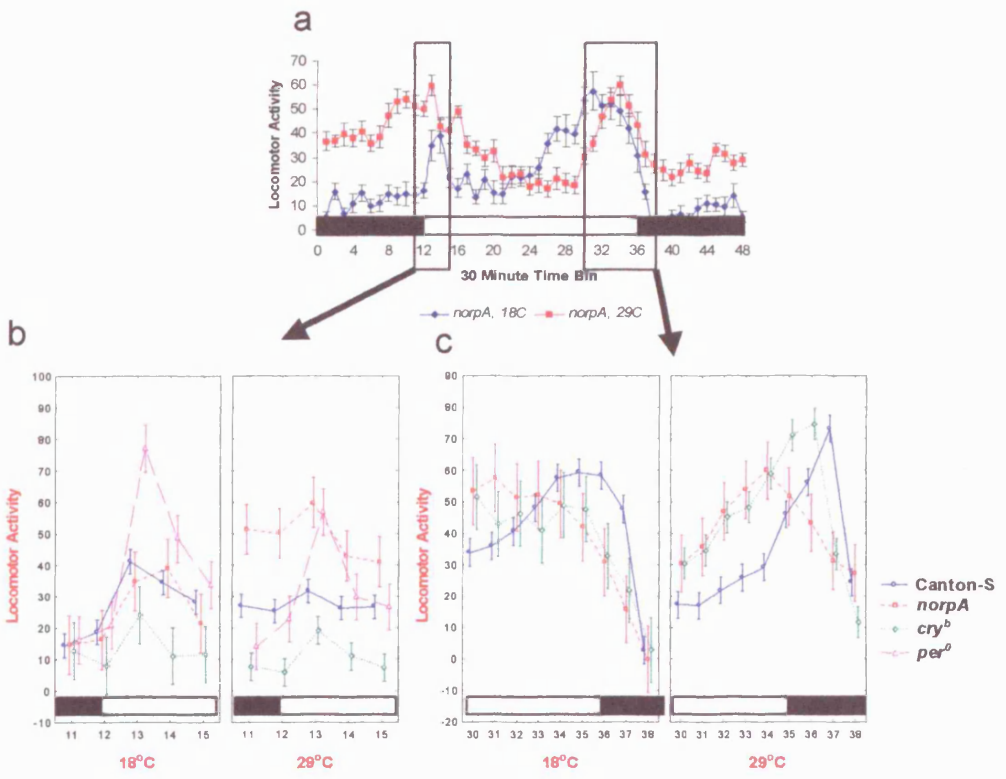


Figure 5.1: Locomotor activity in *norpA*^{P41}. a) 18°C (blue) and 29°C (red), error bars show SEM b). morning peaks of Canton-S (blue), *norpA*^{P41} (red), *cry*^{b} (green) and *per*^{01} (purple) at 18°C and 29°C. c). evening activity peaks of Canton-S, *norpA*^{P41}, *cry*^{b} and *per*^{01}, error bars show 95% confidence intervals

norpA^{P41} flies entrain to LD 12:12, with a bimodal distribution of locomotor activity, which changes in response to different temperatures (figure 5.1a). The morning activity peak is higher at 29°C (A 5.1.4; F=70. 4, p<<<0.001), and peaks earlier (temp. x time, F=4.2, p<0.05). While there is no difference in the level of evening activity at different temperatures (A 5.1.5; F= 1.7, n.s.), the evening peak is earlier at 18 than 29° (temp x time, F=6.0, p<<0.001, figure 5.1a).

At 18°C, the morning activity peak is identical to that of Canton-S flies (A 5.1.6; F=0.83, n.s.), figure 5.1b). At 29°C, the morning peak activity is elevated so that it is more similar in height to *per*^{01}, although there are significant differences in the shape of the peak (A 5.1.7; time x genotype, F= 4.6, p<0.05), 5.1b). Thus the *norpA*^{P41} mutation affects the morning activity

peak in a temperature dependent manner, with its effect only becoming apparent at elevated temperatures.

The evening activity peak occurs earlier than that of Canton-S at both 18 and 29°C (A 5.1.8 - 5.1.9; genotype x time, F=10.12, P<<<0.001; F=20.6, p<<<0.001) and figure 5.1c. The level of evening peak activity is very similar to Canton-S at 18°C (A 5.1.8; F=3.1, n.s.), but $norpA^{P41}$ activity is elevated at 29°C (A 5.1.9; F=20.1, p<<<0.001). At 18°C, the $norpA^{P41}$ evening peak is indistinguishable from that of cry^{b} (A 5.1.10; genotype x time, F=0.78, n.s.). This is not the case at 29°C, where activity peaks earlier in $norpA^{P41}$ than cry^{b} (A 5.1.11; genotype x time, F=8372, p<<<0.001) (figure 5.1c).

$norpA^{P41}$ has an effect on the morning activity peak only at high temperatures, but advances the evening activity peak at both 18 and 29°C.

There is almost no difference in the locomotor behaviour of $norpA^{P41}$; cry^{b} at different temperatures (figure 5.2a). There is some difference in the morning activity peak (A 5.2.3; temp. x time, F= 4.01, p<0.05), with this peak almost eliminated at 29°C. The morning activity peak is indistinguishable from that of cry^{b} at both temperatures (A 5.2.4 - 5.2.5; time x genotype F= 2.2, n.s.; F= 3.1, n.s.). This indicates that the cry^{b} mutation is epistatic to that of $norpA^{P41}$ in terms of morning activity peak determination (figure 5.2b).

There is no difference in the evening activity peak (A 5.2.4; temp. x time, F=1.83, n.s.) at different temperatures (figure 5.2a), and the peak is delayed compared to either single mutant at both 18 and 29°C (A 5.2.7- 5.2.10; all genotype x time, p<<<0.001; figure 5.2c and d).

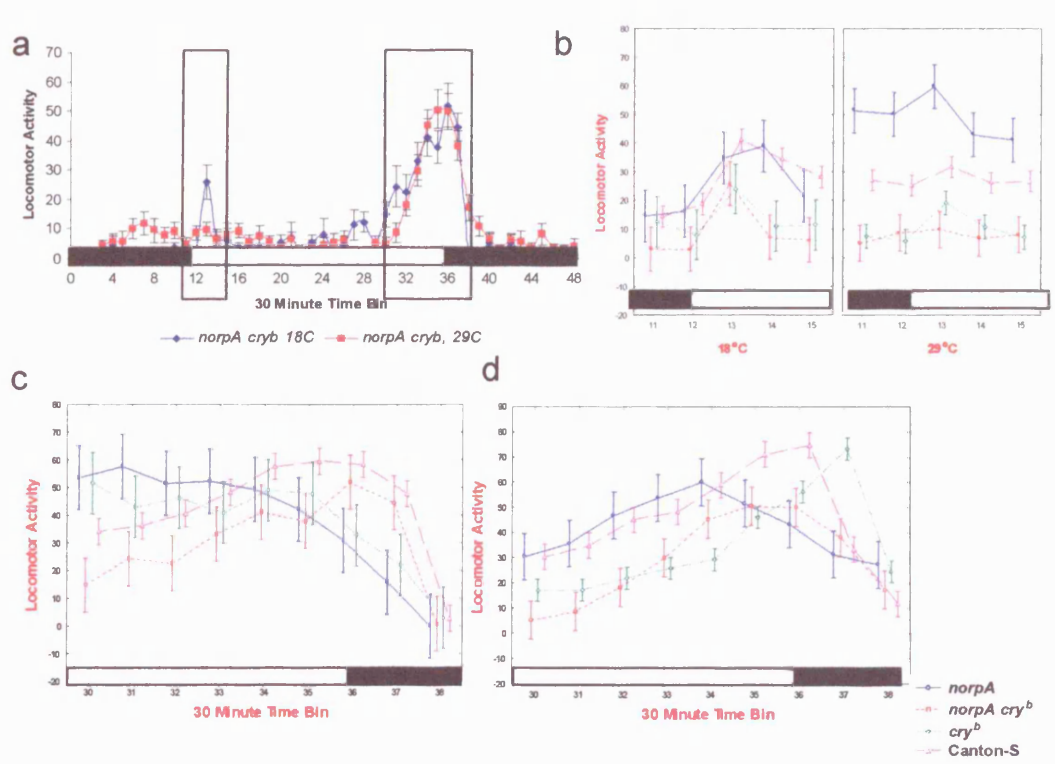


Figure 5.2: Locomotor activity in *norpA^{P41}; cry^{b}*. a). 18°C (blue) and 29°C (red). b). morning activity of *norpA^{P41}; cry^{b}* (red), *norpA^{P41}* (blue), *cry^{b}* (green) and Canton-S (purple) at 18°C and 29°C. c) evening peaks of same genotypes at 18°C d).evening peaks of same genotypes at 29°C.

Therefore the combination of *norpA^{P41}* and *cry^{b}* eliminates the temperature dependent difference in evening activity peak position. It would therefore be expected that there would be no difference in *per* RNA splicing levels at different temperatures in *norpA^{P41}; cry^{b}*.

5.4.2 *per* mRNA splicing in *norpA^{P41}* and *norpA^{P41}; cry^{b}*

The splicing levels of *per* mRNA were also examined around the clock in *norpA^{P41}* and *norpA^{P41}; cry^{b}*, as shown in figure 5.3.

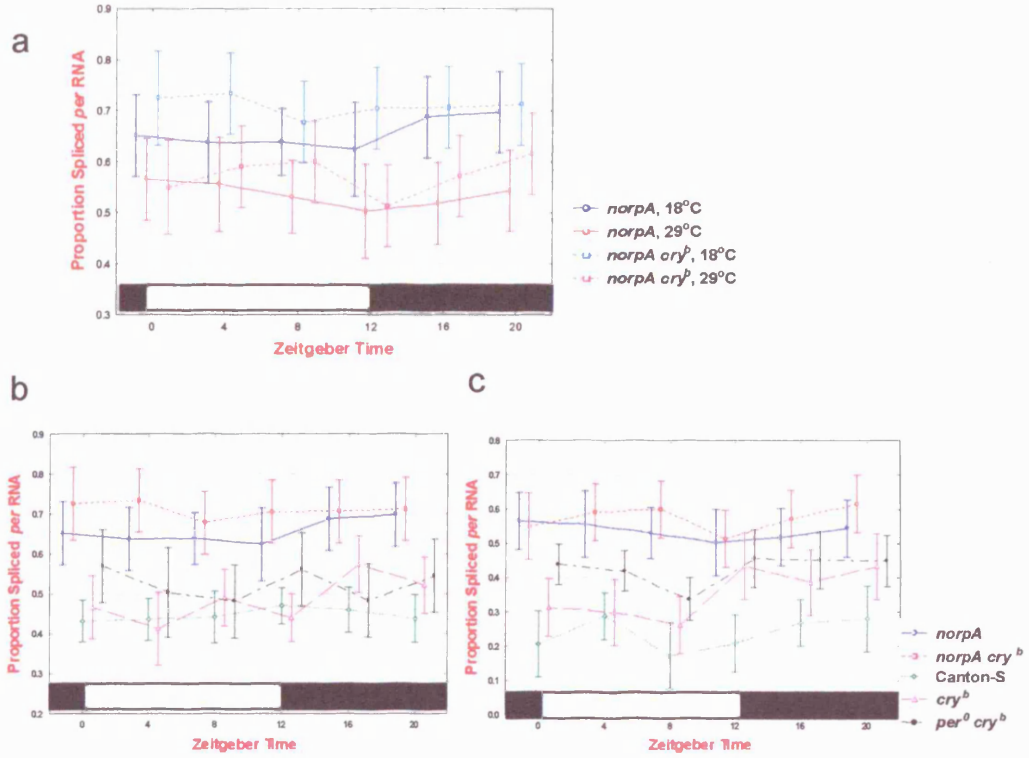


Figure 5.3: Splicing in *norpA*^{P41} and *norpA*^{P41}; *cry*^{b}. a) 18°C (blue) and 29°C (red). b). *norpA*^{P41} (blue), *norpA*^{P41}; *cry*^{b} (red), Canton-S (green), *cry*^{b} (purple) and *per*^{01}; *cry*^{b} (black) at 18°C d). same genotypes at 29°C. Error bars represent 95% confidence intervals.

In both *norpA*^{P41} (A 5.3.2; F=32, p<<0.001) and *norpA*^{P41}; *cry*^{b} (A 5.3.3; F=27.4, p<<0.001) there is more spliced *per* mRNA at 18 than 29°C (figure 5.1a). There is significantly more spliced *per* mRNA in *norpA*^{P41}; *cry*^{b} compared to *norpA*^{P41} at 18°C (A 5.3.4; F=5.2, p<0.05) but not at 29°C (A 5.3.5; F=2.5, n.s.), confirming that *cry* plays a role in the regulation of splicing at low temperatures (Chapter 4). The level of splicing in *norpA*^{P41}and *norpA*^{P41}; *cry*^{b} flies is significantly higher than seen in all the other genotypes analysed (~70% at 18°C and ~65% at 29°C). This is even higher than the high levels seen in *per*^{01}; *cry*^{b} (~55% at 18°C and ~45% at 29°C) (A 5.3.9 - 5.3.10 18°C, F=31.1, p<<0.001; 29°C F=27.1, p<<0.001).

If a mutation eliminates the light input responsible for the repression of splicing, then the level of splicing should be identical to that seen in wild-type in DD. Genotypes carrying *norpA*^{P41} are the only ones analysed where the level of splicing exceeds level seen after a day of DD in wild-type.

So, in *norpA*^{P41} there are higher levels of spliced *per* at both temperatures than Canton-S, which relates to the earlier evening activity peaks observed. However, when *norpA*^{P41} is combined with *cry*^{b} the relationship between splicing levels and evening peak position breaks down as there are differences in splicing but no differences in locomotor behaviour. The splicing level is therefore not the only determinant of evening activity peak position.

5.4.3 Locomotor activity in *gl*^{60j} and *gl*^{60j} *cry*^{b}

The levels of splicing seen at different temperatures in DD reflect the levels of splicing seen in the absence of a light input. Therefore any mutation which eliminates the light input to splicing should leave splicing levels similar to those of Canton-S in DD. The levels of splicing of *norpA*^{P41}mutants are elevated above the levels seen in DD at both 18 and 29°C and temperature dependent differences in locomotor activity are eliminated. Therefore *norpA*^{P41} may have a role in the regulation of splicing beyond its role in the visual system. To determine whether this is the case, the locomotor activity and splicing phenotype of another visual mutant, *gl*^{60j} (and *gl*^{60j} *cry*^{b}), was analysed, as shown in figures 5.4 and 5.5.

There is no morning activity peak in *gl*^{60j} mutants (A 5.4.2; F=1.07, n.s.), and the level of activity is identical at both temperatures (F=1.5, n.s.); figure 5.4a. This indicates that the startle response is lost, and that a visual input is vital to produce a morning activity peak (figure 5.4c). There is also no difference in the position of the evening activity peak at different temperatures (A 5.4.3; temp. x time, F=0.35, n.s.), figure 5.4c. The only difference seen at different

temperatures is an elevated level of activity during the night at 29°C (figure 5.4a).

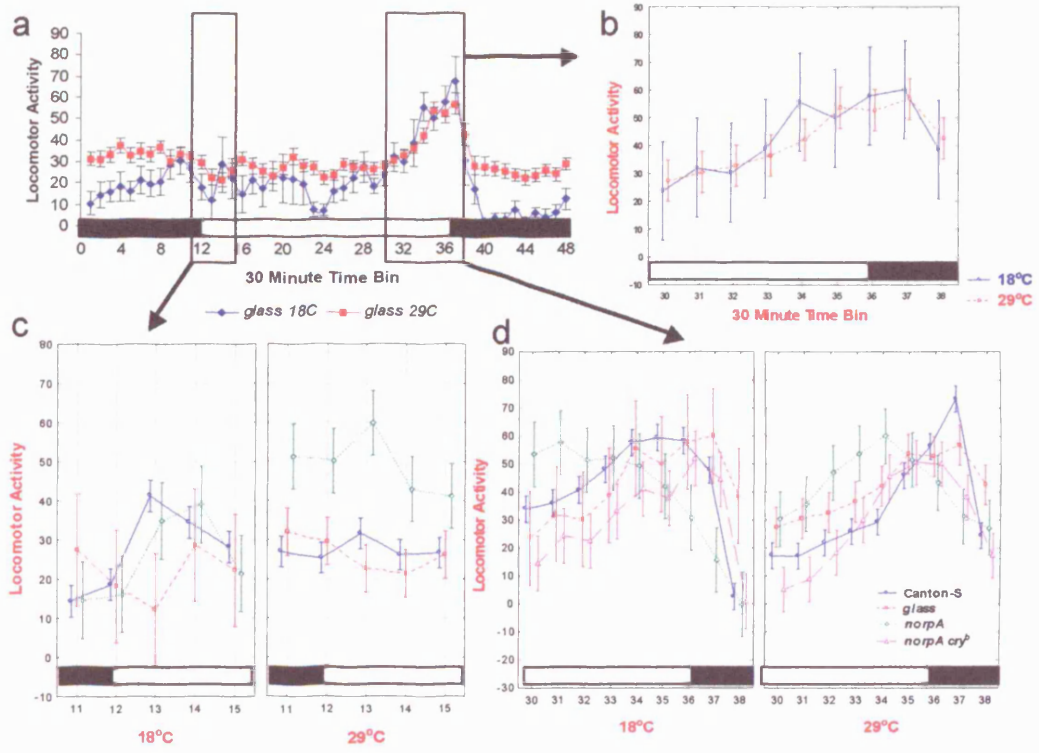


Figure 5.4. Locomotor activity in *gl*^{60j} a). 18°C (blue) and 29°C (red). Error bars represent SEM. b). evening activity peak at 18°C (blue) and 29°C (red). c) morning activity of Canton-S (blue), *gl*^{60j} (red), *norpA*^{P41} (green) and *norpA*^{P41}; *cry*^{b} (purple) at 18°C and 29°C. d). evening peaks in the same genotypes. Error bars show 95% confidence intervals

At 18°C, the evening activity peak occurs at the same time as in the *norpA*^{P41} *cry*^{b} double mutant (A 5.4.9; genotype x time, F=0.79, n.s., figure 5.4d). The peak remains in the same place at 29°C, again most similar to *norpA*^{P41}; *cry*^{b} (figure 5.4d) although there is a difference at 29°C between the two genotypes (A 5.4.10; F=2.892, $p<0.05$)[10]

[10] If the range of the 'evening peak' is narrowed to only include bins 32-27 (A 5.4.11), then this interaction is lost between *glass* and *norpA*^{P41}; *cry*^{b} activity, but not between *glass* and

gl^{60j} eliminates the morning activity peak, and the temperature dependent difference in evening activity peak position.

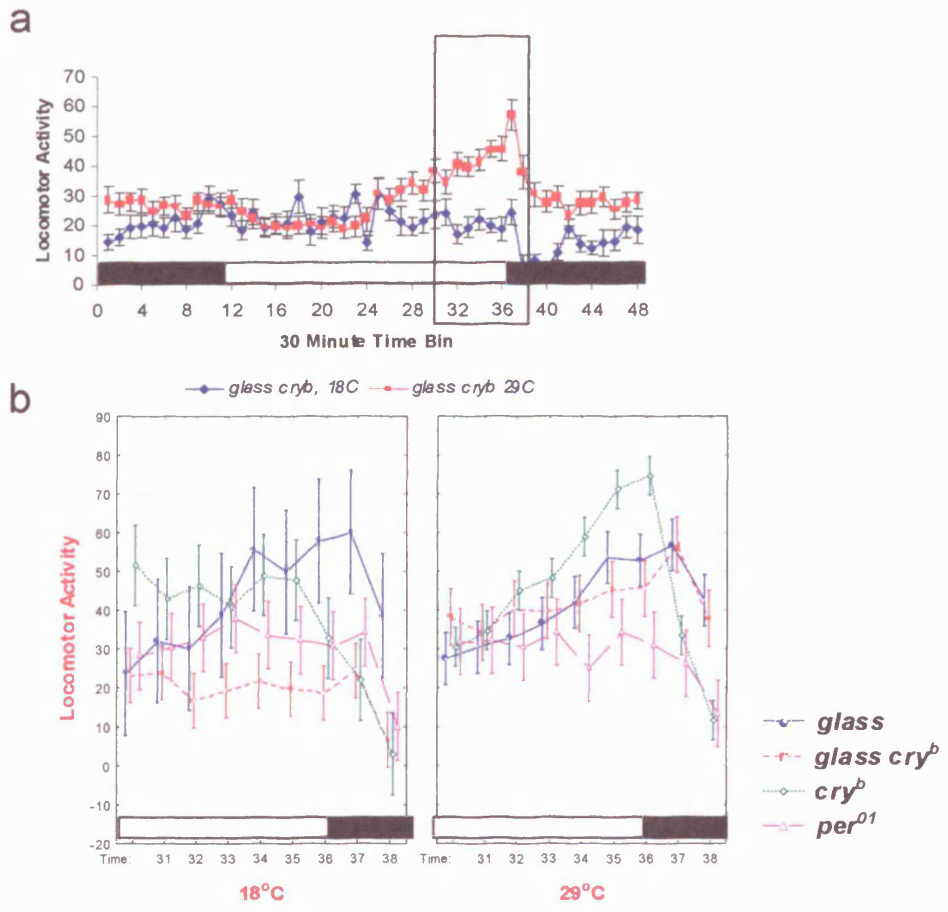


Figure 5.5: Locomotor activity in *gl*^{60j} *cry*^{b}. a). 18°C (blue) and 29°C (red). Error bars show SEM. b). Evening activity peaks of *gl*^{60j} *cry*^{b} (red), *gl*^{60j} (blue), *per*^{01} (purple) and *cry*^{b} (green) at 18 and 29°C. Error bars represent 95% confidence interval.

The locomotor activity profile of *gl*^{60j} *cry*^{b} mutants has previously been reported to be unentrainable to LD cycles at 25°C (Helfrich-Forster et al., 2001). As can be seen from figure 5.5a, this is also the case at 18°C, but not at 29°C. There is no morning activity peak at either temperature, but at 29°C activity begins to rise before 'lights off', indicating entrainment. At 18°C, evening activity is similar in phase to that of *per*^{01} (figure 5.5b) (A 5.5.6;

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any other line, indicating that *glass* activity at both temperatures most closely resembles that of *norpA*^{P41} *cry*^{b}.

genotype x time, F=0.87, n.s.). The level of activity drops immediately after 'lights off' at this temperature; a small rise in activity after 'lights off' at 25°C was attributed to masking (Helfrich-Forster et al., 2001).

Although there is a difference in the locomotor activity profiles of gl^{60j} and gl^{60j} cry^{b} at 29°C (A 5.5.2; F=6.6, $p<0.05$), the evening peak of gl^{60j} cry^{b} at 29°C is indistinguishable from that of gl^{60j} (A 5.5.8; F=0.09, n.s.), figure 5.5c. This suggests that the cry^{b} mutation has little effect on the locomotor behaviour of flies carrying the gl^{60j} mutation at high temperatures. To ensure that the flies were not entrained to temperature cycles produced by the incubator lights, the experiments were repeated using flies kept under the same conditions, but shielded from the light. Despite experiencing the mild temperature fluctuations caused by the incubator light, these flies are not entrained to LD 12:12 cycles (See Appendix 5.7). Therefore, at high temperatures, the gl^{60j} cry^{b} clock cannot be 'blind' to light.

In gl^{60j} the only light input for entrainment comes from CRY. This means that if gl^{60j} cry^{b} flies can entrain to LD cycles, either cry^{b} becomes functional at higher temperatures, or there is an additional, as yet unidentified light input to the clock.

5.4.4 *per* RNA splicing in gl^{60j} and gl^{60j} cry^{b}

The levels of splicing of the intron within the 3' UTR of the *per* transcript of gl^{60j} and gl^{60j} cry^{b} were also examined around the clock at 18 and 29°C, as shown in figure 5.6.

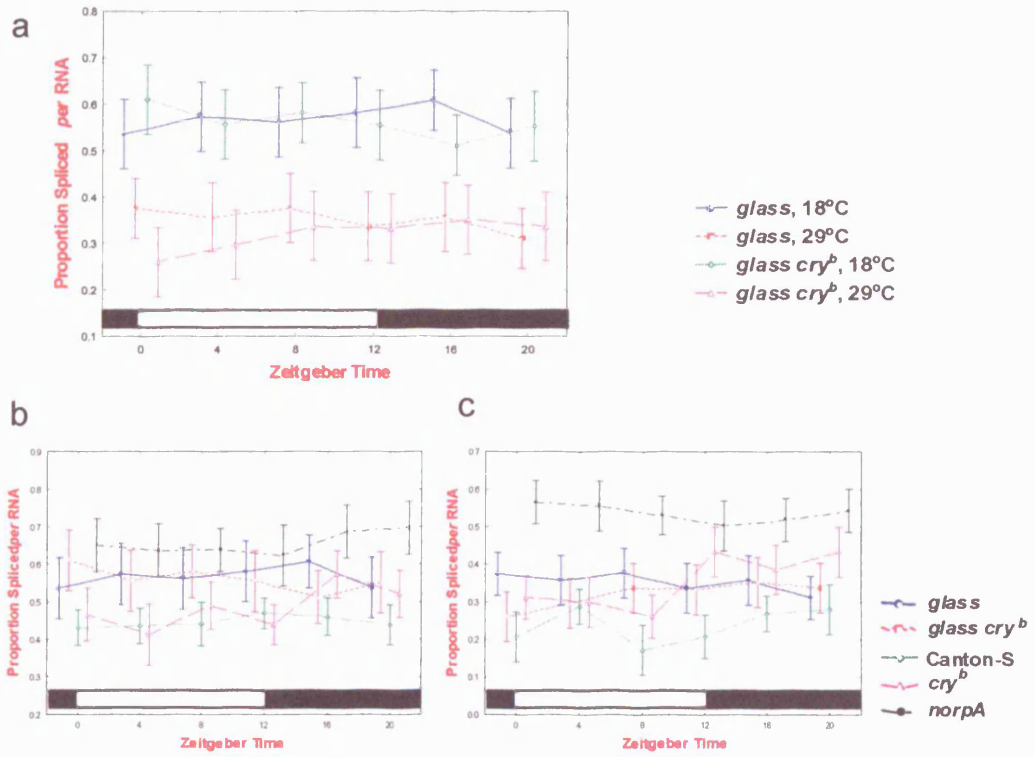


Figure 5.6: Splicing in gl^{60j} and gl^{60j} cry^b a) *gl* (red/blue) and *gl* cry^b (green/purple) at 18°C and 29°C b). gl^{60j} (blue), gl^{60j} cry^b (red), Canton-S (green), cry^b (purple) and $norpA^{P41}$ (black) at 18°C. c). the same genotypes at 29°C. Error bars represent 95% confidence interval.

The splicing levels in gl^{60j} and gl^{60j} cry^b mutants are identical at both 18 and 29°C (A 5.6.1; genotype x temp. x time, F= 1.8, n.s.), figure 5.6a, indicating that the effect of the gl^{60j} mutant is epistatic to that of cry^b in setting the splicing level. In flies carrying gl^{60j}, ~60% of transcripts are spliced at 18°C, and ~40% of transcripts spliced at 29°C, compared to ~45% at 18°C and ~25% at 29°C in wild-type.

At 18°C the level of splicing in gl^{60j} and gl^{60j} cry^b is higher than in Canton-S (A 5.6.2; F=28.6, p<<<0.001) or cry^b (F=10.7, p<<0.001), figure 5.6b. At 29°C, the level of splicing in the gl^{60j} and gl^{60j} cry^b mutants is again higher than that of Canton-S (A 5.6.5; F=28.4, p<<<0.001). In cry^b mutants, the repression of splicing was lost at night at 29°C. There is no difference in the levels of splicing at different times in gl^{60j} and gl^{60j} cry^b (A 5.6.1; F=0.33, n.s.) indicating that the levels of splicing are identical during the day and night. This coupled with the identical splicing levels in *gl* and gl^{60j} cry^b again

indicates that the effect of gl^{60j} on splicing levels is epistatic to cry^{b} (figure 5.6c).

The level of splicing is not as elevated as that of $norpA^{P41}$ mutants at either 18°C or 29°C (A 5.6.6; genotype x temp., F= 9.5, $p<0.05$), figures 5.6b and c. This is surprising as gl^{60j} is a more severe visual mutant than $norpA^{P41}$, and suggests that *norpA* is likely to have a role in the regulation of splicing independent of its role in visual photoreception, especially as the levels of splicing in $norpA^{P41}$ are higher than those seen after a day of DD. A mutation which eliminates the visual input to splicing would be expected to have splicing levels similar to those seen in DD (Chapter 2). If so, the levels of splicing in gl^{60j} would be predicted to be the same as those observed in Canton-S flies in DD. This appears to be the case as shown in figure 5.7.

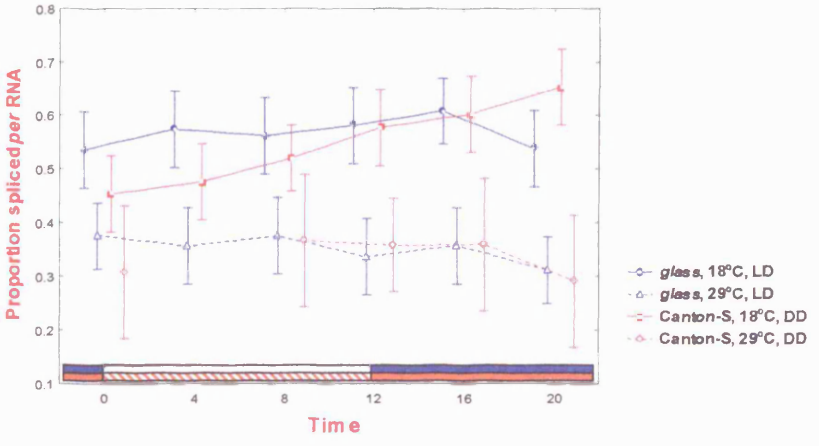


Figure 5.7: Splicing in gl^{60j} in LD compared to Canton-S in DD. Error Bars represent 95% confidence intervals.

The light input that represses splicing appears to mainly come through the visual system

5.5 Discussion

At low temperatures the splicing level is set by the photoperiod, and this does not require a functional clock (Chapter 3). CRY plays some role in the photoperiodic repression of splicing as the level of splicing is increased at night at 18°C in cry^b (and $norpA^{P41}$; cry^b). However, the level of splicing seen in cry^b is lower than that of Canton-S flies after a day in DD (compare figures 3.7 and 4.2). This indicates that an additional light input must be responsible for the remainder of the repression of splicing seen in wild-type flies kept in LD as opposed to DD (Chapters 3 and 4).

During the day at 29°C, splicing is repressed even in per^{01}, tim^{01} or cry^b mutants, and is therefore independent of the clock and CRY. At night levels of splicing in cry^b, per^{01} and tim^{01} mutants are elevated from ~25% to 40% of transcripts being spliced, indicating that PER, TIM and CRY are required to repress splicing at night at 29°C (Chapters 3 and 4). Therefore a separate light input pathway, independent of CRY, must be responsible for the daytime repression of splicing at 29°C (Chapter 4). This light input presumably comes via the visual system, as this known to be involved in entraining the clock to LD cycles (e.g. Helfrich-Forster et al., 2001).

The mutants gl^{60j} and $norpA^{P41}$ both affect visual photoreception. *norpA* is a phospholipase involved in phototransduction (Pearn et al., 1996), and in $norpA^{P41}$ mutants only the H-B eyelets and CRY are available to entrain the clock (Helfrich-Forster et al., 2001). *glass* is a transcription factor which, when knocked out, eliminates the expression of all opsins. As a result, *glass* mutations are more extreme visual mutants than those of *norpA*, and the only photoreceptor remaining in $glass^{60j}$ mutants is CRY. This means that $glass^{60j}$ cry^b double mutants are blind to light (Helfrich-Forster et al., 2001).

Although gl^{60j}, $norpA^{P41}$ and $norpA^{P41}$; cry^b flies entrain to LD cycles, they do so with altered patterns of locomotor activity. gl^{60j}, $norpA^{P41}$ and $norpA^{P41}$; cry^b also alter the response of locomotor activity to temperature changes.

5.5.1 Locomotor activity and splicing in *norpA*^{P41} and *norpA*^{P41}; *cry*^{b}

The *norpA*^{P41} mutation affects both the morning and evening activity peaks in a temperature dependent manner. At 18°C, the morning activity peak is identical to that seen in Canton-S flies. At 29°C, the level of morning activity is greatly increased in the *norpA*^{P41} mutant in comparison to Canton-S (figure 5.1a). Therefore *norpA*^{P41} behaves, in terms of the morning peak, as if it is hypersensitive to increased temperatures. The morning peak at 29°C is more similar to that of *per*^{01} mutants, which also show an elevated response to lights on, albeit at both 18 and 29°C (figure 5.1).

The main focus of work reported here has been to relate the splicing of the *per* intron to the position of the evening activity peak. In *norpA*^{P41}, the evening activity peak occurs earlier at 18 than at 29°C, as seen in wild-type lines. The evening activity peaks are also earlier than the corresponding peaks of Canton-S flies at both temperatures. These differences in evening peak position (between temperatures and between genotypes) correlate with the splicing levels of *per* mRNA.

Higher levels of spliced *per* are associated with earlier evening activity in wild-type flies. In *norpA*^{P41} flies there is more spliced *per* transcript at 18 than 29°C, accounting for the earlier evening peak at low temperatures. At both temperatures there is also more spliced *per* in *norpA*^{P41} than Canton-S, accounting for the earlier evening activity peaks of *norpA*^{P41} compared to Canton-S (figures 5.1 and 5.3).

The evening activity peak in *norpA*^{P41} at 29°C also occurs earlier than in *cry*^{b} mutants, and again, there is also a correspondingly higher level of splicing in *norpA*^{P41} compared to *cry*^{b} (figures 5.1 and 5.3). However, at 18°C, activity peaks at the same time in *cry*^{b} and *norpA*^{P41} flies, even though there is a higher level of splicing in the *norpA*^{P41} (figure 5.1c). This means that the absolute level of splicing of the intron is not the only determinant of evening

peak position. The simplest explanation would be that no matter how high the splicing level, there is a limit on how early the evening activity peak can occur, so there is no difference between *norpA*^{P41} and *cry*^{b}. Splicing alters the accumulation of PER protein, and this is limited by the light-dependent degradation of TIM. Therefore the timing of the evening peak may be limited by this degradation.

In *norpA*^{P41} the evening peaks occur early at both 18 and 29°C, and there is a corresponding elevation in the levels of *per* mRNA splicing compared to wild-type. These are responses associated with low temperatures in wild-type *D.melanogaster* (~45% of transcripts are spliced at 18°C in Canton-S, compared to ~55% in *norpA*^{P41} at 29°C). Therefore *norpA*^{P41} mutants behave as if they have an impaired ability to detect high temperatures. *norpA*^{P41} do still detect temperature changes - witness the altered evening peaks and splicing levels - they just react as if the temperature is colder than it actually is.

norpA^{P41}*; cry*^{b} double mutants are temperature insensitive in terms of locomotor activity, and behave as if it were very hot at all temperatures (they react as if it is hotter than it actually is (figure 5.2). However, even within the double mutant, the splicing machinery remains temperature sensitive, with more spliced *per* mRNA at low temperatures. This means that the splicing levels of *norpA*^{P41} and *norpA*^{P41}*; cry*^{b} are almost identical, with ~65-70% of transcripts spliced at 18°C and ~55-60% spliced at 29°C (figure 5.3). At 18°C, there is slightly more spliced *per* RNA in *norpA*^{P41}*; cry*^{b} than in the *norpA*^{P41} single mutant. This suggests that *cry* plays a small role in the regulation of splicing at low temperatures, supporting the observations reported in Chapter 4. At 29°C, the level of splicing is identical in *norpA*^{P41} and *norpA*^{P41}*; cry*^{b} mutants. There is no elevated level of splicing at night, as seen in the *cry*^{b} mutant. Therefore, *norpA*^{P41} is epistatic to *cry*^{b} at 29°C when it comes to determining the splicing level.

Given that the *per* mRNA splicing level is though to determine evening peak position, $norpA^{P41}$; cry^{b} would be expected to have the same evening activity peaks as $norpA^{P41}$ single mutants at different temperatures, which is not the case (figure 5.3). Therefore, when the $norpA^{P41}$ and cry^{b} mutations are combined, the relationship between splicing levels and evening peak position breaks down. A break down in the relationship between splicing levels and evening activity peak position is also seen in per^{01}; cry^{b} (Chapter 4). However, in per^{01}; cry^{b} the *per* transcript cannot be translated to produce a mature PER protein, and therefore the splicing level can have no bearing on locomotor behaviour (Chapter 3). As a result, the position of the evening activity peak in this mutant may be the default position which is then modified by altering the PER expression profile in response to altered temperatures and photoperiods.

Within the mouse, it has been shown that one role of the *mCry* proteins is to stabilise *mPer1* and *mPer2* (Lee et al., 2001). Within *D.melanogaster,* CRY and PER are also known to interact (Rosato et al., 2001) and if CRY stabilises PER in *D.melanogaster* as well, it may provide an explanation for this. Within *norpA* mutants, the PER-CRY interaction is unaffected, so the evening activity peak remains dependent on the splicing levels of *per* mRNA. If this is combined with cry^{b}, PER is no longer stabilised by CRY, and therefore the regulation of evening activity peak position breaks down. As the early evening activity peak seen at 18°C is though to be due to earlier upswing in PER protein levels, an increase in PER protein degradation would be predicted to delay the evening activity peak. Unfortunately this fails to explain why the splicing level still relates to evening activity peak position in cry^{b} (Chapter 3) or why within gl^{60j} mutants the relationship between splicing levels and evening activity peak position is also lost (below).

Perhaps the simplest explanation is that when $norpA^{P41}$ and cry^{b} are combined, the level of splicing is unable to advance the PER protein cycle, and alter evening activity peak position. The evening activity peak of $norpA^{P41}$; cry^{b} is delayed at 18°C, as would be expected at high temperatures

- they behave as if it were always hot. This is similar to the situation seen in wild-type flies unable to splice the *per* intron, where there is a delayed evening activity peak at both 18 and 29°C (Majercak et al., 1999). The reason given for the delayed evening activity peak in these flies is that the splicing of the intron ultimately advances the PER protein cycle, leading to an advanced evening activity peak with increasing levels of splicing. So, a late evening activity peak can be produced by reducing the amount of splicing, because this delays the PER protein cycle. This suggests that within $norpA^{P41}$; cry^{b} mutants, the PER protein cycle may be delayed compared to wild-type or $norpA^{P41}$.

Both CRY and the visual system play a role in regulating the PER/TIM cycle (Stanewsky et al., 1998). Therefore if $norpA^{P41}$ and cry^{b} are both eliminated the light input to the clock will be much reduced. TIM will then be more stable, and as a result, the repression of *per* and *tim* RNA by the PER/TIM complex will persist for longer (similar to TIM^{UL}, Rothenfluh et al., 2001). This will then delay the *per* RNA cycle, and as a result, the phase of activity will also be delayed, mimicking the effects of high temperatures. This may mean that in $norpA^{P41}$; cry^{b} the PER protein cycle is so impaired by the loss of light inputs that changes in splicing level have little impact.

5.5.2 Locomotor activity and splicing in gl^{60j} and gl^{60j} cry^{b}

The most obvious difference between the locomotor activity profiles of Canton-S and gl^{60j} is the absence of a morning activity peak in gl^{60j} mutants (figures 5.4 and 5.5). This demonstrates that detection of 'lights on' by the visual system is required to produce the morning activity peak. The role of the clock is then to modify the startle response in a temperature dependent manner.

There is no variation in the level of activity throughout the day and night in gl^{60j}, except for the evening activity peak. The evening peak is delayed at both temperatures in comparison to most other lines and occurs at the same time at both 18 and 29°C (figure 5.2d). This means that the total elimination

of visual photoreception eliminates most of the responses of locomotor activity to temperature changes.

As there is no difference in the position of the evening activity peak at different temperatures in gl^{60j}, it would be predicted that there would also be no difference in splicing levels at 18 and 29°C (figure 5.4). As the evening peak is delayed in comparison to Canton-S, it would also be predicted that the level of splicing would be reduced. This is not the case as there is still a difference in splicing levels at different temperatures with more spliced *per* RNA at 18 than at 29°C (figure 5.6). Additionally, the level of splicing at both temperatures in gl^{60j} is higher than in Canton-S (~60% vs. ~45% at 18°C, and ~40% vs. ~25% at 29°C) (figure 5.6a). Again, the level of splicing is not the only factor that determines the position of the evening activity peak.

In both $norpA^{P41}$; cry^{b} and gl^{60j} there is a difference in splicing levels at different temperatures, and the evening peak is delayed in comparison to Canton-S despite the elevated level of splicing. As gl^{60j} is more severe in terms of effect on the visual system than $norpA^{P41}$, the simplest explanation is that gl^{60j} has a similar effect in reducing the light input to the clock as the elimination of both *norpA* and *cry.* This would mean that the H-B eyelets and *cry* remaining in $norpA^{P41}$ are together sufficient to maintain the PER cycle. If only one of these photoreceptors is present (*cry* in gl^{60j} mutants, or the H-B eyelets in $norpA^{P41}$; cry^{b} mutants) the clock, or PER protein cycle, is sufficiently impaired so that changes in temperature no longer have an effect on evening activity peak position. As a result, in both gl^{60j} and $norpA^{P41}$; cry^{b} the evening activity peak is delayed at both temperatures in these mutants. To confirm this, it may be worthwhile examining the *per* mRNA and/or protein cycles of gl^{60j} at 18 and 29°C, and see if there is a significant delayed compared to Canton-S.

Like $norpA^{P41}$; cry^{b} mutants, gl^{60j} have locomotor activity which is relatively insensitive to temperature changes, but the splicing machinery still responds to temperature changes. This again reveals a separation between the temperature input which sets activity and that which sets the splicing levels.

If all the light inputs to the splicing machinery are eliminated, then levels of splicing would be expected to be the same as those seen in wild-type flies in DD. This is the case with gl^{60j}, suggesting that the visual system provides the majority of the light input that sets the splicing level (figure 5.7)[11]. It also indicates that temperature sets a maximum level of splicing that is then repressed by the photoperiod (figure 5.7). At 18°C this is ~60% of all *per* transcripts, and at 29°C, ~40% of all *per* transcripts. These levels are only exceeded in $norpA^{P41}$ that supports the idea that norpA plays a role in regulating splicing levels independent of its role in photoreception.

In per^{01}, tim^{01} and cry^{b} mutants at 29°C, splicing is repressed during the day but not at night (Chapters 3 and 4). In gl^{60j}, like $norpA^{P41}$, there is no difference in splicing levels between the day and night at 29°C. The levels of splicing seen gl^{60j} at 29°C at all times are similar to the (elevated) night-time levels of splicing seen in per^{01}, tim^{01} and cry^{b}, with ~40% of transcripts being spliced.

This suggests that during the day at 29°C, the light input received through the eyes sets the splicing level, and the clock maintains this repression at night. If the visual input is removed then the level of splicing remains constant (and high) during the day and night ($norpA^{P41}$ and gl^{60j}, figures 5.3 and 5.6). If the clock or CRY are eliminated, then the visual input is still able to repress splicing during the day, but there is no repression of splicing at night because the clock is not available to maintain repression (Chapters 3 and 4). If both PER and CRY are removed, this repression of splicing during the day by light is also lost (Figure 4.4). Therefore the visual input requires either PER or CRY (but not both) in order to repress splicing during the day.

[11] It should be noted that although the splicing phenotype of *glass* flies is identical to that of Canton-S in DD, the locomotor activity is not. In Canton-S flies in DD there is a single, early activity peak, whereas in *glass* flies, the evening peak is delayed. Therefore CRY plays an important role in entrainment, but not such an important role in setting the splicing level.

5.5.3 Rhythmicity of gl^{60j} cry^{b} mutants at 29°C

The circadian clock of gl^{60j} cry^{b} flies was previously reported to be blind, as they have no known photoreceptors (Helfrich-Forster et al., 2001). Individual gl^{60j} cry^{b} flies are rhythmic, but their behaviour cannot be entrained to LD cycles. To see whether there was any effect of temperature on the level of locomotor activity in gl^{60j} cry^{b} mutants, the locomotor activity profiles were analysed in LD 12:12 at 18 and 29°C.

At 18°C, behaviour is similar to that previously reported with this line. These mutants show no morning activity peak, and no anticipation of lights off. gl^{60j} cry^{b} behaviour at 18°C is similar to that of per^{01} flies, with a constant level of activity throughout the day, followed by an immediate drop in activity immediately after lights off (ZT 12). In per^{01} flies, the level of activity remains low until lights on, whereas in gl^{60j} cry^{b} flies, the level of activity rises again a couple of hours after lights off (figure 5.5a). At 25°C, there is a small peak in locomotor activity immediately after lights off which was attributed to masking (Helfrich-Forster et al., 2001) as there is no regulated PER/TIM protein cycle. Masking could therefore account for this small fall in activity seen at 18°C here.

Surprisingly it was found that at 29°C gl^{60j} cry^{b} flies are entrainable to LD cycles, with a clear evening activity peak anticipating 'lights off'. The pattern of behaviour at 29°C of gl^{60j} cry^{b} closely resembles that of gl^{60j} single mutants at 29°C (figure 5.5). This means that cry^{b} has no phenotypic effect on locomotor behaviour when combined with gl^{60j}, at elevated temperatures. The only known photoreceptor available in gl^{60j}, which must therefore be used to entrain behaviour, is *cry.* For gl^{60j} cry^{b} mutants to behave like gl^{60j} at high temperatures, some photoreceptor function must be gained at high temperatures. The most parsimonious explanation would be that as cry^{b} is not a null mutation, it becomes 'active' at high temperatures, allowing gl^{60j} cry^{b} to entrain to LD cycles. The only alternative explanation is that there is an additional light input to the clock which has not yet been characterised –

the existence of an additional light input driving circadian rhythmicity has recently been postulated (Malpel et al., 2002). It cannot be because gl^{60j} becomes functional at high temperatures as *gl* encodes a transcription factor required for the formation of all photoreceptor structures during development (Moses et al., 1989; Helfrich-Forster et al., 2001). There is some evidence to support the idea that cry^b is not a complete loss of function mutant and is temperature sensitive, notably the altered behaviour of per^s cry^b at different temperatures (Rosato et al., 2001). However, in this example rhythmicity breaks down at high temperatures, as does the CRY-TIM interaction in yeast two hybrid assays.

Observations of other lines carrying the cry^b mutation do not support the idea that cry^b can detect light at higher temperatures. Both $norpA^{P41}$; cry^b and per^{01}; cry^b show very similar patterns of behaviour at 18 and 29°C. By analogy to gl^{60j} cry^b behaviour resembling that of gl^{60j} at high temperatures, per^{01}; cry^b should resemble per^{01} and $norpA^{P41}$; cry^b resemble $norpA^{P41}$ at 29°C, which is not the case. It may be that cry^b is always able to detect light at high temperatures, but that this only has a phenotypic effect when there is no other light input available as in gl^{60j} cry^b but not per^{01}; cry^b or $norpA^{P41}$; cry^b.

5.5.4 Morning activity peak determination

The presence of a morning activity peak is dependent on the visual photoreception system as it is lost in gl^{60j}. One role of the clock is to modulate the size of the startle response to 'lights on'. The morning activity peak in Canton-S flies is higher at 18 than 29°C. In cry^b flies this difference is lost and the morning peak is reduced in height, presumably because cry^b is less sensitive to light. At high temperatures, the light intensity is also likely to be higher, and therefore it makes sense if the clock is less sensitive to light, so a higher light intensity is required to elicit the same response.

In tim^{01} mutants, the size of the morning peak is increased compared to Canton-S, but there is no difference in peak height at different temperatures. In contrast, in per^{01} flies, the size of the morning activity peak is increased to the same extent as tim^{01}, but there is a larger startle response at low temperatures. Therefore a functional clock reduces the startle effect, but only CRY and TIM are required to produce a difference in morning peak size at different temperatures (Chapter 4).

$norpA^{P41}$ behaves as if it were hypersensitive to 'lights on' at 29°C, with a morning peak as high as that of per^{01} mutant flies. In the $norpA^{P41}$; cry^{b} double mutant the height of the morning peak is reduced to the same size as in the cry^{b} single mutant at both temperatures. Therefore, the $norpA^{P41}$ mutation increases the startle effect, and the cry^{b} mutation reduces it. cry^{b} is epistatic to $norpA^{P41}$ in determining the size of the morning activity peak.

There is no morning activity peak at either 18 or 29°C in gl^{60j} and gl^{60j} cry^{b} mutants. This peak is present in $norpA^{P41}$ at 18 and 29°C, and in $norpA^{P41}$; cry^{b} mutants at 18°C, so it seems that the light input from the H-B eyelets alone is sufficient to produce a morning activity peak at 18°C, but at 29°C, an additional light input, from *cry,* is also required. The light input from CRY alone is not sufficient to produce this peak, as there is no peak in gl^{60j}.

5.5.5 Upswing of locomotor activity

Normally, the level of locomotor activity begins to rise earlier at 18 than 29°C. CRY and the clock operate antagonistically to determine when this rise occurs as this difference is lost in cry^{b} mutants but partially restored in per^{01} cry^{b} double mutants (Chapter 4). This difference is maintained in $norpA^{P41}$, but lost from $norpA^{P41}$; cry^{b}, so *norpA* seems to have no role in determining the timing of this upswing. This difference is eliminated by the gl^{60j} mutation. This may mean that a light input from the H-B eyelets is required to produce this earlier upswing. Alternatively, it may be lost owing to some other defect of the clock in gl^{60j} mutants - for instance, there is no morning activity peak or

temperature difference in activity levels during the day, and the evening peak position is not related to the splicing levels.

5.5.6 Evening activity peak determination

The regulation of evening peak position in a temperature dependent manner seems to be the key to avoiding mid day activity. The position of the peak of evening activity is controlled by the splicing levels of the *per* 3' UTR intron, under the control of the photoperiod, clock and CRY (Chapter 4 and 5). Analysis of the behaviour of gl^{60j} and $norpA^{P41}$ visual mutants reveals that the light input received by the eyes is involved in setting the *per* mRNA splicing level.

In general, the level of splicing determines when the peak level of locomotor activity will occur, as previously reported (Majercak et al., 1999).
Observations of wild-type, $norpA^{P41}$ and cry^{b} flies fit with the idea that the level of splicing of the *per* 3' UTR determines evening peak position. In all of these cases, there is an earlier evening activity peak and more spliced *per* RNA at 18 than 29°C. Additionally, at both 18 and 29°C, the lowest level of splicing and latest peak is observed in Canton-S flies, and the highest level of splicing and earliest evening peak are found in $norpA^{P41}$ flies. At 18°C, there is more spliced *per* in $norpA^{P41}$ than in cry^{b} even though the evening peak occurs at the same time. There is therefore probably a limit to how early the evening activity peak can occur, no matter what the *per* splicing level. That this could be an effect of altered *per* protein and RNA cycles in the different mutants cannot be discounted as the role of splicing regulation is simply to modify the pre-existing RNA cycle.

The level of splicing of the *per* intron is not the only determinant of evening peak position as $norpA^{P41}$; cry^{b} and gl^{60j} flies have different levels of splicing but no difference in the evening peak position at different temperatures. The evening activity peak also occurs at a similar time in both gl^{60j} and $norpA^{P41}$; cry^{b} mutants, even though the levels of splicing are very different in the two

lines. The most likely explanation is that in these flies the light input to the clock is so impaired that the degradation of TIM and/or the negative feedback loops are disrupted. As a result, the effect of altered splicing on the cycle of *per* RNA is so minor as to have no effect on locomotor activity. The only problem with this model is that it has previously been suggested that even within the eye, the visual system is not required for the regulation of the levels of PER/TIM (Suri et al., 1998). However the visual system must drive the entrainment PER/TIM cycle within the LN_vs as cry^b flies are rhythmic because of this cycle (Stanewsky et al., 1998).

5.5.7 How light and temperature set the *per* mRNA splicing level: Conclusions to Chapters 3-5

At 18°C, analysis of Canton-S, per^{01}, tim^{01} and cry^b strains indicated that photoperiod was the primary determinant of splicing level. The detection of light by the splicing machinery appears to be independent of PER, TIM and CRY. The clock is not required to set the splicing level in response to altered photoperiods (Chapter 3; J. Majercak, personal comm.) and instead, the repression of splicing by light at low temperatures is mediated by the visual system. Even if the splicing level is not always related to the evening peak position, the analysis of splicing levels in visual mutant lines helps uncover the pathway by which photoperiod sets the clock.

CRY seems to have a small role in setting the splicing level at 18°C. In cry^b mutants at 18°C, the level of splicing is elevated at night compared to Canton-S. Also, the level of splicing of $norpA^{P41}$; cry^b is slightly higher than that of $norpA^{P41}$ at 18°C. These differences between lines with and without the cry^b mutation are small, even at 18°C and the effect of visual mutants alone on the splicing levels at 18°C is far greater. CRY plays no role in the light input to the splicing machinery at 29°C as the splicing levels in both *norpA* cry^b and gl^{60j} cry^b flies are identical to those of $norpA^{P41}$ and gl^{60j} flies respectively. Therefore at both 18 and 29°C, the visual system is responsible for detecting the photoperiod, and this information then sets the

splicing level. This is consistent with the observed splicing levels in reduced photoperiods fits this observation where the clock and *cry* are not required for the photoperiod setting of splicing levels (J. Majercak, pers. com.; Chapter 3).

Although *norpA* and *glass* have different roles in determining locomotor behaviour, knocking out either one, while leaving the fly entrainable, prevents photoperiod information reaching the splicing machinery and the level of splicing is elevated. This means that although CRY or the H-B eyelets are sufficient for entrainment, more than one photoreceptor is required for the photoperiod to set the splicing level.

Temperature seems to set a maximum level of splicing which is then repressed by light. This level is seen in DD, or in gl^{60j} and gl^{60j} cry^{b} mutants. It is ~60% spliced *per* at 18°C and ~40% spliced *per* at 29°C (See figures 3.6 and 5.6; 5.7). At high temperatures, the repression of splicing at night requires the presence of PER, TIM and CRY. If any of these proteins is eliminated, then the level of splicing at 29°C at night rises to ~45% of *per* transcripts, a similar level to that seen in wild-type in DD at 29°C. The fact that splicing is still repressed at 29°C in comparison to 18°C even in visual mutants suggests that temperature sets the splicing level which is then repressed further by the photoperiod, fine-tuning behaviour to different seasonal conditions. To confirm that this is the case, the splicing level after more than one day of DD needs to be examined to confirm this limit.

The only line in which these limits are exceeded are those carrying the $norpA^{P41}$ mutation. The levels of *per* splicing in $norpA^{P41}$ mutants are higher than those of gl^{60j} at both 18 and 29°C. Also, the difference between the 18 and 29°C splicing levels is reduced in $norpA^{P41}$ mutants in comparison to gl^{60j} (Figure 5.8).

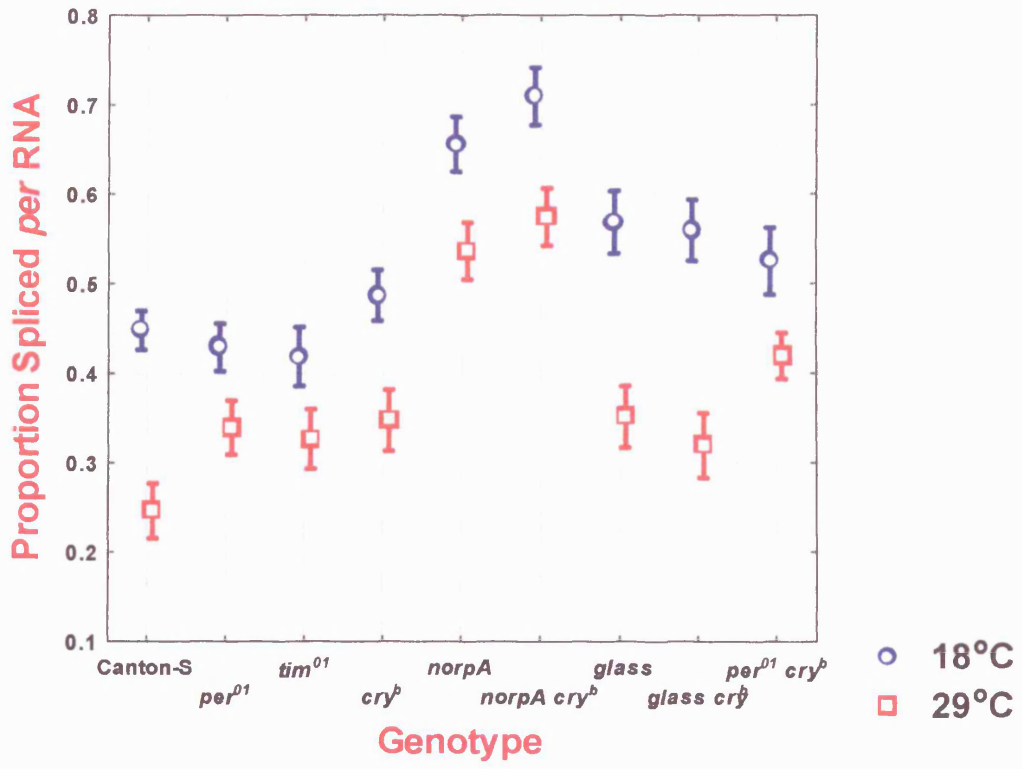


Figure 5.8: Differences in splicing levels in different genotypes at different temperatures. Error bars are 95% confidence intervals.

There is evidence that *norpA* is involved in a temperature input pathway to the splicing machinery, and perhaps the clock in general. The elevated level of splicing and reduced temperature difference of $norpA^{P41}$ cannot be solely due to the role of *norpA* in photoreception as gl^{60j} is a more severe visual mutant but has less of an effect on splicing levels. This suggests that *norpA* is possibly a signalling molecule in the temperature sensing pathway. This seems to be confirmed by the decreased sensitivity of *norpA* to increased temperatures in terms of splicing levels.

The patterns of locomotor activity also indicate that *norpA* is involved in temperature sensing. The $norpA^{P41}$ single mutant seems to be more sensitive to high temperatures than wild-type lines. The level of behaviour at night through to the morning activity peak is elevated, and the morning

activity peak is similar in size to that of per^{01} flies. This suggests that per^{01} and $norpA^{P41}$ flies are more sensitive to the 'startle effect' of lights on than other lines, and that both a functional clock and a signalling pathway involving *norpA* are required to modulate morning peak activity. While *per* is required to modulate this response at all temperatures, *norpA* is only required at high temperatures. In wild-type flies, the size of the startle response is repressed in a temperature dependent manner, and *norpA, cry* and the clock are all involved in this action. This extreme startle effect is attenuated at high temperatures by the cry^b mutation.

Although *norpA* mutant flies show altered patterns of entrainment, little work has been done to connect the function of *norpA* with circadian rhythms as its effect is though to be primarily because *norpA* mutants are blind. It takes longer for *norpA* mutants to entrain to new LD cycles (Emery et al., 2000b; Wheeler et al., 1993), and they show altered patterns of behaviour, including a period ~1 h shorter than normal (Dushay et al., 1990). The elevated splicing levels may explain this reduced period as high levels of splicing are though to advance the PER protein cycle (Majercak et al., 1999). An advanced PER protein cycle might then be predicted to speed up the clock, and as a result to reduce the free-running period.

A role for *norpA* in the clock outside of the visual system has previously been reported in the malpigian tubules (MT) where the level of TIM in $norpA^{P41}$; cry^b mutant flies is higher than that of the cry^b single mutant (Ivanchenko et al., 2001). This fits nicely with a temperature sensing role for *norpA* as the clock is also involved in this, and if the two proteins interact, it becomes possible for one to regulate the other. Recently, it has been demonstrated that NORPA is able to interact with TIM (Sehgal; SRBR Conference 2002), indicating that there could be a role for *norpA* signal transduction in circadian rhythms outside the visual system.

The Phospholipase C (PLC) encoded by the *norpA* gene of *D.melanogaster* is required for inositol signalling within the phototransduction pathway. Inositol signalling is not confined to phototransduction, and is found in a wide

range of cellular processes. *norpA* is primarily expressed within the rhabdomeres of the compound eye (Schneuwly et al., 1991), and the photoreceptors of severe *norpA* mutants show no electrophysiological response to light. In the photoreception pathway, NORPA is activated by photoactivated rhodopsin through a G-protein (Bacigalupo et al., 1995). *norpA* is expressed in the retina and in axons of photoreceptor cells (McKay et al., 1995) and in *norpA* mutants. PLC activity is lost within the head (Yohsioka et al., 1995; McKay et al., 1995). *norpA* could therefore be used in different signalling roles in different tissues.

Any role which *norpA* may have in the clock is independent of its role in photoreception as the light induced degradation of TIM within the eye is unaffected in the *norpA* mutant line (Suri et al., 1998) and PER also cycles normally (Zerr et al., 1990). This would then preclude the regulation of TIM levels by the visual system in cry^b mutants, yet they are entrainable. To account for this, there must be redundancy between CRY and the visual system in the regulation of TIM degradation and the entrainment of the clock.

In contrast, *gl* seems to have no role outside the visual system in regulating splicing levels, and splicing remains temperature sensitive, with splicing levels similar to those achieved under DD. It seems that the clock receives information on the photoperiod through the eyes rather than through CRY and the role of CRY seems to be limited to its interactions with PER/TIM and controlling the temperature sensitive upswing in activity levels and morning peak height.

5.5.8 A model as to how light and temperature set the splicing level

Figure 5.9 shows a possible model of how light and temperature may set the splicing level of the clock, and determine the evening peak position.

The regulation of splicing differs at 18 and 29°C. How temperature is detected is not known, but it seems that *norpA* plays a role in the pathway which transmits information about the temperature to the clock, as $norpA^{P41}$ mutants seem to be less temperature sensitive.

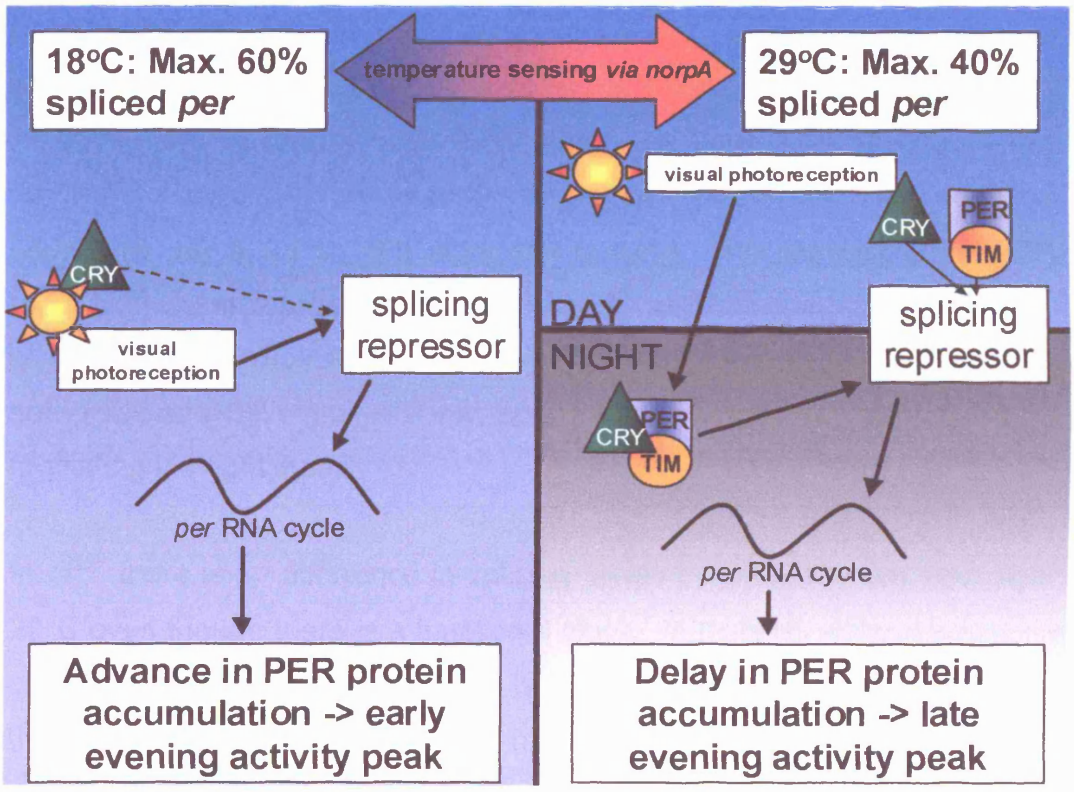


Figure 5.9: A model of how the splicing level is set by light and temperature

Temperature appears to set a maximum level of splicing which is then further repressed by the photoperiod, so the splicing level, *per* RNA cycle and ultimately behaviour can be fine-tuned to any given conditions. This maximum level of splicing for a given temperature is that which is observed in DD, or in gl^{60j} visual mutants.

At low temperatures, the splicing level is set by the photoperiod. The photoperiod is mainly detected by the visual system, but CRY also seems to play a small role in detection. The splicing level then modifies the *per* RNA cycle. This produces an early upswing in PER protein, generating an early

evening activity peak. It may be that the visual system also regulates the *per* mRNA and/or protein cycle. This is shown on the left-hand side of figure 5.9.

At high temperatures, the regulation of splicing is more complex, and involves the circadian clock (figure 5.9, right-hand side). This is probably because it is more important to regulate behaviour at high temperatures, and avoid midday desiccation. The primary determinant of the splicing level is still light, and splicing is repressed during the day. This light input to the splicing machinery comes from the visual system, with no input from CRY. During the day at 29°C, light represses splicing. This requires either PER or CRY as there is repression of splicing per^{01} and cry^{b} single mutants but not when they are combined in per^{01}; cry^{b}. However, during the day the levels of both PER and CRY are very low, so it is unclear what role they can play in mediating the light input.

In gl^{60j}, there is no difference in splicing levels between the day and night at 29°C even though there is a functional clock. In mutants without a functional clock, the level of splicing at night is higher than during the day. Therefore the clock cannot repress splicing at night unless there is a light input from the visual system. The level of splicing set during the day by this light input is then 'remembered' by the clock; in the absence of PER, TIM or CRY there is no repression of splicing at night at 29°C. The role of CRY in this is probably because CRY is a core clock component in peripheral clocks like the eye, and the situation may be different in the LN_vs.

Chapter six: Temperature regulation of *Drosophila pseudoobscura per* RNA

6.1 Introduction

D.melanogaster and *D.pseudoobscura* are two closely related species that diverged approximately 25 Million years ago. The geographical distribution of *D. pseudoobscura* is limited to a region extending from British Columbia, down through the West Coast of North America, to Mexico and Guatemala (Wang and Hey, 1996), whereas *D.melanogaster* has an almost universal distribution. The two organisms display very different patterns of locomotor activity. This is particularly apparent in DD, when the behaviour of *D.melanogaster* peaks early in the day, while the evening activity peak of *D.pseudoobscura* is delayed (Petersen et al., 1988; Tauber et al., 2003).

Rhythmic, species-specific behaviour is known to be encoded by the *per* gene (Petersen et al., 1988). A construct containing the coding region of *D.pseudoobscura* and the untranslated and intronic regions of *D.melanogaster* called *mps1* rescues rhythmicity in per^{01} flies, albeit rather poorly as only 10-30% of flies are rhythmic with two copies of the transgene in DD (Petersen et al., 1988; Piexoto et al., 1998). The period of these transformants is longer than either wild-type species, and behaviour more closely resembled that of *D.pseudoobscura,* with one major activity peak. In LD cycles, activity of the homozygous *mps1-*per^{01} transformant has a sharp evening activity peak post-lights off (Petersen et al., 1988; Piexoto et al., 1998). If *mps1* is transformed onto a *D.melanogaster* per^{+} background, the resultant transformant behaves like *D.pseudoobscura* but has robust 24 h rhythms, perhaps reflecting the stronger nature of *D.pseudoobscura* rhythms

(Petersen, et al., 1988; Tauber et al.,2003). A modified chimeric construct, *mps3*, shows a much higher rescue of rhythmicity and a 24 h period, but the pattern of locomotor activity still resembles that of *D.pseudoobscura* (Peixoto et al., 1998; Hennessy 1999). The structure of the *mps3* construct is shown in figure 6.1.

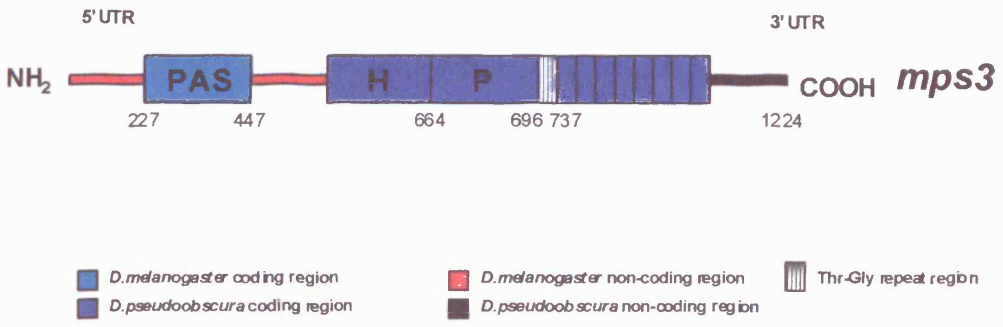


Figure 6.1: The *MPS3* construct. The *MPS3* Construct contains the 5' end of the *D.melanogaster* coding region. The remainder of the coding region and the 3' UTR are from *D.pseudoobscura*.

The higher level of rhythmicity and 24 h period of per^{01} flies transformed with the *mps3* construct indicates that if the N-terminal *D.melanogaster* regions of the protein are heterospecific (e.g. *mps1*) then rhythmicity appears to be reduced and the period extended. The N-terminal is also responsible for determining the species-specific pattern of behaviour. In *mps3* flies, the cycle of PER protein was found to be very similar to that of *D.melanogaster* at 25°C with the level of PER rising around ZT12 (lights off) in both cases. However, PER seems to persist for longer during the night in the *mps3* transformant (compare So & Rosbash, 1997 with Peixoto et al., 1998).

In the wild, *D.pseudoobscura* and *D.melanogaster* often co-exist in the same environments. In the wild, *D.pseudoobscura* mating activity peaks just before darkness, while that of *D.melanogaster* peaks earlier in the evening (Partridge et al., 1997). In wild-type flies in the laboratory in DD, the maximum level of mating is reached about 3 h earlier in *D.melanogaster* than *D.pseudoobscura,* reflecting the earlier phase of circadian activity.

If the *mps1* construct is put on a *D.melanogaster per*$^{+}$ background, behaviour resembles that of *D.pseudoobscura,* but with a 24 h period. This then allows the comparison of transformant flies to see whether species-specific behaviour affects mating. In this way it was demonstrated that transformant *D.melanogaster* favour matings with individuals carrying the *per* gene from the same species (e.g. *mps1* favour matings with other *mps1* transformants. The cycle of mating activity lags behind the cycle in locomotor activity by several hours in both species, so although both cycles may be run by the clock, they are not identical (Tauber et al., 2003).

Another interesting difference between *D.melanogaster* and *D.pseudoobscura* is in their response to increased temperatures. At 29°C in DD, *D.melanogaster* has a clearly defined activity peak in the subjective night. In contrast, locomotor activity of *D.pseudoobscura* is arrhythmic under the same conditions (Hennessey, 1999). Additionally, the number of rhythmic *D.pseudoobscura* flies decreases from ~70-90% at 12°C to ~60% at 29°C. In contrast the level of rhythmicity in *D.melanogaster* the opposite over this temperature range. Additionally, the free running period of *D.pseudoobscura* is close to 24 h at temperatures up to ~25°C, but increases considerably, to ~26 h at 29°C (Henessey 1999).

This suggests that the two species may have altered abilities to cope with increased temperatures, with *D.melanogaster* appearing to be better adapted to warmer and *D.pseudoobscura* to colder climes. Little has been done on the relationship between temperature, RNA cycling and locomotor activity in species other than *D.melanogaster.* The only study that looked for

alternative splicing is in *Bactrocera tryoni* and *Bactrocera neohimeralis*, two sibling tephritid fruit fly species native to Australia (An et al., 2002). Although they are much more closely related to each other than *D.melanogaster* and *D.pseudoobscura,* they also show differences in behaviour. Like *D.melanogaster* and *D.pseudoobscura* they show differentiation by mating behaviour (e.g. Tauber et al., 2003) and *B.tryoni's* ecological range extends into much colder climes than *(B.neohimeralis)* (An et al., 2002).

In these *Bactrocera*, there are no differences in the PER protein sequence between the two species, so the only way in which *per* could be involved in determining species specific behaviour would be if the expression pattern of the gene is altered. The intron of the *period* 3' UTR intron is alternatively spliced within these species and appears to be under temperature control but unlike *D.melanogaster,* splicing is repressed at low temperatures. The RNA cycle is also under temperature control, with *per* levels peaking ~3 h earlier at 18°C than at 29°C, but the level of *per* remains constant at different temperatures. In this study there was no difference found between the RNA cycling and splicing levels of the two species, so the regulation of *per* expression cannot explain the greater climactic range of *B.tyroni* or the differences in mating behaviour (An et al., 2002).

6.2 Aims

In DD at 18°C, *D.melanogaster* has an earlier locomotor activity peak than *D.pseudoobscura* (Tauber et al., 2003). At 29°C, the peak of *D.melanogaster* occurs at a similar time as the *D.pseudoobscura* 18°C peak, meaning that at low temperatures, *D.pseudoobscura* behaves like *D.melanogaster* at high temperatures (Henessey, 1999). This may then indicate that *D.pseudoobscura* is hypersensitive to high temperatures. Species specific differences in behaviour between *D.melanogaster* and *D.pseudoobscura* have already been demonstrated to be encoded by the *per* gene (e.g. Petersen et al., 1998; Tauber et al. 2003). The mechanism which allows *D.melanogaster* to adjust locomotor behaviour to different

temperatures involves the regulation of PER protein levels by altering the splicing of the intron within the 3' UTR of the *per* gene. It could therefore be that species-specific differences in behavioural temperature sensitivity also involve the splicing of the *per* gene. 3' RACE was therefore used to determine whether alternatively spliced *per* mRNA transcripts also exist in *D.pseudoobscura.* In addition, the *per* mRNA and protein cycles of *D.pseudoobscura* were also analysed at different temperatures. Finally, the *mps3* transgene contains the promoter region from *D.melanogaster,* so the expression of the RNA should be normal, although altered properties of this chimeric protein could alter the negative feedback loops which control *per* RNA expression. The 3' UTR comes from *D.pseudoobscura,* so analysis of the splicing levels of *per* within this line may reveal whether conserved RNA sequence is required for the regulation of splicing.

6.3 Methods

Canton-S data is from Chapter 3, *D.pseudoobscura Ayala* strain.
mps3-65c transformants had previously been generated, as described by J. Henessey (1999). These flies were then crossed onto a per^{01} background, as described in Chapter 2. Flies were collected and analysed as in Chapter 3, with Western blots of proteins as in Chapter 2.

6.4 Results

6.4.1 Temperature compensation in *D.pseudoobscura*

A comparison of free running periods in *D.melanogaster* and *D.pseudoobscura*, as shown in figure 6.2

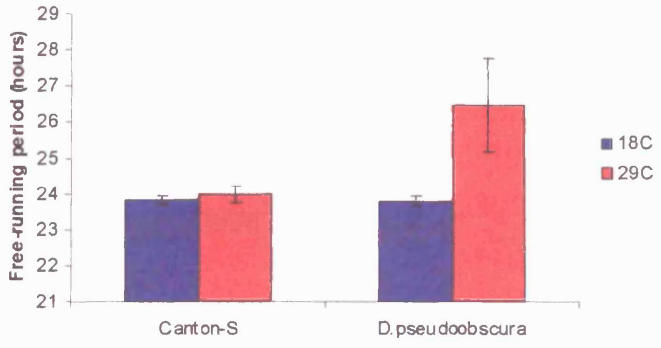


Figure 6.2: Temperature compensation in *D.melanogaster* and *D.pseudoobscura* free-running periods of *D.melanogaster* (Canton-S) and *D.pseudoobscura (Ayala)* at 18°C (blue) and 29°C (red) as determined by spectral analysis. Error bars represent SEM.

The period of *D.melanogaster* is very close to 24 h at both 18 (23.84+/-0.12 h) and 29°C (23.99+/- 0.25 h), as is the free-running period of *D.pseudoobscura* at 18°C (23.8+/- 0.14 h). At 29°C, fewer *D.pseudoobscura* flies remain rhythmic in DD, and of those which do, the period rises to ~26.47+/-1.3 h. This replicates the observations of Henessey (1999), and indicates that the clock of *D.melanogaster* is better temperature compensated than that of *D.pseudoobscura* over this temperature range.

6.4.2 Locomotor activity *D.pseudoobscura*

The locomotor activity profile of *D.pseudoobscura* compared to *D.melanogaster* at 18 and 29°C is shown in figure 6.3.

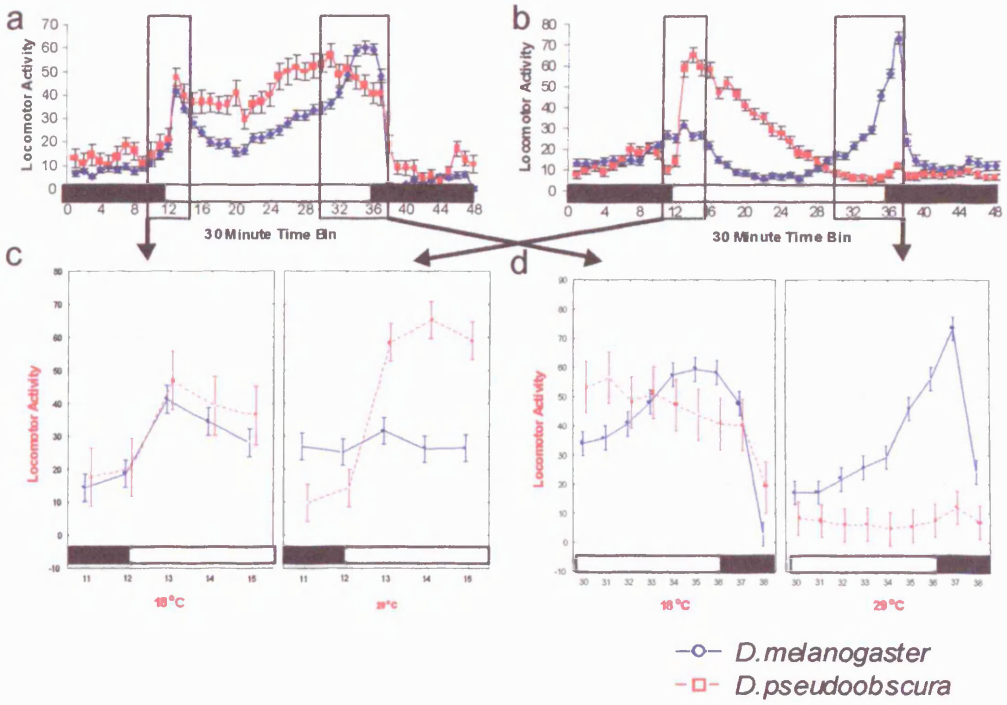


Figure 6.3: Locomotor activity of *D.pseudoobscura Ayala* and *D.melanogaster* Canton-S. a). *D.melanogaster* (blue) and *D.pseudoobscura* (red) at 18°C. Error bars show SEM. b). 29°C. c) expanded morning activity peaks at 18°C and 29°C. d). expanded evening activity peaks at 18°C and 29°C. Error bars represent 95% confidence interval

In LD 12:12 the locomotor activity patterns of both *D.melanogaster* and *D.pseudoobscura* alter in response to temperature changes. At 18°C, the locomotor profiles of both species are bimodal and the levels of activity are similar (6.2a). There is no difference in the morning activity peaks (A 6.3.5; time x genotype, F=0.11, n.s.) between the two species at 18°C. At 18°C, the evening activity peak in *D.pseudoobscura* is broader than that of *D.melanogaster;* the peak begins to rise at ZT 4 (Time Bin 20), peaks around ZT 9.5 (Time Bin 31) and then drops rapidly after lights off (ZT 12, Time Bin 26). In contrast, the evening peak of *D.melanogaster* begins to rise (ZT 9.5) and peak (ZT 11) later, and then falls rapidly after lights off. As a result, *D.pseudoobscura* locomotor activity is advanced compared to *D.melanogaster* at 18°C (6.3a and 6.3d; A 6.3.7, genotype x time, F=7.8, p<<<0.01). (F=7.801, p<<<0.05).

The difference in locomotor activity is much more apparent at 29°C. In *D.melanogaster,* the clock drives the shift of locomotor activity from the middle of the day, so most activity occurs around the morning and particularly the evening activity peaks. In contrast, locomotor activity in *D.pseudoobscura* is almost unimodal, with a large burst of activity at lights on, followed by a gradual decline in activity during the day. At the time where the evening activity peak occurs in *D.melanogaster,* locomotor activity in *D.pseudoobscura* has returned to the low levels seen throughout the night (6.3b). There is a significant effect of genotype x time at both the morning (A 6.3.6; $F=51.6$, $p<<<0.001$) and evening (A 6.3.8; $F=31.5$, $p<<<0.001$) activity peaks, reflecting this difference.

***D.pseudoobscura* is less well temperature compensated than *D.melanogaster,* and at higher temperatures there is no evening locomotor activity peak.**

6.4.3 *per* mRNA splicing in *D.pseudoobscura*

In *D.melanogaster,* locomotor activity occurs earlier at 18 than 29°C, whereas the opposite is true of *D.pseudoobscura* (figure 6.3). Therefore, if splicing also regulates the evening activity peak in *D.pseudoobscura,* there should be a higher level of spliced *per* mRNA at 29 than 18°C. This would be similar to *Bactrocera,* where the level of splicing is elevated at the higher temperature, rather than at the lower temperature seen in *D.melanogaster*, although this has not as yet been linked to behavioural differences (An et al., 2002).

Alternatively, there may be no temperature regulation of splicing in *D.pseudoobscura,* and species specific differences in locomotor behaviour may be due to some other factors. In order to determine whether alternatively spliced *per* transcripts were present in *D.pseudoobscura,* 3' RACE was carried out on the 3' UTR of the *per* gene, as described in Chapter 2. Two

products were generated and their sequences compared to the genomic *D.pseudoobscura per* sequence. This indicated that transcripts of *D.pseudoobscura per* differing in the splicing of a 3' UTR intron exist *in vivo*. The sequence of the 3' UTR was then compared to that of *D.melanogaster per* to see whether there were any conserved elements which could indicate a conserved 3' UTR function, as shown in figure 6.4.

Comparison of the sequence of the 3' RACE products of *D.pseudoobscura per* identify an intron within the 3' UTR of *period* analogous (but sharing no homology to) the 3' UTR intron which undergoes alternative splicing within *D.melanogaster.* The recovery of 3' RACE products both with and without this intron raises the possibility that *D.pseudoobscura* may also alternatively splice this intron in response to temperature changes.

If the 3' UTR sequences of *D.melanogaster* and *D.pseudoobscura* are compared, there is low (~50%) shared sequence identity between species, as might be expected for a non-coding region of DNA. Up to the start of the first intron within *D.melanogaster* the sequences share 65.5% identity. Most of this is accounted for a highly similar sequence starting 13 bases downstream of the stop codon, extending to the first 7 bp at the start of the *D.melanogaster* intron. A BLAST search found a high degree of conservation of these 131 bp within the *Drosophilids D.yakuba* (90% identity), and *D.simulans* (98%) but the sequence is not found in *D.virilis, Musca Domestica* or *Antheraea pernyi.*

While both species have an intron within the 3' UTR there is no homology in either their or their flanking sequence beyond the 7 bp mentioned above. The introns are almost equidistant from the stop codon (122 bp in *D.melanogaster* and 130 bp in *D.pseudoobscura*) but the intron within *D.melanogaster* is larger at 89 bp than that of *D.pseudoobscura* (53 bp).

The poorly conserved 3' UTR sequence does not rule out the possibility that splicing is regulated in both species by the same machinery, so both the

splicing level and *per* level were assayed in *D.pseudoobscura* around the clock as shown in figures 6.5 and 6.6.

```
D.pseudoobscura 3'UTR: 363 (53 bp Intron)
D.melanogaster 3'UTR: 507 (89 bp Intron)

 Percent Similarity: 49.587   Percent Identity: 49.587
```

Figure 6.4: GAP alignment of 3' UTR Sequences from *D.melanogaster* and *D.pseudoobscura* *D.pseudoobscura* and *D.melanogaster* 3' UTR sequences were aligned using GAP (GCG)

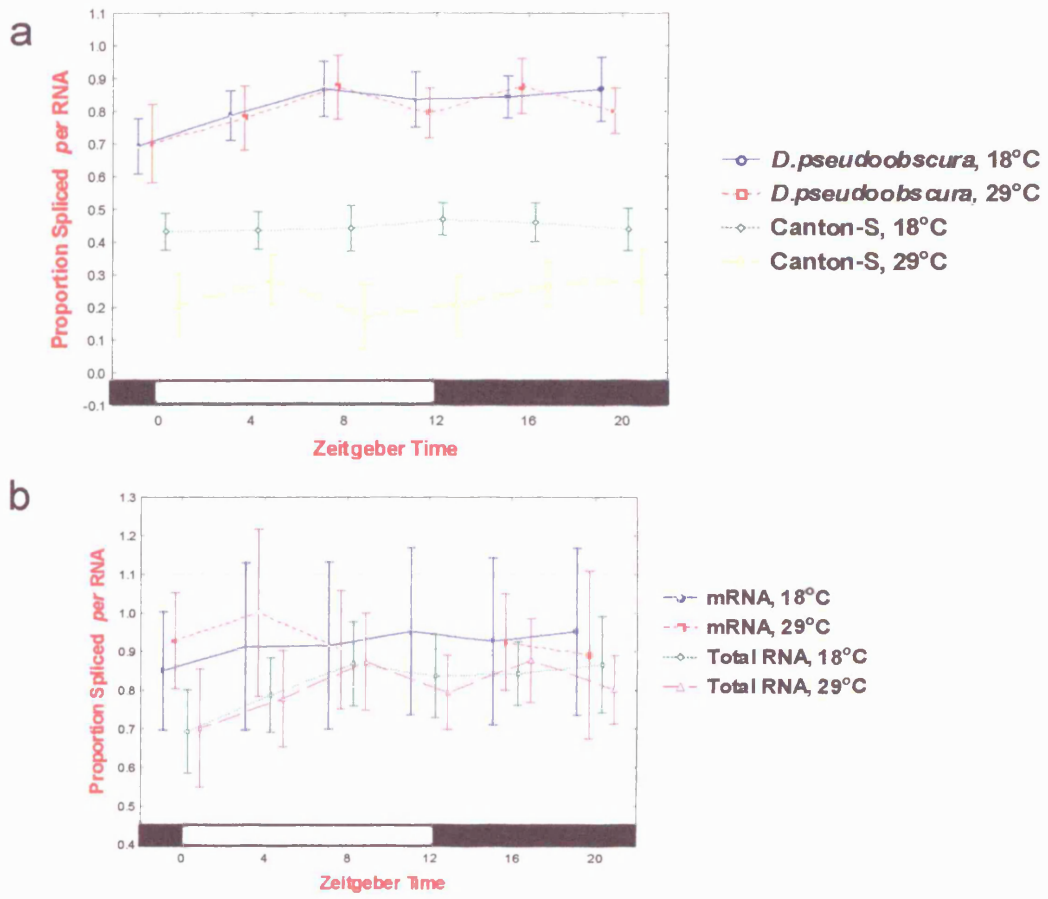


Figure 6.5: *per* mRNA splicing in *D.pseudoobscura*. a). *D.pseudoobscura* at 18°C (blue), 29°C (red), *D.melanogaster* Canton-S at 18°C (green) and 29°C (yellow). Error bars represent 95% confidence intervals. b). splicing levels of *per* poly-A selected mRNA at 18°C (blue) and 29°C (red) and total mRNA (green/purple) in *D.pseudoobscura*

In *D.pseudoobscura,* unlike *D.melanogaster,* there is no significant difference in splicing levels at 18 and 29°C (A 6.5.1; F=0.11, n.s.). There is also no effect of time on the splicing level of *D.pseudoobscura* (F=2.05, n.s.), figure 6.5a. The level of splicing in *D.pseudoobscura* is significantly higher than in *D.melanogaster* at both 18 and 29°C (A 6.5.2; F=776, p<<<0.001; figure 6.5a), with ~80% of all transcripts being spliced in *D.pseudoobscura*.

To confirm that there was no regulation of splicing by temperature in *D.pseudoobscura,* the level of splicing in polyA- selected RNA was also assayed. In *D.melanogaster,* splicing is thought to stimulate polyadenylation which then produce an earlier upswing in PER protein levels at low temperatures (Majercak et al., 1999). This means that a difference in splicing levels could conceivably become manifest at the polyadenylated mRNA level. In *D.pseudoobscura,* there is no difference in the splicing level in polyadenylated RNA at either temperature, and the level of splicing is identical to that seen in total RNA (figure 6.5b).

Therefore, temperature seems to play no role in regulating the splicing of the *per* 3' UTR intron in *D.pseudoobscura.*

6.4.4 *per* RNA cycling in *D.pseudoobscura*

Although the positioning of the evening locomotor activity peak at different temperatures is absolutely dependent on the ability to splice the *per* 3' UTR intron in *D.melanogaster,* temperature also alters the cycling of *per* RNA, with an earlier and higher peak at low temperatures (Majercak et al., 1999). Therefore, the differences in behaviour at different temperatures in *D.pseudoobscura,* and the differences in behaviour between species may be a result of altered RNA cycles. The cycling of *period* RNA in *D.melanogaster* and *D.pseudoobscura* is shown in figure 6.6.

In *D.melanogaster, per* RNA levels peak at ~ZT12 at 18°C and ~ZT16 at 29°C (A 6.6.1; time x temperature, $F=9.2$, $p<<0.001$) and the level of *per* mRNA is higher at 18 than 29°C ($F=9.2$, $p<0.05$), (figure 6.6a). This is the same result as previously reported (Majercak et al., 1999).

In *D.pseudoobscura* at 18°C the level of *per* mRNA begins to rise around ZT 8, peaking around ZT 12-16, and falls after ZT20. At 29°C, the RNA peak is much narrower and peaks later, at ZT 16 (A 6.6.2; time x temperature, $F=2.6$,

p<0.05). Again, like *D.melanogaster,* the level of *per* mRNA is higher at 18 than 29°C (F=14.05, p<0.05) (figure 6.6b)

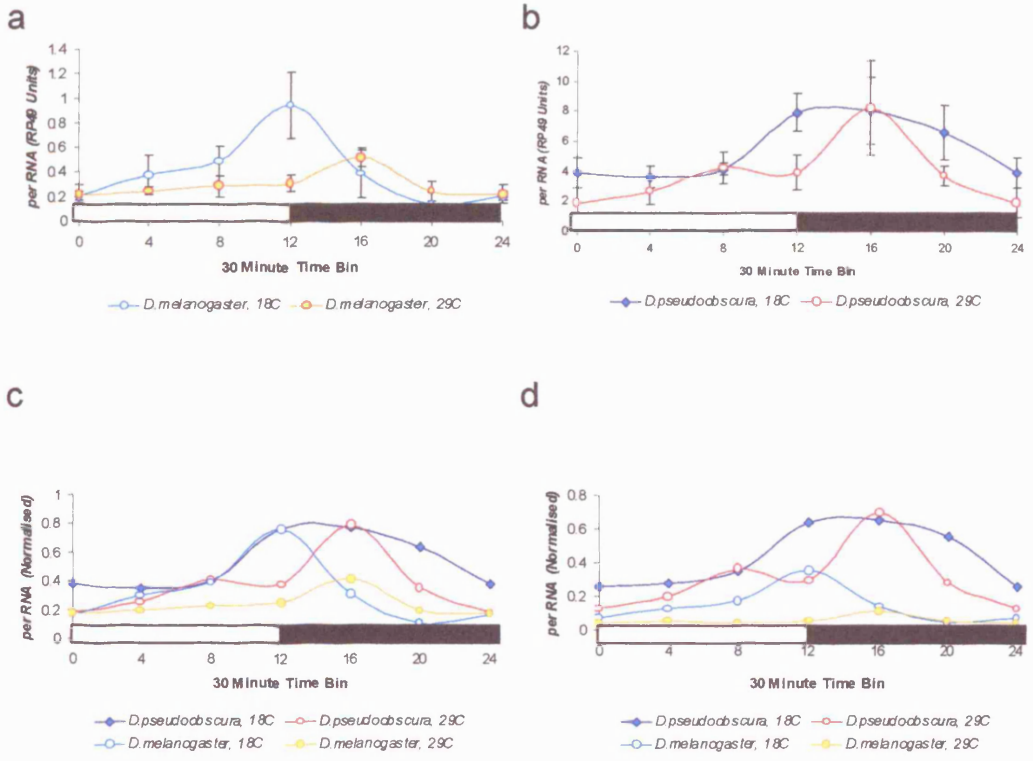


Figure 6.6: *per* mRNA cycles in *D.pseudoobscura* and *D.melanogaster*

a). *D.melanogaster per* at 18°C (light blue) and 29°C (orange). b). *D.pseudoobscura* at 18°C (dark blue) and 29°C (red). Error bars represent standard deviation. c). The levels of *per* RNA are relative to RP49 so in order to compare the cycles of *per* in *D.melanogaster* and *D.pseudoobscura,* the levels of *per* were normalised with the highest value seen for each species set to 1. d) normalised *per* RNA levels multiplied by the % of *per* splicing at each time point.

The cycles between the two species differ at 18°C (A 6.6.3; genotype x time, F=5.0674, p<0.05) with the level of *per* mRNA rises at the same time in both species, but peak levels of *per* RNA persist for longer in *D.pseudoobscura* (figure 6.6c). At 29°C the RNA peak occurs at the same time in both species, at ZT 16 (A 6.6.4; genotype x time; F=0.08, n.s.). Because different sets of

primers are used in each species, it is not possible to directly compare the overall levels of *per* RNA.

If the splicing levels are then superimposed onto the *per* levels as shown in figure 6.6d, the difference in RNA cycles between the species is exaggerated. In *D.melanogaster* there is much more spliced RNA at low than high temperatures, driving the earlier upswing in PER levels and phase of locomotor activity. In *D.pseudoobscura,* the only difference remains the longer time peak levels of *per* RNA are around at low temperatures.

6.4.5 *D.pseudoobscura* PER cycles

If *per* RNA levels are controlling the pattern of locomotor activity, then they must do so by altering the pattern of PER expression. *D.melanogaster per* protein and RNA cycle in phase with each other, with the cycle of RNA leading that of the protein by ~3-4 h (So and Rosbash, 1997). Therefore, PER levels in *D.pseudoobscura* would be expected to show a broad peak of expression at 18°C, lagging the RNA cycle, and a pattern of expression similar to *D.melanogaster* at 29°C. Previously no cycle in PER protein levels in *D.pseudoobscura* PER at 25°C was found (Hennessy 1999) but it is possible that the protein cycles only at low temperatures as the *D.pseudoobscura* clock seems to lose function at high temperatures. Western blots of PER protein in the heads of *D.pseudoobscura* at 18 and 29°C were analysed, as shown in figure 6.7.

Although there appeared to be a shallow protein cycle on some individual blots, overall there is no cycle in PER protein levels at either temperature, and the level of PER is the same at 18 and 29°C (A6.7.1; F=4, n.s.). As previous studies also failed to find a cycle in PER protein levels, it is likely that PER does not cycle in whole head extracts, which is surprising given the cycle of *per* RNA levels.

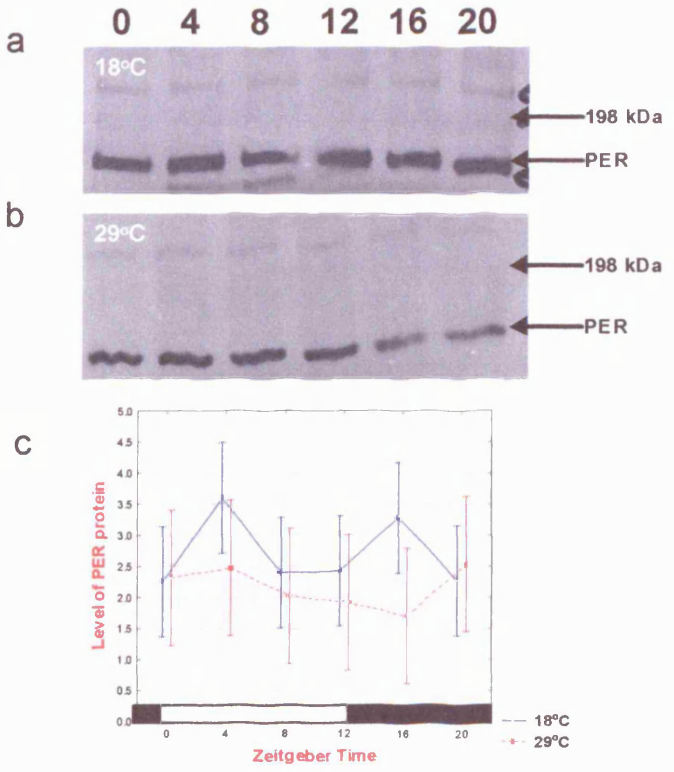


Figure 6.7: *D.pseudoobscura* PER at 18 and 29°C. a) typical 18°C western blot. b) 29°C western blot. c). Average levels of PER of three blots at each temperature. Error bars represent 95% confidence level.

6.4.6 Locomotor activity of the *mps3* transformant

Comparisons of the responses of *D.melanogaster* and *D.pseudoobscura* to altered temperatures revealed some interesting differences between the species. In order to further understand the differences in behaviour and the relationship to *per* RNA cycling, the behaviour and splicing of the *mps3* construct (Piexoto et al., 1999; Henessey, 1999) was examined at 18 and 29°C.

The temperature compensation of the *mps3* transformant is shown in figure 6.8.

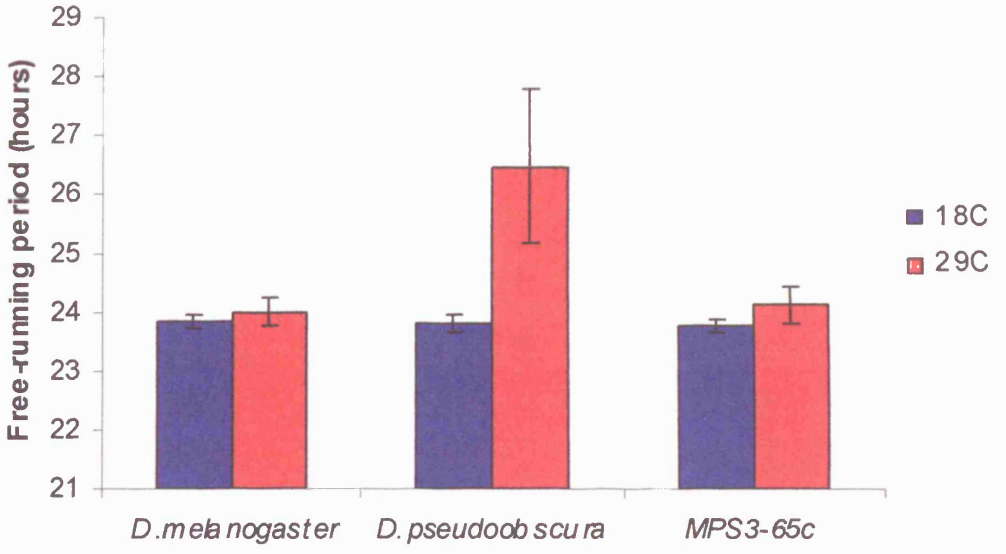


Figure 6.8: Temperature compensation in *D.melanogaster*, *D.pseudoobscura* and *per*$^{-01}$; *mps3*. at 18°C (blue) and 29°C (red). Error bars represent SEM. Free-running period was determined by spectral analysis.

The free running period of *mps3* is more similar to that of *D.melanogaster* than *D.pseudoobscura* at both 18 and 29°C. This indicates that *mps3* shows the temperature compensation of *D.melanogaster* over this range of temperatures.

The locomotor activity profiles of the *mps3* transformants are shown in figure 6.9.

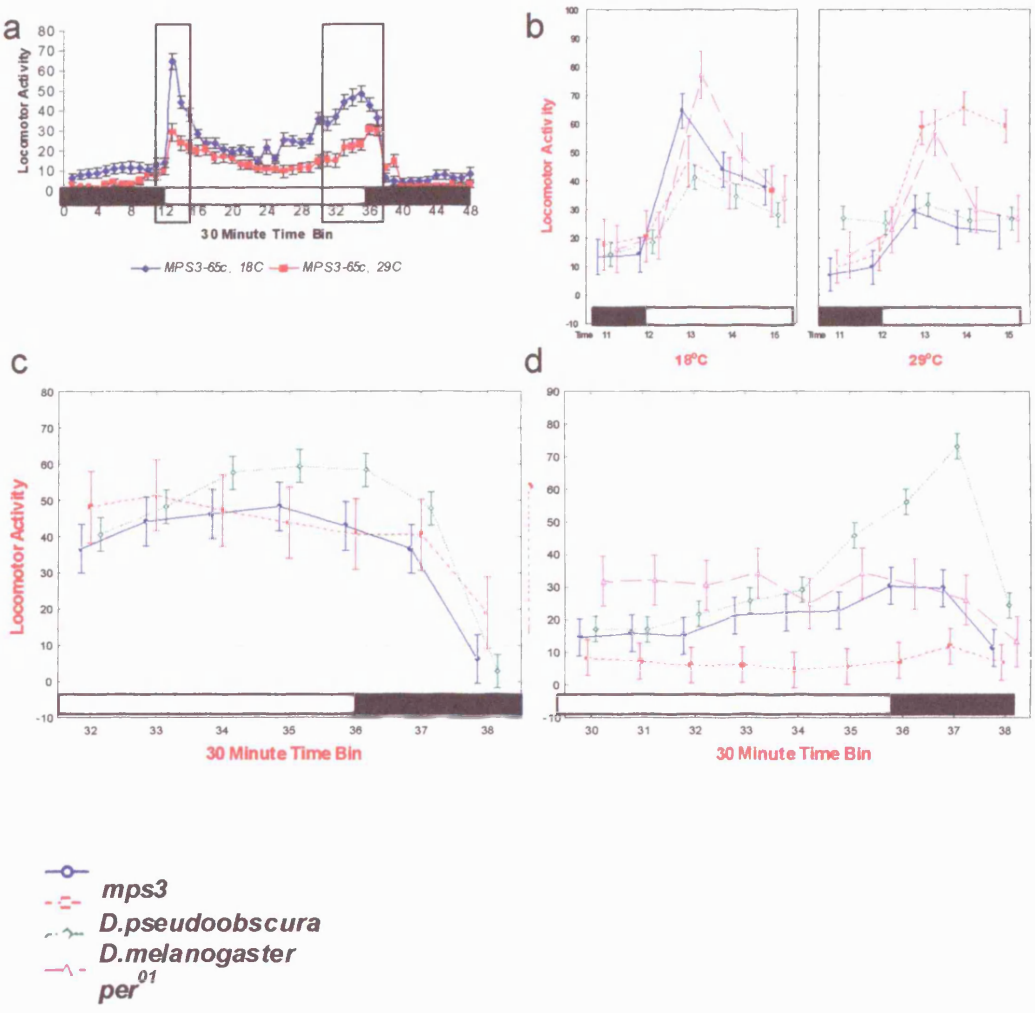


Figure 6.9: Locomotor activity in *per*^{01}; *mps3* transformants a). 18°C (blue) and 29°C (red). Error bars are SEM. b). Expanded morning activity peaks of *per*^{01}; *mps3* (blue), *D.pseudoobscura* (red), *D.melanogaster* (green) and *per*^{01} (purple). c) expanded 18°C evening peak of *per*^{01}; *mps3*, *D.pseudoobscura* and *D.melanogaster*. d). expanded 29°C evening peaks of *per*^{01}; *mps3*, *D.pseudoobscura*, *D.melanogaster* and *per*^{01}. Error bars are 95% confidence intervals.

The locomotor activity of *mps3* transformants is bimodal, with clear differences in behaviour at different temperatures, as can be seen in figure 6.9a (A 6.9.1; temperature x time; $F=5.1$, $p<0.01$). There is a larger morning activity peak at 18 than 29°C (A 6.9.2; $F=67.2$, $p<<<0.001$). The evening activity peak occurs earlier at 18 than 29°C (A 6.9.3; temp. x time, $F=4.5$, $p<<0.001$).

In LD, the locomotor activity profiles of *mps3* transformants show some aspects of *D.melanogaster* behaviour, and some of *D.pseudoobscura* behaviour. At 18°C, the morning activity peak is most similar to per^{01} (A 6.9.7; time x genotype, $F=1.34$, n.s.). At 29°C, the morning activity peak is reduced in comparison to per^{01} (A 6.9.8; $F=3.3$, $p<0.05$), but is also significantly different from wild-type *D.melanogaster* (A 6.9.9, $F=5.1$, $p<0.001$; figure 6.9b) suggesting that the *mps3* transgene cannot rescue the morning activity peak.

If the time bins 30-38 are taken to contain the 'evening activity peak' as is the case for most of the work reported here, then *mps3* behaviour is statistically different from both *D.melanogaster* (A 6.9.10; $F=2.5$, $p<0.05$) and *D.pseudoobscura* (A 6.9.11; $F=2.5$, $p<0.05$). Previously it has been shown that *mps3* evening peak activity resembles that of *D.pseudoobscura* rather than *D.melanogaster* in LD. This is because *D.melanogaster* locomotor activity rises until lights off, by which time the locomotor activity of both *D.pseudoobscura* and *D.melanogaster* has begun to fall (Henessey, 1999). If the time bins around the transition from lights to dark (35-38) are analysed separately, then *mps3* locomotor activity more closely resembles that of *D.pseudoobscura* than *D.melanogaster* (genotype x time; A 6.9.14; $F=1.8$, n.s.; A 6.9.15; $F=4.0$, $p<0.05$). This confirms that, to a certain extent, *mps3* behaviour is *D.pseudoobscura*-like in LD cycles (figure 6.9c).

The locomotor activity profile of *mps3* at 29°C differs from *D.pseudoobscura* as it shows an anticipatory evening activity peak (compare figures 6.2a and 6.9a). The level of evening activity of *mps3* is not as high as is seen in

D.melanogaster, and peak activity occurs earlier (A 6.9.12; genotype x time; F=15.2, p<<<0.001). There is no interaction between genotype and time in a comparison between *mps3* and *D.pseudoobscura* (A 6.9.13; F=1.76, n.s.). However, the level of locomotor activity in *mps3* is much higher than that of *D.pseudoobscura* (F=144.2, p<<<0.001). Therefore at 29°C, locomotor activity does not closely resemble either parent species, but is most clearly different from *D.pseudoobscura* where there was no well-defined evening activity peak.

Interestingly, locomotor activity of the *mps3* transgenic line resembles that of *D.melanogaster* rather than *D.pseudoobscura* in DD (Henessey, 1999) as shown in figure 6.10.

In DD, the locomotor activity profiles of *mps3* transformant flies show an early peak of activity at 18°C and a delayed peak at 29° (figure 6.10a) which is similar to the situation seen in wild-type Canton-S flies (figure 6.10c), and the peak at 18°C occurs earlier than the corresponding peak of activity in *D.pseudoobscura* (figure 6.10b) as has previously been reported (Tauber et al., 2003).

The behaviour of the *mps3* transformant differs depending on whether there is a LD cycle or not; in LD at 18°C *mps3* behaves like *D.pseudoobscura,* whereas in DD *mps3* behaves like *D.melanogaster.*

As the regulation of splicing responds to photoperiod, all the remaining work reported here was done in LD cycles, where the *mps3* fly shows *D.pseudoobscura* behaviour at 18°C, and an intermediate behavioural phenotype at 29°C.

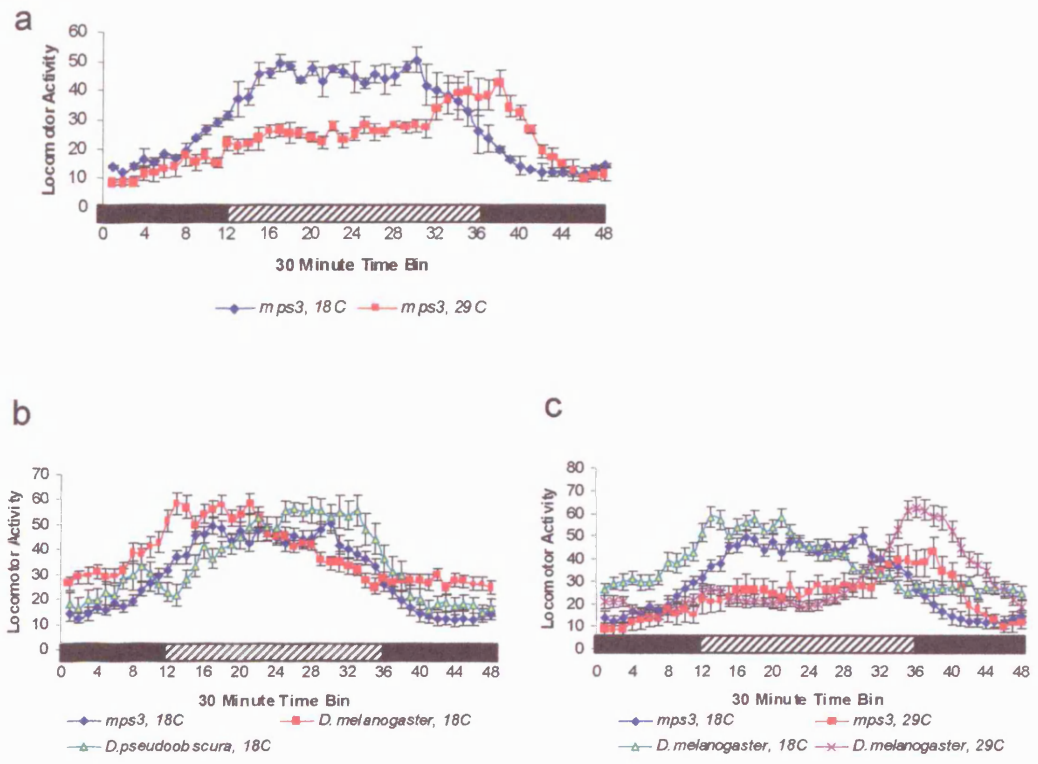


Figure 6.10: Locomotor activity of *mps3* transformants in DD a). 18°C (blue) and 29°C (red). b). 18°C *mps3* (blue), *D.melanogaster* (red) and *D.pseudoobscura* (green). c). same genotypes at 29°C. all error bars represent SEM

The *mps3* transformant shows an earlier evening peak at 18 than 29°C, as controlled by the alternative splicing of the intron within the 3' UTR of *per* in *D.melanogaster.* In this construct, the 3' UTR sequence comes from *D.pseudoobscura,* which raises the possibility that the splicing of the 3' UTR intron from *D.pseudoobscura per* can be regulated by the splicing machinery of *D.melanogaster* in a temperature dependent manner despite the lack of splicing regulation in *D.pseudoobscura.* If this is the case, then a lower level of splicing would be expected at high temperatures in *mps3* transformants. The splicing profile of *mps3* flies is shown in figure 6.11.

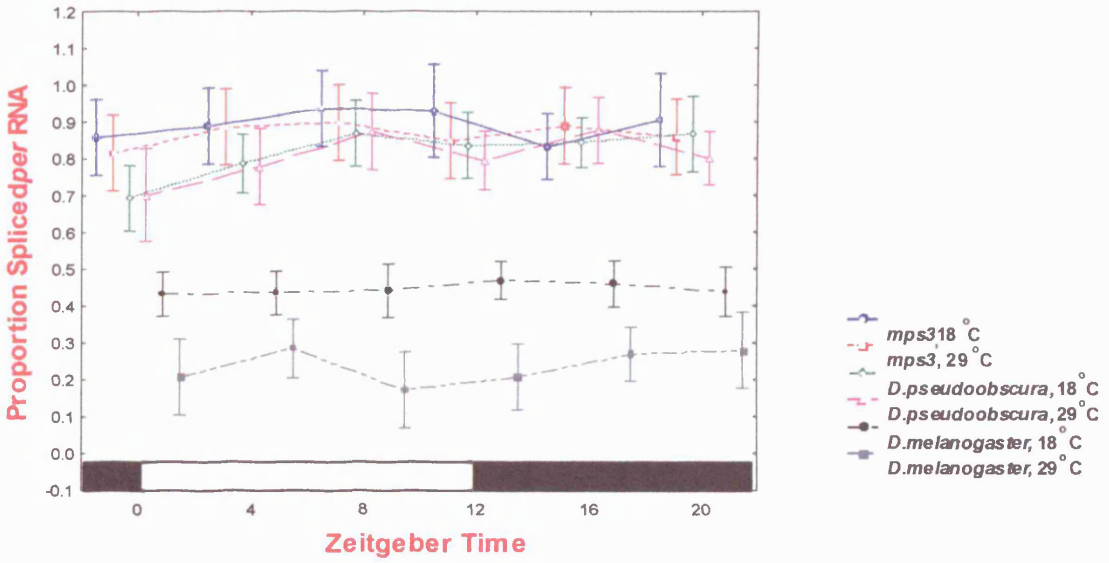


Figure 6.11: *mps3 per* RNA splicing levels. *mps3* (blue/red), *D.pseudoobscura* (green/purple) and *D.melanogaster* (black/grey) at 18°C/29°C. Error bars represent 95% confidence intervals

As can be seen in figure 6.11, there is no difference in the *per* RNA splicing level at different temperatures in *mps3* transformant flies (A 6.11.1; F=0.47, n.s.). Despite the lack of difference in splicing levels in *mps3,* the evening activity peak of these transformants occurs earlier at 18 than 29°C (figure 6.9). The level of splicing of *mps3* is similar, but not identical to that of *D.pseudoobscura* (A 6.11.2; F=7.6, $p<0.05$; genotype x temp. x time, F=0.079, n.s.). This level is far higher than that seen in wild-type *D.melanogaster* but activity is not greatly advanced in *mps3* compared to wild-type *D.melanogaster* (figure 6.9).

In *mps3* there is a difference in the position of the evening activity peak, but no corresponding difference in *per* splicing levels

If splicing levels are not responsible for the change in evening peak position at different temperatures in *mps3*, then it is likely that this is due to differences in the RNA cycle. To see if this is the case, the level of *per* RNA in *mps3* flies was measured around the clock and the cycle compared to *D.melanogaster* and *D.pseudoobscura* (figure 6.12).

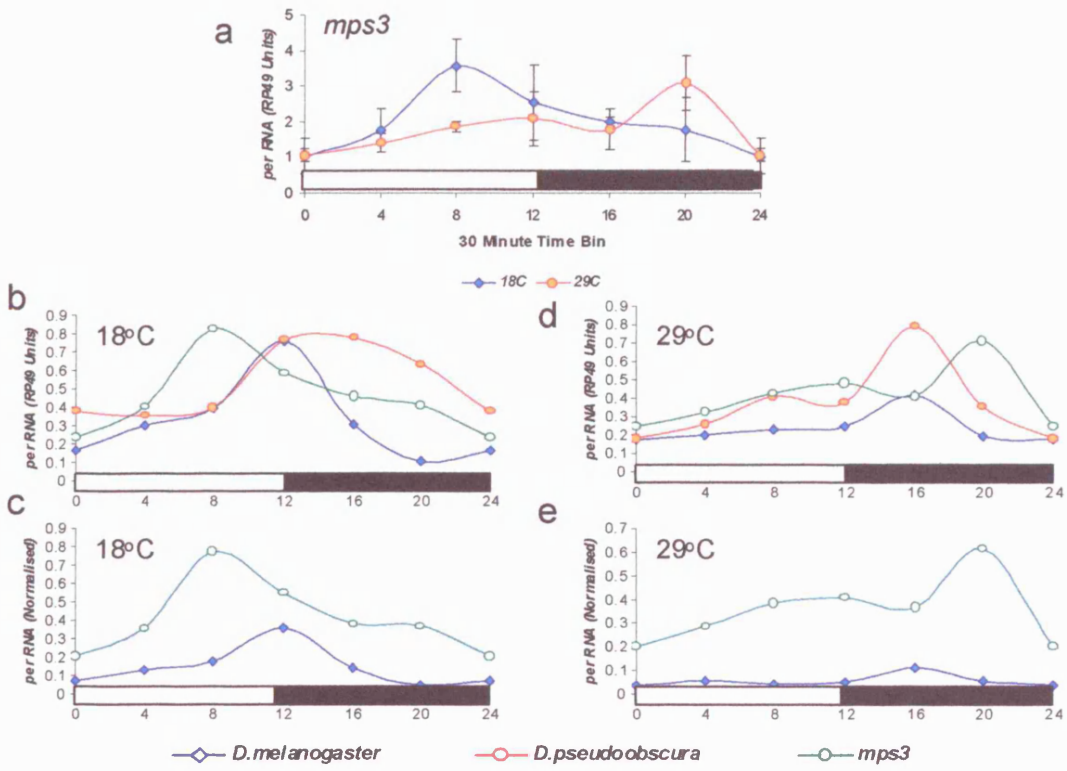


Figure 6.12: RNA cycles in *per*^{01}; *mps3* transformant, *D.melanogaster* and *D.pseudoobscura* a). *per*^{01}; *mps3* at 18°C (blue) and 29°C (orange). Error bars show SEM b). *per* levels in *per*^{01}; *mps3* (green), *D.pseudoobscura* (orange) and *D.melanogaster* (blue) at 18°C. c). at 29°C d). levels of spliced *per* mRNA in *per*^{01}; *mps3* and *D.melanogaster* at 18°C. e). at 29°C. In 6.12 b-e, the highest level of *per* for each genotype has been normalised to 1.

The timing of the peak level of *per* RNA in the *mps3* transformant occurs at ZT8 at 18°C and ZT 20 at 29°C (figure 6.12a). At 18°C, the *per* RNA peak is advanced in comparison to both *D.melanogaster* and *D.pseudoobscura* (figure 6.12b) and at 29°C, the peak is delayed in comparison to the two parent lines (figure 6.12c). Assuming that the RNA cycle controls evening peak position, this difference in the positioning of peak *per* RNA levels can account for the earlier evening activity peak seen at 18°C in *mps3* flies in the absence of regulated *per* RNA splicing.

By superimposing the *per* splicing levels onto the RNA cycle at 18°C (figure 6.12c) and 29°C (figure 6.12d) it can be seen that this exaggerates the difference in *per* levels between wild-type and *mps3* transformant lines. Although the level of *per* peaks earlier in wild-type *D.melanogaster* than in *mps3* flies at 29°C, the evening activity peak occurs around the same time in both lines, so presumably the elevated level of splicing of *mps3 per* compensates for the delayed evening activity peak.

Differences in the *per* RNA cycle of *mps3* transformants seem to correspond to changes in evening activity peak position.

6.5 Discussion

6.5.1 *D.pseudoobscura* locomotor activity

The comparison of behaviour of *D.pseudoobscura* and *D.melanogaster* at different temperatures suggests that *D.pseudoobscura* is better adapted to cold climates, whereas *D.melanogaster* is better suited to the warm. At low temperatures, high numbers of *D.pseudoobscura* flies remain rhythmic in DD while fewer *D.melanogaster* are. The opposite is true at 29°C, where only ~60% of *D.pseudoobscura* are rhythmic, as opposed to ~95% of *D.melanogaster* (Henessey, 1999). Over the 18 - 29°C temperature range, *D.melanogaster* are well temperature compensated, with a period close to 24 h at both temperatures. Under the same conditions, *D.pseudoobscura* has a 24 h period at 18°C, but a 26 h period at 29°C (Henessey, 1999 and figure 6.2).

The locomotor activity profile of *D.pseudoobscura* in LD at 29°C also reflects its reduced capacity to cope with elevated temperatures compared to *D.melanogaster.* At 18°C, *D.pseudoobscura* locomotor activity, like that of *D.melanogaster,* is bimodal with a clearly defined evening activity peak. The evening activity peak of *D.pseudoobscura* is earlier and broader than the corresponding peak of *D.melanogaster* in LD at 18°C. The difference

between the two species becomes more apparent at 29°C. At this temperature, *D.melanogaster* locomotor activity is bimodal, with the evening activity peak shifted later into the night. In contrast, in *D.pseudoobscura* there is almost no evening activity peak and locomotor activity is concentrated around the morning activity peak (figure 6.3).

In order to avoid being active at mid-day at high temperatures, *D.melanogaster* concentrates most of its activity into a late evening peak. One possibility is that *D.pseudoobscura* avoids mid-day activity by concentrating most activity immediately after dawn, so that it is almost inactive by mid-day. This would then account for the large morning activity peak. This is unlikely for a number of reasons. In comparison to *D.melanogaster,* the locomotor activity of *D.pseudoobscura* at 'mid-day' (ZT 6, time bin 24) at 29°C is relatively high in comparison to that of *D.melanogaster* (figure 6.2b). The morning activity peak of *D.melanogaster* is present in per^{01} and tim^{01} mutants, indicating that it is not primarily under clock control (see Chapter 3) making it less likely that the distribution of locomotor behaviour is controlled in this way.

The role of the clock seems to be to attenuate the size of the startle effect (see Chapters 3 and 4). The morning peak at 18°C in *D.pseudoobscura* is the same size as that of *D.melanogaster* but at 29°C it is considerably higher, approaching the levels seen in per^{01} flies (figure 6.2c). This could therefore indicate that the clock is not functioning to repress the startle effect in *D.pseudoobscura* at high temperatures. The lack of temperature compensation and reduced levels of rhythmicity at 29°C support the idea that the *D.pseudoobscura* clock is less functional at high temperatures. This makes it less likely that the distribution of locomotor activity in *D.pseudoobscura* at 29°C is an adaptive response.

As aspects of behaviour which are known to involve clock regulation in *D.melanogaster* are impaired at high temperatures in *D.pseudoobscura,* it seems most likely that the *D.pseudoobscura* clock is unable to properly entrain behaviour to LD cycles at high temperatures. As a result

environmental cues, such as dawn, rather than the clock dictate when peak activity occurs.

The distribution of *D.melanogaster* locomotor activity is determined by the *per* RNA cycle (Qui and Hardin, 1996). This is altered in response to different temperatures, leading to changes in the pattern of behaviour. As *D.pseudoobscura* and *D.melanogaster* show different patterns of activity, they might also be expected to have different *per* RNA cycles.

6.5.2 D.pseudoobscura per RNA

In *D.melanogaster* at low temperatures the abundance of *per* RNA is increased and the timing of the *per* RNA peak advanced. Further modifications of *per* mRNA then 'fine-tune' locomotor activity to different seasonal conditions (Majercak et al., 1999). In *D.melanogaster,* low temperatures lift the repression of the splicing of this intron, which advances the rise in PER protein, producing an earlier evening activity peak. The evening activity peak of *D.pseudoobscura* occurs earlier than that of *D.melanogaster* at 18°C. As a result, *D.pseudoobscura* would be predicted to have a higher level of splicing of this intron and/or an advanced cycle of PER protein at low temperatures.

The level of splicing in *D.pseudoobscura* is elevated above the level of splicing seen in *D.melanogaster* with ~80% of all *per* transcripts spliced at both 18 and 29°C. However, *D.pseudoobscura* does not regulate the splicing of this intron in a temperature dependent manner, because there is no difference in the splicing levels at 18 and 29°C (figure 6.5). As there is no clear evening activity peak at 29°C in *D.pseudoobscura* it is not possible to determine whether this splicing event is related to the evening peak position in this species. The higher level of splicing of this intron at 18°C compared to *D.melanogaster* could account for the earlier phase of activity of *D.pseudoobscura* at low temperatures.

The splicing of this intron modifies the *per* RNA cycle of *D.melanogaster* in response to altered photoperiods and temperatures. Therefore a comparison of the *per* RNA cycles at 18 and 29°C with the locomotor activity profiles of both species should indicate the role of the *per* RNA cycle in determining species-specific patterns of locomotor activity.

In *D.melanogaster* at 18°C the *per* RNA cycle peaks ~ZT 12, and at 29°C ~ZT 16, and there is more *per* RNA at 18 than 29°C; figure 6.6. This confirms the reported cycles of Majercak et al. (1999). In *D.pseudoobscura,* the RNA cycle peaks from ~ZT 12-ZT 20 at 18°C and ZT 16 at 29°C. At both 18°C and 29°C, the RNA cycle peaks at around the same time in both species but In *D.pseudoobscura,* unlike *D.melanogaster,* the peak level of *per* RNA is the same at both 18 and 29°C. The peak level of *per* is maintained for longer in *D.pseudoobscura* than *D.melanogaster* at 18°C. The broader *per* RNA peak of *D.pseudoobscura* could be due to increased RNA stability, but if this were the case, a broader peak might also be expected at 29°C. Therefore it is more likely that *per* RNA transcription continues for longer at low temperatures in *D.pseudoobscura*. At 29°C, the two RNA peaks occur at the same time.

If the levels of splicing are then superimposed onto the *per* RNA cycles, the differences in *per* levels between the two temperatures are exaggerated in *D.melanogaster* but not *D.pseudoobscura* (compare figure 6.6c and 6.6d). Because of the limitations of RT-PCR, the *per* RNA levels in each different species are not directly comparable. If it is assumed that the levels of *per* transcription of the two species are relatively similar, then as the level of splicing is so much higher in *D.pseudoobscura,* a high level of *per* RNA will be reached earlier in *D.pseudoobscura.* This should then produce an earlier phase of PER protein and explain why *D.pseudoobscura* locomotor activity peaks earlier than *D.melanogaster* at 18°C. The extended period of *per* transcription in *D.pseudoobscura* at 18°C may also be involved in generating this earlier phase of activity and/or the broader evening activity peak.

If the distribution of locomotor activity into a large morning peak in *D.pseudoobscura* at 29°C were a clock controlled response to high temperatures, then it would be predicted that it would be controlled by the *per* RNA cycle. If this were the case then a higher level of splicing and/or an earlier peak in RNA levels would be predicted at high temperatures. Neither of these is true - the level of splicing is the same at both 18 and 29°C and the peak level of RNA occurs earlier at 18°C. Therefore the distribution of locomotor activity at 29°C does not seem to be controlled by *per* RNA levels.

6.5.3 PER protein cycles

If the RNA cycle is driving locomotor activity, then it must do so by altering the expression of PER protein. Western blots of PER protein from *D.pseudoobscura* reveal no protein cycle at either 18 or 29°C, as previously reported (Hennessey 1999), and the level of PER seems to be the same at both temperatures (figure 6.7). This is a surprise given the clear *per* RNA cycle of *D.pseudoobscura.*

The situation in *D.pseudoobscura* could be analogous to that of the *D.melanogaster* cry^b mutant, where PER and TIM do not cycle in the head. In the cry^b mutant, cycling of PER and TIM only within the small lateral neurons (LN_Vs) is sufficient to drive rhythmic behaviour (Stanewsky et al., 1998). Alternatively it may be that the cycle in RNA levels leads to a cycle of PER phosphorylation states. To distinguish between these possibilities, immunocytochemistry could be used to look at the LN_Vs, and altered phosphorylation states by 2D protein gel electrophoresis.

A similar result has been obtained for *Musca domestica*, where PER does not seem to cycle in whole head extracts (Piccin, 1998). As the location of the pacemaker neurons is not known in *M.domestica*, it has not been possible to see if the levels of PER cycle within these structures (V. Codd, pers. com.). The role of PER in the clock also differs in the silk moth *Antheraea Pernyi,* where PER does not enter the nucleus (Sauman &

Reppert, 1996), suggesting that the cycle of PER expression is not necessarily vital to generate circadian rhythmicity.

6.5.4 *mps3* locomotor activity

The evidence from the comparative analysis of *D.melanogaster* and *D.pseudoobscura* indicates that the *per* RNA cycle may account for some species-specific behavioural differences. In *D.melanogaster,* the modification of the *per* RNA cycle in response to different temperatures determines the position of the evening activity peak. As both the *per* RNA and evening activity peaks of *D.pseudoobscura* are broader than those of *D.melanogaster* at 18°C, it is possible that an altered *per* RNA cycle is responsible for the altered behaviour. Species-specific differences in locomotor activity have previously been demonstrated to be specified by the *per* gene as *D.melanogaster* per^{01} flies transformed with *D.pseudoobscura per* show *D.pseudoobscura* type behaviour (Petersen et al., 1998).

To help elucidate the link between RNA cycle and locomotor activity, both features of the *mps3* transformant were examined at 18 and 29°C. *mps3* transformant flies carry the N-terminal coding sequence of *D.melanogaster* PER, the C-terminal sequence and 3' UTR of *D.pseudoobscura,* so can also help understand the species-specificity of alternative splicing.

Unlike *D.pseudoobscura, mps3* is well temperature compensated at both 18 and 29°C (figure 6.8). This is probably because *D.melanogaster* is better temperature compensated than *D.pseudoobscura* over this temperature range. All the genes involved in temperature compensation with the exception of *per* come from *D.melanogaster* in the *mps3* transformant. Additionally, the PAS domain of the *mps3* transgene comes from *D.melanogaster,* so interactions with other proteins are likely to be relatively normal, and the transformant retains *D.melanogaster* temperature compensation. This is not the case for *mps1,* which contains the PAS domain from *D.pseudoobscura* and displays long free-running periods *mps1*

at both 18 and 29°C, suggesting that the period is altered if the PAS domain does not match the genetic background (Henessey, 1999).

The evening activity peak of the *mps3* transformant at 18°C in LD cycles has previously been shown to resemble *D.pseudoobscura* more closely than *D.melanogaster* (Henessey, 1999), as shown in figure 6.9. This indicates that the species-specificity of behaviour must be dependent on the C-terminal of the PER protein. In contrast, at 29°C the behaviour of these flies differs significantly from both *D.pseudoobscura* and *D.melanogaster*.

The pattern of locomotor activity of *mps3* at 29°C is bimodal, with a clear morning and evening activity peak in LD (figure 6.9). One interesting aspect of *mps3* behaviour is that the species specificity of the profile depends on whether the fly is in LD or DD conditions. In LD, *mps3* behaves somewhat like *D.pseudoobscura,* at least at 18°C, while in DD, *mps3* behaviour clearly resembles that of *D.melanogaster* (Henessey, 1999; and figures 6.9 - 6.10 above). So the C-terminal of the protein seems to determine the species specificity of behaviour in LD, while the N-terminal of the protein, determines species-specific behaviour in DD.

The most likely explanation is that the behaviour seen in LD may not truly be *D.pseudoobscura*-like, and therefore the behaviour seen in DD is a more accurate reflection of the species specificity of behaviour. The resemblance of *mps3* behaviour to *D.pseudoobscura* rather than *D.melanogaster* is only evident at 18°C in LD 12:12, and then only over few time bins. At this temperature, both *D.melanogaster* and *D.pseudoobscura* are bimodal (see figure 6.3), and the levels of activity are very similar between the two species. At 29°C in LD, the evening activity peak of *mps3* is intermediate between the parental strains, so the resemblance of behaviour to *D.pseudoobscura* breaks down at elevated temperatures.

6.5.5 *mps3 per* RNA

In *mps3* flies the evening activity peak occurs earlier at 18°C than at 29°C. As in *D.melanogaster* this is dependent on the alternative splicing of the intron within the 3' UTR of *per,* it would be predicted that the regulation of this splicing event would be 'normal' for *D.melanogaster* in the *mps3* transformant. This turns out not to be the case as there is no regulation of splicing in *mps3*, with a level of splicing identical to that of *D.pseudoobscura.* As the level of splicing of this intron is similar in *mps3* and *D.pseudoobscura* flies, the splicing is likely to be unregulated in *D.pseudoobscura* lines, with the level of splicing observed being the maximum possible.

This result helps explain how splicing is regulated in different species. Whatever represses splicing within *D.melanogaster* is unable to repress the splicing of the 3' UTR intron from *D.pseudoobscura.* The 3' UTR of the *mps3* transformant is from *D.pseudoobscura* and a comparison of the 3' UTRs of the two species revealed little conservation of sequence (figure 6.4). Therefore it is perhaps not surprising that the splicing of the intron was not regulated in the transformant, or it could be because per^{01} still produces an RNA transcript with the wild-type *D.melanogaster per* 3' UTR which could titrate out all of the splicing repressor.

This may also explain why the locomotor activity of *mps3* in DD resembles *D.melanogaster* but in LD resembles *D.pseudoobscura*. It could be that the *mps3* chimeric protein has problems transducing information about the LD regime to the negative feedback loop of the clock. In *mps3*, there is no regulation of the splicing of the *per* 3' UTR intron. In *D.melanogaster* this is under the control of the photoperiod and appears to be one way in which photoperiod determines the phase of activity. As the behaviour of *mps3* in LD resembles that of *D.pseudoobscura,* it suggests that there is some kind of 'default' pattern of behaviour which is shared between *D.melanogaster* and *D.pseudoobscura.* The regulation of splicing of the *per* intron then allows *D.melanogaster* to modify this pattern of behaviour. If it cannot splice this

intron, behaviour instead resembles that of *D.pseudoobscura* (or defective *D.melanogaster* behaviour). In DD, there is no LD cycle, so the requirement for PER to transmit light information to the clock is lost, and *mps3* PER functions as well as wild-type PER, producing a normal pattern of *D.melanogaster* behaviour.

The difference in evening peak position observed at different temperatures in *mps3* flies cannot be under the control of alternative splicing as there is none. Therefore, even though alternative splicing is responsible for delaying evening peak activity at high temperatures in *D.melanogaster,* altering the PER protein can have a similar effect. As the role of alternative splicing is to alter the *per* RNA cycle, the most parsimonious explanation is that the *per* RNA cycle is altered at different temperatures in *mps3* transformants.

As can be seen in figure 6.12, the *per* RNA cycle of *mps3* is altered in comparison to wild-type *D.melanogaster.* At 18°C, the RNA cycle of *mps3* peaks at ZT 8 (wild-type = ZT 12) and at 29°C, it peaks at ZT 20 (wild-type = ZT 16). Therefore the *per* RNA cycle is advanced at 18°C and delayed at 29°C in comparison to wild-type *D.melanogaster.* The advanced RNA cycle at 18°C and delayed RNA cycle at 29°C could then compensate for the lack of regulated splicing, and produce the different phases of activity at 18°C and 29°C.

The *mps3* transgene includes the promoter region from *D.melanogaster per* so it would be expected that the RNA cycle would resemble that of *D.melanogaster.* However, the cycle of *per* RNA is dependent on the negative autoregulatory feedback loop which includes the PER protein, and in this case a large proportion of the PER protein, enough to confer the activity of a different species, is from *D.pseudoobscura.* Although the cycle of *per* RNA does not resemble that of *D.pseudoobscura,* the altered interactions of this chimeric protein are presumably enough to advance the RNA cycle at 18°C and delay the RNA cycle at 29°C.

If this is the case, then it raises the question as to why *D.melanogaster* modifies the splicing of the *per* intron when altered *per* RNA cycles alone are enough to shift the evening peak position. This is most likely because altering the *per* RNA cycle is rather a crude response to altered temperatures. The regulation of alternative splicing allows the fine-tuning of the *per* RNA cycle to altered temperatures and photoperiods so that behaviour is optimised for any given conditions.

In order for the RNA cycle to alter the behaviour, it must also alter the cycle of PER protein. Within *mps3* flies, the cycle of PER protein was found to be very similar to that of *D.melanogaster* at 25°C with the level of PER rising around ZT12 (lights off) in both cases. However, PER seems to persist for longer during the night in the *mps3* transformant (compare So & Rosbash, 1997 with Peixoto et al., 1998). This may reflect increased stability of *D.pseudoobscura* PER protein and/or *per* RNA, as indicated by the *per* RNA cycle of *D.pseudoobscura.*

In the experiments reported here, RNA levels were examined at 18°C and 29°C. 25°C is in the middle of these two temperatures, and as a result the RNA peak at 25°C might be expected to lie between ZT8 and ZT 20, or ~ZT 14. At 25°C, *per* cycles in *D.melanogaster* peak at around ZT 12-14 (So & Rosbash), so it may be that at 25°C the *per* cycle of *mps3* appears normal. Although this data is encouraging, more work needs to be done to confirm the link between species specific patterns of RNA expression and species specific patterns of behaviour.

6.5.6 Conclusions

Regulation of splicing in different species

The work on *Bactrocera* species suggests that the mechanisms of temperature regulation of *per* RNA through alternative splicing is not unique to *D.melanogaster.* As there is no regulation of alternative splicing in

D.pseudoobscura, this is probably not a common mechanism for coping with temperature changes. This is supported by the analysis *per* transcripts identified by 3' RACE in *D.virilis.* The two isoforms of *per* transcript in *D.virilis* differ not by alternative splicing but by alternative polyadenylation (see Chapter 7). As both *D.pseudoobscura* and *D.virilis* are more closely related to *D.melanogaster* than the *Bactrocera* species it seems more likely that the regulation of splicing has evolved independently in these species. Once other components of the splicing regulatory machinery are uncovered, it should become clear whether this is the case.

The data reported here supports the hypothesis that *D.pseudoobscura* is cold adapted and *D.melanogaster* warm adapted. Over a range of 18 - 29°C, *D.melanogaster* is well temperature compensated, has a high percentage of rhythmicity and is bimodal in activity in LD cycles.

The clock of *D.melanogaster* is regulated by light and temperature, through the regulation of the *per* RNA cycle. Low temperatures produce an earlier *per* RNA peak and an elevated level of *per* transcription. This cycle is then modified by light and temperature through the repression of the splicing of the intron within the 3' UTR of the *per* transcript. In this way, *D.melanogaster* is able to fine-tune behaviour to a wide range of environmental conditions. The *mps3* construct demonstrates that it is the cycle of *per* RNA rather than the splicing which sets the evening peak position - the role of altered splicing is to modify the RNA cycle so that it then alters locomotor activity.

Over the same temperature range, *D.pseudoobscura* is poorly temperature compensated, and shows reduced levels of rhythmicity at high temperatures. In addition, at 29°C, the clock of *D.pseudoobscura* seems to have a reduced role in determining the pattern of locomotor activity which is concentrated into a large morning activity peak. *D.pseudoobscura* also modifies its *per* RNA cycle in response to altered temperatures. At 18°C the *per* RNA peak seems to be related to the pattern of locomotor activity. At 29°C, despite the altered *per* cycle, the clock does not appear to regulate behaviour. Therefore factors

other than *per* must prevent *D.pseudoobscura* from living happily at elevated temperatures. At low temperatures (12°C) *D.pseudoobscura* has a higher level of rhythmicity than *D.melanogaster,* so it may be worth repeating some of these experiments at this temperature.

The regulation of alternative splicing of *per* gives *D.melanogaster* an additional level of control over the *per* RNA cycle which *D.pseudoobscura* does not have. *D.melanogaster* uses the regulation of splicing to amplify the pre-existing difference in *per* RNA levels at different temperatures. Because *D.pseudoobscura* is unable to regulate the splicing of the *per* intron, it is less able to modify the *per* RNA cycle in response to altered temperature and photoperiod. *D.pseudoobscura* may possess an as yet undiscovered mechanism for responding to altered temperatures. On the other hand it may be that the reduced ability of the *D.pseudoobscura* clock to cope with elevated temperatures can at least partially be accounted for by its reduced ability to temperature regulate the *per* RNA cycle.

For *D.melanogaster,* it is assumed that the regulation of locomotor activity at high temperatures is more important than at low temperatures. In the heat it is essential to avoid activity in the mid-day sun, whereas in the clock, the regulation is less important. The regulation of splicing is a response to elevated temperatures, as splicing is repressed by heat and light. This is supported by the role of the clock in regulating *per* splicing at high but not low temperatures (Chapter 3). *D.pseudoobscura* does not posses this mechanism for coping with elevated temperatures. Indeed, locomotor activity in *D.pseudoobscura* appears to be largely unregulated by the clock at 29°C. As the lack of regulation of behaviour in response to elevated temperatures is likely to be bad news for survival in certain conditions, this again supports the hypothesis that *D.pseudoobscura* is a cold-adapted species.

Chapter seven: temperature regulation of *per* RNA in *D.virilis*

7.1 Introduction

The discovery of a mechanism by which *D.melanogaster* adjusts locomotor activity to different seasonal conditions (Majercak et al., 1999) raises the possibility that this system may also determine species specific patterns of locomotor activity. The splicing of this intron seems to be regulated by temperature in two *Bactrocera* species native to Australia, but as yet it has not been demonstrated that this plays a role in determination of behaviour in these species (An. et al., 2002). An examination of the splicing levels of *per* mRNA in *D.pseudoobscura* indicated that there is no regulation of splicing within this species, as reported in Chapter 6.

The comparison of the locomotor activity and *per* RNA cycles between *D.melanogaster, D.pseudoobscura* and the *mps3* transformant suggested that the cycle of *per* RNA determines the pattern of locomotor activity at different temperatures. The role of the regulation of the splicing of the intron in *D.melanogaster* is to alter the *per* RNA cycle, fine-tuning locomotor activity to different seasonal conditions (Chapter 6).

7.2 Aims

Drosophila Virilis is a species which is native to Northern Europe, and therefore in general inhabits colder climes than *D.melanogaster*. The 3' UTR of the *per* gene of *D.virilis* was characterised to see whether different transcripts exist *in vivo*, and *D.virilis* locomotor activity and *per* RNA cycles were examined to investigate how this species responds to changes in temperature.

7.3 Methods

Strains: *D.virilis* (from stock centre); *D.melanogaster* (Canton-S strain from Chapter 3)

D.virilis were entrained to LD 12:12, and collected every four hours.
3' RACE and locomotor activity experiments were carried out as described in Chapter 2.

Because the *per* transcripts differ not through the presence and absence of an intron, but through different polyadenylation signals, RT-PCR as used for *D.melanogaster* and *D.pseudoobscura* could not be used to measure the levels of the different transcripts. This is because the transcripts differ only by the sequence at one end, and therefore a single set of primers will amplify both transcripts. A one step RT-PCR approach is therefore unsuitable, and Real-time PCR was used instead, as described in Chapter 2.

7.4 Results

7.4.1 *D.virilis* locomotor activity

In order to determine how well *D.virilis* copes with different temperatures, the free running period of *D.virilis* was measured at 18 and 29°C, as shown in figure 7.1.

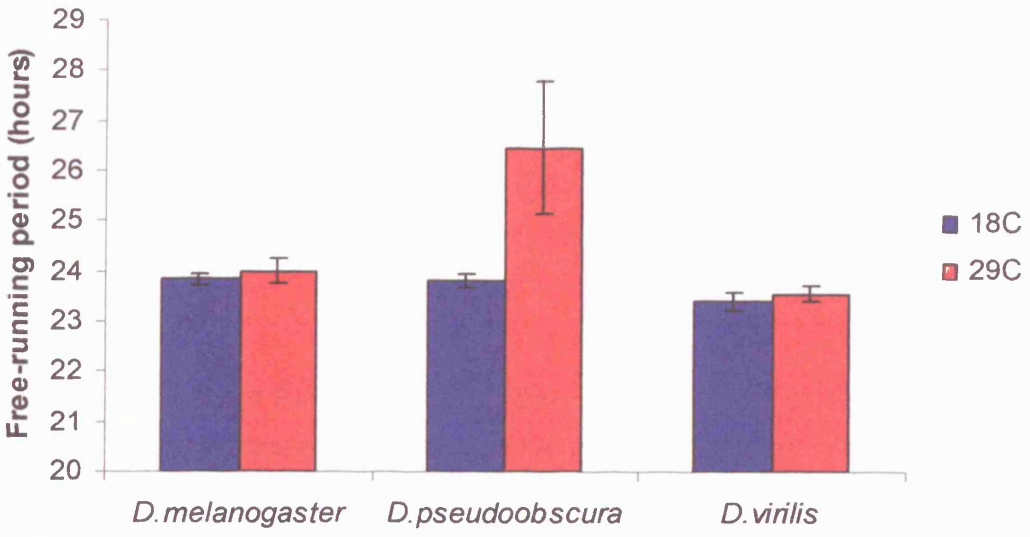


Figure 7.1 Temperature compensation of *D.virilis* at 18°C (blue) and 29°C (red) (*D.melanogaster* (Canton-S) and *D.pseudoobscura* taken from figure 6.2; error bars show SEM)

D.virilis is well temperature compensated over the temperature range of 18 - 29°C, as shown in figure 7.1. At 18°C, the period is 23.4+/-0.19 h and at 29°C, it rises slightly to 23.56+/-0.16 h. The period is therefore slightly shorter than the Canton-S strain shown in figure 7.1, but is comparable to the periods of some other *D.melanogaster* strains over the same temperature range (see Chapter 7 and Sawyer et al., 1997).

***D.virilis* is clearly better temperature compensated than *D.pseudoobscura* between 18 and 29°C.**

The analysis of the locomotor activity of *D.virilis* recorded in LD 12:12 and the seven subsequent days of DD at 18 and 29°C is shown in figure 7.2

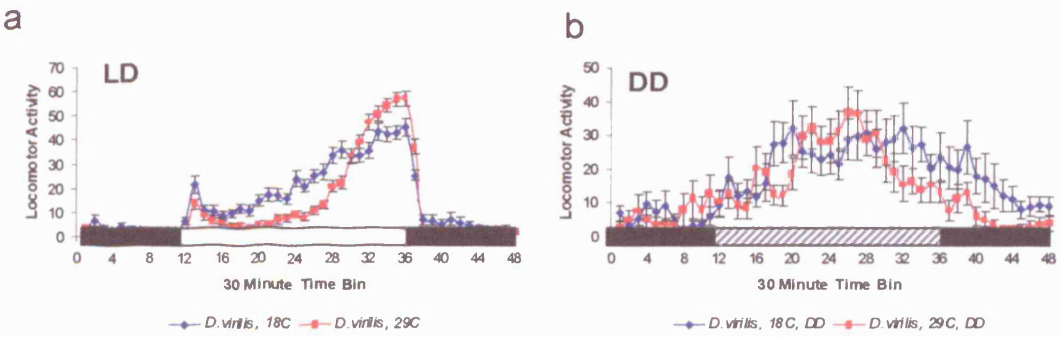


Figure 7.2 Locomotor Activity of *D.virilis* a). in LD 12:12 at 18°C (blue) and 29°C (red). b) in DD after prior entrainment to LD 12:12. Error bars represent SEM

Like *D.melanogaster, D.virilis* shows a clear difference in locomotor activity profiles at different temperatures in LD (figure 7.2a). In LD, the level of the morning activity peak is higher at 18 than 29°C (A 7.2.1; $F=10.6$, $p<0.05$). During the day (time bins 20-28) the level of activity rises earlier at 18 than 29°C (A 7.2.2; $F=137$, $p<<<0.001$, time x temperature ($F=0.5521$, n.s.). The evening activity peak reaches a peak level earlier, and is broader at 18°C (A 7.1.3; time x temperature, $F=2.565$, $p<<0.001$) (figure 8.2a).

In DD, there is no difference in the distribution of activity between the two temperatures (A 7.2.4; temperature x time $F=1.3$, n.s.). This suggests that temperature can only control the locomotor activity profile of *D.virilis* in the presence of a light input to the clock.

From the locomotor activity profile, it appears that *D.virilis* avoids being active in the middle of the day at high temperatures. It does this by reducing the level of locomotor activity and delaying evening activity at high temperatures.

This is similar to the behaviour of *D.melanogaster* - in LD *D.melanogaster* also has an earlier evening activity peak at 18°C. To determine whether *D.virilis* and *D.melanogaster* respond in the same way to increased

temperatures, their LD and DD locomotor activity profiles were compared, as shown in figure 7.3. A similar comparison with *D.pseudoobscura* was not carried out, as at 29°C there is no evening activity peak in this species (Chapter 6).

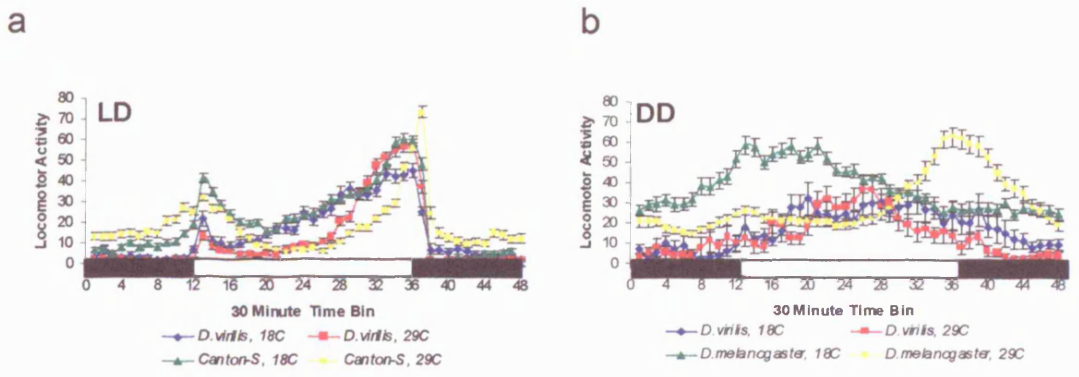


Figure 7.3 Comparison of *D.virilis* and *D.melanogaster* locomotor activity a). LD 12:12 in *D.virilis* (blue/red) and *D.melanogaster* (green/yellow) at 18°C/29°C. b). DD. Error bars represent SEM

In LD, the morning activity peak of *D.melanogaster* is higher than that of *D.virilis* at both temperatures (A 7.3.1, 7.3.4; 18°C, F=152.6, p<<<0.001; 29°C, f=317, p<<<0.001). During the day, the level of activity is not significantly different between the two species (A 7.3.2; F=2.2, n.s.) at 18°C. At 29°C, the levels of activity are similar, but the evening peak begins to rise earlier in *D.virilis* than *D.melanogaster* (A 7.3.5; species x time, F=3.7, p<0.05).

At 18°C, the evening activity peak of *D.virilis* occurs earlier than that of *D.melanogaster* (A 7.3.3; species x time, F=5.5, p<<0.001), figure 7.3a). The level of evening peak activity is higher in *D.melanogaster* than *D.virilis* (F=47, p<<<0.001). At 29°C, evening activity again peaks earlier in *D.virilis* than *D.melanogaster,* and the evening activity peak of *D.virilis* is broader (species x time, A 7.3.6; F=41.340, p<<<0.001), figure 7.3a.

Therefore in LD cycles, although both *D.melanogaster* and *D.virilis* show a later phase of activity at high temperatures, the evening activity peak of *D.virilis* is earlier than that of *D.melanogaster* at both temperatures. This is the most obvious difference in behaviour between the two species as during the day the level of activity is similar at both temperatures until the level of evening peak activity begins to rise.

In DD, the peak of activity of *D.melanogaster* occurs in the subjective morning at 18°C, and the subjective evening at 29°C (figure 7.3b). In contrast, the peak of *D.virilis* in DD occurs at the same time at both temperatures, and is distinct from both peaks of *D.melanogaster* (A 7.3.7 - 7.3.8; species x time, 18°C $F=598.5$, $p<<<0.001$; 29°C $F=442.7$, $p<<<0.001$).

7.4.2 *D.virilis* 3' UTR

3' RACE of *D.virilis per* identified two different *per* RNA transcripts. These were sequenced and aligned to the genomic *per* sequence of *D.virilis* using GCG. There are no introns within the 3' UTR sequence of *D.virilis per.* A comparison of the two transcripts is shown in figure 7.4

D.virilis 3' RACE products

```
GCCCTTATTCAGGGATCCGAATTCTGGCAACAGCGATGACATGGATGGCTCTAGCTTCTC 60

ATCCTTCTACTCATCCTTCATCAAAACCACCGACGGCTCTGAGAGTCCGCCCGACAATGA 120

GAAGGAGACGAAAGTTCACAAGCTCAAGCCCATCGTTGAGCATCCGGAGGAGGACCAGAC 180
                  STOP
GCAGCACGGAGATGGATAGCATTCCAAAGGACGGCTCTAGTTGTGTCGAATCCTGACATT 240

CAGTTGCAGCAACAACCCAAGCCGCAAGGCCCGCACCCAGACGAGCTTCTCTTGGACACC 300

CAAACCAAACGCTTGAGCATTATTTAGAGTTTTCCATTTCGTGTGTAACTACATAAATTT 360

Polyadenylation Signal 1
ATTTTCTGATCAAATTCAATTTTGCAAGTGGAGAAGACAACGTTCAAAAAAAAAAAAAAA 420

Polyadenylation Signal 2
ATTTTCTGATCAAATTCAATTTTGCAAGTGGAGAAGACAACGTTCAAAAAACAAAAACAA 420

AAAATACATTTACAACAAATTATGTGCCCAAGCGRCAAATTTTCATATCCCAATAAACGC 480

TGCGATTACAGAAAGATCYGWGATGGCAAAATAAATAAAAGAAAAAAAAAAAAAAAAA   540
```

Figure 7.4 Sequences of the two 3' RACE products of *D.virilis period* The polyadenylated ends of the two transcripts are highlighted in yellow.

The two *per* RNA transcripts identified by 3' RACE differ in size by ~120 bp. These products are referred to throughout as the 'long' and 'short' *per* transcripts. It is worth noting that that there is no consensus polyadenylation signal immediately upstream of the polyA tail of the short *per* RNA product. As a result, it may be that the short product is an artefact of some sort, such as a breakdown product of the longer RNA transcript. As both products were isolated from RNA by 3' RACE, and the short product is more abundant than the long product (below), it seems probable that both exist *in vivo* and may therefore have different functions.

7.4.3 *D.virilis per* RNA cycling

Real time PCR was used to determine the relative levels of the two *per* RNA transcripts, as shown in figure 7.5.

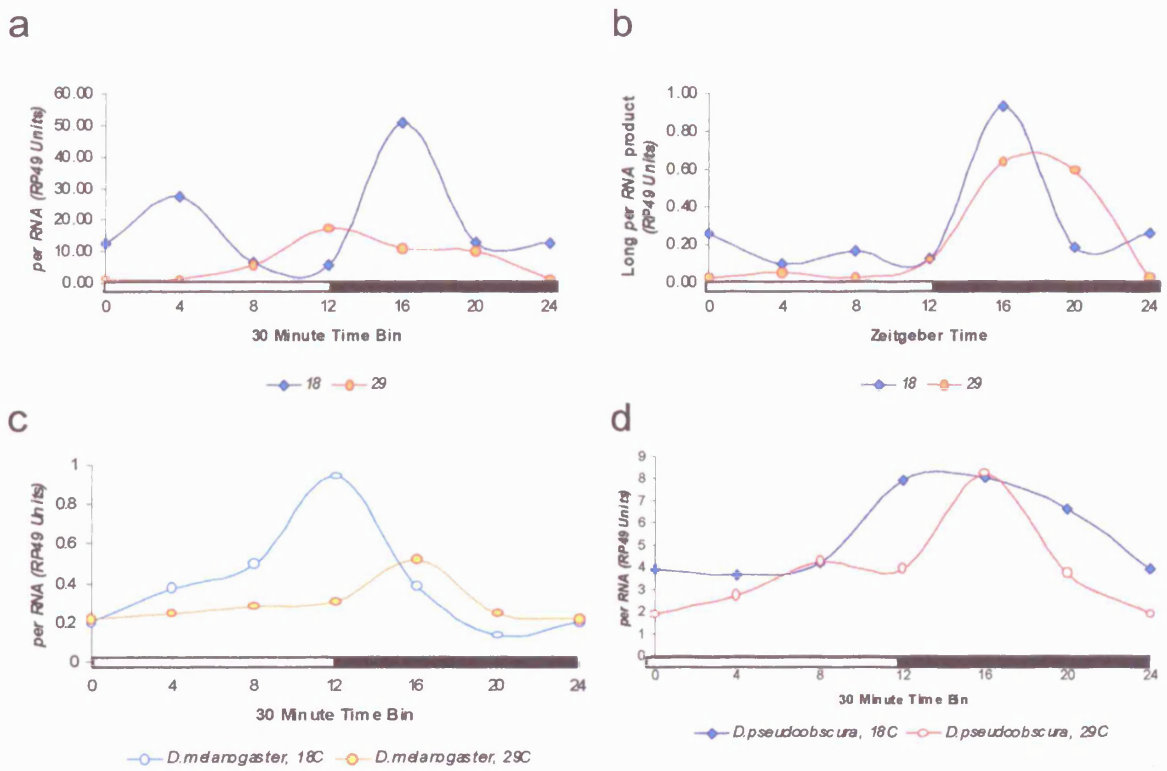


Figure 7.5 *per* RNA cycles in *D.virilis, D.melanogaster* and *D.pseudoobscura* a). total *per* RNA at 18°C (blue) and 29°C (orange) in *D.virilis*. b). long *per* RNA only. c). *per* RNA cycle of *D.melanogaster* at 18°C (blue) and 29°C (red). d). *D.pseudoobscura* at 18°C (blue) and 29°C (red). c and d are taken from figure 6.6. Only two sets of cDNA have been analysed for figures 7.5a and 7.5b, and therefore no error bars are shown.

3' RACE revealed two *per* transcripts which differ by the polyadenylation site in *D.virilis.* This means that a set of primers designed to amplify the short product will also amplify the long product. To determine the amount of the short product, the level of *per* amplified by the long primers has to be subtracted from that amplified from the short primers. It can be seen by comparing the Y-axis of figures 7.5a and 7.5b, that the level of the long *per* RNA transcript is orders of magnitude lower than that of the short transcript. So, the *per* RNA cycle in *D.virilis* is primarily the cycle of the short *per* product, as shown in figure 7.5a.

At 18°C, the cycle of the 'short' product peaks around ZT 16, while at 29°C it peaks from around ZT 12-20 (figure 7.5a). This is the opposite of

D.melanogaster (figure 7.5c) and *D.pseudoobscura* (figure 7.5d) where the *per* RNA peaks earlier at 18 than 29°C. However, this needs to be repeated as there is too much variation between the samples analysed to be sure that this accurately represents the *per* RNA cycles. The cycle of the long *per* RNA product shows far less variation that the total RNA cycle. Although the level of the long RNA product is far lower than the level of total *per* RNA, it seems to match more closely the cycle of *per* RNA seen in *D.melanogaster* and *D.pseudoobscura.* The cycle peaks at ~ZT 16 at 18°C, and between ZT 16 and ZT 20 at 29°C (compare figures 7.5b, 7.5c and 7.5d).

Therefore both the short and long products levels peak at ZT 16 at 18°C. At 29°C, there is a broader peak level of both products, so that the peak level of RNA is maintained later into the night. This means that the phase of the RNA cycle is delayed in comparison to both *D.melanogaster* and *D.pseudoobscura.*

7.5 Discussion

7.5.1 *D.virilis* locomotor activity

In LD cycles, both *D.melanogaster* and *D.virilis* have a later phase of activity at high temperatures. The two species also show different patterns of locomotor activity from each other. The evening activity level rises and peaks earlier in *D.virilis* than *D.melanogaster.* It is thought that the delayed evening activity peak seen in *D.melanogaster* at 29°C is required to avoid desiccation in the mid-day sun. As the phase of locomotor activity of *D.virilis* means that it becomes active earlier in the day, it may be that *D.virilis* is less well suited to hot climates than *D.melanogaster.* This fits with the natural distribution of these two species, where *D.virilis* is native to Northern Europe, whereas the origins of *D.melanogaster* seem to be from Africa.

Despite this, *D.virilis* is better equipped for high temperatures than *D.pseudoobscura.* At 29°C, *D.pseudoobscura* is poorly temperature

compensated, and the clock does not seem to control the distribution of locomotor activity (Chapter 6). In contrast, *D.virilis* is well temperature compensated at 29°C, and locomotor activity shows a (clock controlled) evening activity peak.

In DD, the difference in peak position in *D.melanogaster* is exaggerated, with a peak in the subjective morning at 18°C and in the subjective evening at 29°C. This seems to be unique to *D.melanogaster* as at 18°C in *D.pseudoobscura* the peak occurs in the subjective evening (Hennessey, 1999). This is also the case in *D.virilis,* where the peak occurs in the middle of the day at both temperatures. Therefore some unique feature of the free-running clock of *D.melanogaster* must be responsible for this difference in peak position at different temperatures in DD.

7.5.2 *D.virilis per* RNA cycles

3' RACE of *D.virilis per* identified two alternative *per* transcripts which differed in terms of the position of the polyA signal. In *D.melanogaster* (Majercak et al., 1999), *D.pseudoobscura* (Chapter 6) and *Bactrocera* (An. et al., 2002) *per* transcripts differ in terms of the splicing of an intron within the 3' UTR. The alternative splicing of this intron in *D.melanogaster* has been shown to be functionally important in *D.melanogaster* (Majercak et al., 1999), and this may also be the case in *Bactrocera* where the level of splicing changes with temperature (An, X. et al., 2002). This regulation is not shared by *D.pseudoobscura* (Chapter 6) or *D.virilis,* both of which are more closely related to *D.melanogaster* than *Bactrocera.* This could be an ancestral regulatory mechanism which has been lost by *D.virilis* and *D.pseudoobscura,* or it could have arisen independently in *D.melanogaster* and *Bactrocera.*

The evening activity peak of *D.melanogaster* at 29°C is delayed until just after lights off, later than the corresponding peak of *D.virilis.* This suggests that *D.melanogaster* has stricter regulation of behaviour at high temperatures, possibly because it has this splicing mechanism resulting in

later activity. It may be that the need to regulate locomotor activity at high temperatures has selected for the alternative splicing of the intron in *D.melanogaster* (and poteintially *Bactrocera* (An et al., 2002) but not *D.virilis* or *D.pseudoobscura.*

If this is the case, then it would be expected that there would be no temperature regulation of the alternatively polyadenylated transcripts of *D.virilis.* This seems to be the case as at both 18 and 29°C there is 30-60 times as much of the short *per* transcript as the long transcript. For the long transcript to play an important role, it would be predicted that it would be present in levels comparable to those of the short transcript.

In Chapter 6, it was suggested that differences in the *per* RNA cycle are responsible for species-specific differences in behaviour. The phase of the *per* RNA cycle of *D.virilis* lags behind that of *D.melanogaster* at both 18 and 29°C, yet the locomotor profile is in advance of *D.melanogaster.* It may be that differences in the dynamics of the clock between different species alter the way in which the RNA cycle determines the pattern of locomotor activity. For instance, *per* may be translated at different rates in the two species, so if *per* were translated at a higher level in *D.virilis* it could compensate for the differences in peak RNA position. Additionally, the *per* RNA cycle in *D.melanogaster* is retarded through the repression of the splicing of the 3' intron of *per* which does not occur in *D.virilis.*

This explanation is less satisfactory when trying to determine why the evening peak is earlier at 18 than 29°C in *D.virilis* as the peak level of the 'short' abundant *per* RNA product appears to occur earlier at 29 than 18°C. It could be that the higher peak level of *per* RNA at 18°C seen in *D.virilis* it is responsible for the earlier evening activity peak. The data is not good enough to draw any firm conclusions.

As mentioned in the results section, there is too much variation in the levels of the short *per* RNA product to be sure that the cycle detected is accurate.

This is less of a problem with the cycle of the long *per* transcript where the level of *per* RNA peaks earlier at 18 than at 29°C. If this is the true picture, then *D.virilis,* like *D.melanogaster* and *D.pseudoobscura* has an earlier peak of *per* RNA at 18 than 29°C, which then produces an earlier evening activity peak. This needs to be repeated to confirm that the cycle of *per* RNA in figure 7.5 is accurate.

Therefore the regulation of the splicing of the *per* 3' UTR intron is not a universal mechanism. It also cannot solely account for species specific differences in behaviour as the *per* RNA products of *D.virilis* differ by polyadenylation signal.

To determine whether there is a functional difference between the two *per* mRNA transcripts in *D.virilis,* it would be interesting to see whether flies able to produce either only the short transcript, or only the long transcript displayed different patterns of locomotor activity. Within a species like *D.melanogaster,* where null *per* mutants (per^{01}) exist, it would be relatively straightforward to create transgenic flies which could only produce one or other of the transcripts. Unfortunately these null mutants of *per* do not exist in *D.virilis,* but it might be possible to use RNAi to eliminate the longer transcript, and see the effect of expressing only one of the *per transcripts.*

Chapter eight: final conclusions

8.1 The regulation of the splicing of *per* RNA

The alternative splicing of an intron within the 3' UTR of the *per* gene determines the position of the evening locomotor activity peak of *D.melanogaster.* The regulation of activity in this way is one way in which *D.melanogaster* avoids desiccation in the mid-day sun. This mechanism seems to be unique to *D.melanogaster* and possibly very closely related species, as although there is a comparable intron within the same gene of *D.pseudoobscura* the level of splicing does not seem to be under temperature control, while in *D.virilis,* there is no such intron, and *per* RNA transcripts differ only by polyadenylation signal.

D.melanogaster is found in a wide range of environments, from Africa right into northern Europe, but appears to primarily be a warm adapted domestic species, whereas both *D.virilis* and *D.pseudoobscura* both tend to occupy colder regions. *D.virilis* is more active during the middle of the day at 29°C, while at this temperature the behaviour of *D.pseudoobscura* appears no longer to be clock regulated. Therefore, the ability to regulate locomotor activity by the regulation of *per* splicing may be one reason why *D.melanogaster* appears to be better suited to milder climates.

In *D.melanogaster* the level of splicing of this intron is determined by the temperature and by the photoperiod. At 18°C ~45% of all transcripts are spliced, and if the temperature is raised splicing is repressed so that at 29°C only ~25% of *per* transcripts are spliced in wild-type flies. If wild-type flies are placed in DD then these levels rise to ~60% and ~40% respectively, indicating the role of the photoperiod in repressing splicing. As the levels of splicing in DD are comparable to those seen in the visually blind gl^{60j} mutant, it seems reasonable to assume that the light input for repressing splicing is

received through the visual system, particularly as mutations of the circadian photoreceptor CRY have little impact on splicing levels at low temperatures.

At high temperatures the regulation of behaviour to avoid mid-day activity becomes vitally important, and, as a result, the regulation of splicing of this intron becomes much stricter. The clock becomes involved in repressing splicing, and at 29°C the elimination of *per, tim* or *cry* removes the repression of splicing during the night. Therefore it seems that at high temperatures the photoperiod sets the splicing level during the day, and the role of the clock is to maintain the repression of splicing at night. In the absence of these clock components the levels of splicing at night at 29°C rise to levels comparable with those of wild-type flies at 18°C.

Like those of *gl,* mutations of the *norpA* gene produce visually blind flies. In fact gl^{60j} mutation is more severe than $norpA^{P41}$, yet the levels of splicing in $norpA^{P41}$ are elevated even above the levels of splicing seen in wild-type flies in DD. This indicates that as well as playing a role in the visual photoreception system $norpA^{P41}$ may also be involved in the regulation of splicing in some other way. The difference between splicing levels at 18°C and 29°C is maintained between gl^{60j} and wild-type, but is greatly reduced in flies carrying the $norpA^{P41}$ mutation. Perhaps the most logical explanation is that as *norpA* encodes a signalling molecule, a pathway involving this protein is involved in the signalling of temperature information to the splicing machinery and/or the clock. This may also explain why the locomotor activity profiles of $norpA^{P41}$ appear to be very sensitive to increased temperatures.

Generally the regulation of splicing is related to the position of the evening activity peak. However, in *D.melanogaster* carrying the gl^{60j} mutation, the relationship between the splicing level and the position of the evening activity peak breaks down, and the high levels of splicing in this mutant do not advance evening activity. Similarly, in *mps3* transformant *D.melanogaster,* there is no regulation of the splicing of the *per* intron, yet locomotor activity peaked earlier at 18°C than at 29°C. The role of the regulation of the splicing

of this intron is to modify the underlying *per* RNA cycle so that the PER protein cycle, and ultimately locomotor activity can be 'fine-tuned' to seasonal and climatic changes in conditions. In *mps3* transformants, the cycle of *per* RNA appears to be advanced at 18°C, and delayed at 29°C in comparison to wild-type *D.melanogaster* and therefore the regulation of splicing is not required to produce the differences in evening activity. So, when there is no relationship between splicing level and evening activity peak position, it may be that this can be attributed to differences in the underlying RNA cycle which make the splicing level irrelevant in determining behaviour.

8.2 Splicing and the production of mature *per* mRNA

The production of a mature mRNA transcript requires that the mRNA acquires a 5' cap, is spliced, and a polyA tail added at the 3' end. These three processes are interlinked and influence each other's efficiency (Proudfoot et al., 2002). With the *D.melanogaster per* 3' UTR intron, it was demonstrated that it is the act of splicing itself rather than the presence or absence of the 3' UTR intron that determines the timing of evening activity (Majercak et al, 1999). Therefore the act of splicing must in some way advance the production of mature *per* mRNA and/or its translation. They suggested that the increased splicing of this intron at low temperatures may stimulate the production of the mature 3' end of the mRNA through advanced cleavage and polyadenylation (Majercak et al, 1999).

Splicing and polyadenylation

The association between splicing and polyadenylation is well established and understood. Splicing Regulatory (SR) proteins are recruited to the exons through interactions with exonic enhancer sequences. These proteins then recruit the snRNPs U1 and U2 to the RNA. This then determines the exon structure. However, in the final exon, the end of the gene is determined by the polyadenylation signal, with U2AF65 of the splicing complex interacting with the C-terminal domain of the poly(A) polymerase. Therefore

polyadenylation and splicing either side of this final exon are inextricably linked and strongly enhance each other (Reviewed in Proudfoot et al, 2002). It is therefore likely that by regulating the splicing of the final intron, the polyadenylation of the RNA will also be regulated. Thus, in the case of *per*, lower levels of splicing of the final intron will delay polyadenylation, and the production of mature mRNA transcripts. Additionally, it is known that the binding of binding of PolyA-binding proteins (PAPBs) to the mRNA tail prevents degradation (Dreyfus and Regnier, 2002), and therefore it may be that higher levels of splicing result in advanced polyadenylation and therefore more stable *per* mRNA at lower temperatures. A higher level of stable RNAs could then determine the earlier translation of *per*.

However, polyadenylation is not the only step of the journey from transcription to translation which is affected by the splicing of the mRNA, as splicing is also intrinsically linked to nuclear export (Reed and Hurt., 2002). In yeast and mammals, the *Sub2/UAP56* is involved in both spliceosome assembly and mRNA export. RNAi has been used to demonstrate that this protein is required for the nuclear export of mRNAs, including those lacking introns. *Sub2/UAP56* is therefore thought to be freed from the spliceosome prior to nuclear export (Reviewed in Reed and Hurt., 2002), so stimulating splicing will lead to this occurring earlier, and advance nuclear export.

Interestingly, RNA processing has recently been shown to be involved in the timing of seasonal flowering in *Arabidopsis thaliana* (Simpson et al., 2003; Quesada et al., 2003). Therefore organisms as diverse as *Arabidopsis* and *D.melanogaster* control seasonal 'behaviour' through the post-transcriptional regulation of mRNA. In the case of *Arabidopsis,* the timing of flowering depends on the ratio of several differentially polyadenylated transcripts of FCA, an RNA binding protein (Quesada et a., 2003). The proportions of these transcripts are controlled by FCA and FY, its partner protein (Simpson et al., 2003). Thus the model is that FCA protein recruits FY and the rest of the polyadenylation machinery to a polyadenylation site within intron 3, generating the truncated mRNA. This mRNA produces a truncated FCA protein that can no longer bind its own RNA, thereby setting up an

autoregulatory loop (Simpson et al., 2003; Quesada et al., 2003). This is the first example of non-core polyadenylation proteins controlling polyadenylation (Simpson et al., 2003; Quesada et al., 2003). It may be that there is a somewhat similar situation in *D.melanogaster per,* with a non-splicing protein controlling the splicing of the *per* 3' intron.

It is the splicing of the most 3' intron which impinges on polyadenylation/nuclear export (reviewed in Proudfoot et al., 2002) so it is unsurprising that this is the only intron which is alternatively spliced in *per.* It is known that the only different forms of *per* transcript *in vivo* differ only by the presence of this intron - other introns are spliced as normal (Cheng et al., 1998). Therefore something must ensure that the splicing of this intron is regulated, so that it is left until last.

The simplest explanation would be that some RNA binding protein, under temperature, light and clock control binds only to this intron, preventing splicing. Then as this protein unbinds, the mRNA is freed for splicing, and nuclear export, 3' end formation. In the future it may prove profitable to go looking for this 'splicing regulator' to further understand how temperature regulates the clock. One possible candidate, identified by at least two separate microarray analyses as being under clock regulation is CG17386 (Claridge-Chang et al, 2001; Lin et al., 2002). It so far has no known function, but is under clock control. A BLAST search reveals that this protein contains an RNA binding domain that shares characteristics of the *Trypanosome* LA-motif containing proteins that, amongst other things, are involved in posttranslational modification.

Otherwise it may be possible to identify such an RNA binding protein through a technique such as yeast-3-hybrid (SenGupta et al., 1996) or T7 phage display (Danner and Belasco, 2001).

8.3 Unusual behaviour

During the course of this work, two remarkable observations of the behaviour of double mutants carrying the cry^b mutation were made. The gl^{60j} cry^b double mutant has been reported to be blind and unentrainable to LD cycles (Helfrich-Forster et al., 2001), yet at 29°C it appears to anticipate 'lights off'. This recovery of light sensitivity at high temperatures either indicates the existence of an as yet unidentified photoreceptor in *D.melanogaster*, or that cry^b is able to gain function at higher temperatures.

Even more intriguingly, the per^{01}; cry^b double mutant is also able to anticipate 'lights off' even though per^{01} mutants are unable to do this. As tim^{01}; cry^b behave like tim^{01} single mutants - they do not anticipate lights off - the simplest explanation is that in the absence of both PER and CRY TIM is able to enter the nucleus on its own, and drive the negative feedback loop which provides the basis of the clock. In per^{01} in LD the levels of TIM cycle, and it may be that TIM is almost fully degraded by CRY during the light phase. In the absence of CRY, these low TIM levels may then become sufficient to drive the clock in the absence of PER. As in DD and LL per^{01}; cry^b flies are arrhythmic, this must require the daily degradation of TIM by light within the lateral neurons, which is something that needs to be investigated in the future. Thus it is possible that TIM is able to fulfil many clock functions on its own. It has already been established that TIM is able to inhibit the dCLK/CYC complex *in vitro* even in the absence of PER (Lee et al., 1999), and it may be that this is what occurs *in vivo* in the per^{01}; cry^b double mutant.

Thus it the analysis of splicing allied to behaviour has made it possible to develop a greater understanding of how locomotor activity is entrained to different light and temperature conditions. This is particularly important in illuminating the role of the visual system, separate from that of *cry* and the clock, in entraining evening activity. The analysis of behaviour and *per* 3' splicing at two different temperatures has clearly demonstrated that *norpA*

has a circadian role outside the visual system. Perhaps most importantly, the discovery of anticipatory behaviour in LD cycles in the per^{01}; cry^{b} double mutant suggests that there is a PER-independent role for TIM in the *D.melanogaster* clock.

Bibliography

Akashi, M., Tsuchiya, Y., Yoshino, T., and Nishida, E. (2002). Control of intracellular dynamics of mammalian PERIOD proteins by casein kinase Iε (CKIε) and CKIδ in cultured cells. Mol Cell Biol *22*, 1693-1703.

Akiyama, M., Kouzu, Y., Takahashi, S., Wakamatsu, H., Moriya, T., Maetani, M., Watanabe, S., Tei, H., Sakaki, Y., and Shibata, S. (1999). Inhibition of light- or glutamate-induced *mPer1* expression represses the phase shifts into the mouse circadian locomotor and suprachiasmatic firing rhythms. J Neurosci *19*, 1115-1121.

Albrecht, U., Zheng, B., Larkin, D., Sun, Z. S., and Lee, C. C. (2001). *mPer1* and *mPer2* are essential for normal resetting of the circadian clock. J Biol Rhythms *16*, 100-104.

Allada, R., White, N. E., So, W. V., Hall, J. C., and Rosbash, M. (1998). A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of period and timeless. Cell *93*, 791-804.

An, X., Wilkes, K., Bastian, Y., Morrow, J. L., Frommer, M., and Raphael, K. A. (2002). The *period* gene in two species of tephritid fruit fly differentiated by mating behaviour. Insect Mol Biol *11*, 419-430.

Bacigalupo, J., Bautista, D. M., Brink, D. L., Hetzer, J. F., and O'Day, P. M. (1995). Cyclic-GMP enhances light-induced excitation and induces membrane currents in *Drosophila* retinal photoreceptors. J Neurosci *15*, 7196-7200.

Bae, K., Lee, C., Hardin, P. E., and Edery, I. (2000). dCLOCK is present in limiting amounts and likely mediates daily interactions between the dCLOCK-CYC transcription factor and the PER-TIM complex. J Neurosci *20*, 1746-1753.

Bae, K., Lee, C., Sidote, D., Chuang, K. Y., and Edery, I. (1998). Circadian regulation of a *Drosophila* homolog of the mammalian *Clock* gene: PER and TIM function as positive regulators. Mol Cell Biol *18*, 6142-6151.

Bao, S., Rihel, J., Bjes, E., Fan, J. Y., and Price, J. L. (2001). The *Drosophila double-time*^{S} mutation delays the nuclear accumulation of PERIOD protein and affects the feedback regulation of *period* mRNA. J Neurosci *21*, 7117-7126.

Baylies, M. K., Bargiello, T. A., Jackson, F. R., and Young, M. W. (1987). Changes in abundance or structure of the *per* gene product can alter periodicity of the *Drosophila* clock. Nature *326*, 390-392.

Beaver, L. M., Gvakharia, B. O., Vollintine, T. S., Hege, D. M., Stanewsky, R., and Giebultowicz, J. M. (2002). Loss of circadian clock function decreases reproductive fitness in males of *Drosophila melanogaster*. Proc Natl Acad Sci U S A *99*, 2134-2139.

Belvin, M. P., Zhou, H., and Yin, J. C. P. (1999). The *Drosophila* dCREB2 gene affects the circadian clock. Neuron *22*, 777-787.

Benna, C., Scannapieco, P., Piccin, A., Sandrelli, F., Zordan, M., Rosato, E., Kyriacou, C. P., Valle, G., and Costa, R. (2000). A second *timeless* gene in *Drosophila* shares greater sequence similarity with mammalian *tim*. Curr Biol *10*, R512-513.

Berson, D. M., Dunn, F. A., and Takao, M. (2002). Phototransduction by retinal ganglion cells that set the circadian clock. Science *295*, 1070-1073.

Blau, J., and Young, M. W. (1999). Cycling *vrille* expression is required for a functional *Drosophila* clock. Cell *99*, 661-671.

Bunger, M. K., Wilsbacher, L. D., Moran, S. M., Clendenin, C., Radcliffe, L. A., Hogenesch, J. B., Simon, M. C., Takahashi, J. S., and Bradfield, C. A. (2000). Mop3 is an essential component of the master circadian pacemaker in mammals. Cell *103*, 1009-1017.

Ceriani, M. F., Darlington, T. K., Staknis, D., Mas, P., Petti, A. A., Weitz, C. J., and Kay, S. A. (1999). Light-dependent sequestration of TIMELESS by CRYPTOCHROME. Science *285*, 553-556.

Chan, R. C., Chan, A., Jeon, M., Wu, T. F., Pasqualone, D., Rougvie, A. E., and Meyer, B. J. (2003). Chromosome cohesion is regulated by a clock gene paralogue TIM-1. Nature *424*, 1002-1009.

Cheng, Y., Gvakharia, B., and Hardin, P. E. (1998). Two alternatively spliced transcripts from the *Drosophila period* gene rescue rhythms having different molecular and behavioural characteristics. Mol Cell Biol *18*, 6505-6514.

Cheng, Y., and Hardin, P. E. (1998). *Drosophila* photoreceptors contain an autonomous circadian oscillator that can function without *period* mRNA cycling. J Neurosci *18*, 741-750.

Claridge-Chang, A., Wijnen, H., Naef, F., Boothroyd, C., Rajewsky, N., and Young, M. W. (2001). Circadian regulation of gene expression systems in the *Drosophila* head. Neuron *32*, 657-671.

Costa, R., and Kyriacou, C. P. (1998). Functional and evolutionary implications of natural variation in clock genes. Current Opinions in Neurobiology *8*, 659-664.

Costa, R., Peixoto, A. A., Barbujani, G., and Kyriacou, C. P. (1992). A latitudinal cline in a *Drosophila* clock gene. Proc R Soc Lond B Biol Sci *250*, 43-49.

Costa, R., Peixoto, A. A., Thackeray, J. R., Dalgleish, R., and Kyriacou, C. P. (1991). Length Polymorphism in the Threonine-Glycine-Encoding Repeat Region of the *period* Gene in *Drosophila*. J Mol Evol *32*, 238-246.

Curtin, K. D., Huang, Z. J., and Rosbash, M. (1995). Temporally regulated nuclear entry of the *Drosophila* PERIOD protein contributes to the circadian clock. Neuron *14*, 365-372.

Cyran, S. A., Buchsbaum, A. M., Reddy, K. L., Lin, M. C., Glossop, N. R., Hardin, P. E., Young, M. W., Storti, R. V., and Blau, J. (2003). vrille, *Pdp1*, and *dClock* form a second feedback loop in the *Drosophila* circadian clock. Cell *112*, 329-341.

Danner, S., and Belasco, J. G. (2001). T7 phage display: a novel genetic selection system for cloning RNA-binding proteins from cDNA libraries. Proc Natl Acad Sci U S A *98*, 12954-12959.

Darlington, T. K., Wager-Smith, K., Ceriani, M. F., Staknis, D., Gekakis, N., Steeves, T. D., Weitz, C. J., Takahashi, J. S., and Kay, S. A. (1998). Closing the circadian loop: CLOCK-induced transcription of its own inhibitors PER and TIM. Science *280*, 1599-1603.

Dembinska, M. E., Stanewsky, R., Hall, J. C., and Rosbash, M. (1997). Circadian cycling of a PERIOD-beta-galactosidase fusion protein in *Drosophila*: evidence for cyclical degradation. J Biol Rhythms *12*, 157-172.

Dockendorff, T. C., Su, H. S., McBride, S. M., Yang, Z., Choi, C. H., Siwicki, K. K., Sehgal, A., and Jongens, T. A. (2002). *Drosophila* lacking *dfmr1* activity show defects in circadian output and fail to maintain courtship interest. Neuron *34*, 973-984.

Dreyfus, M., and Regnier, P. (2002). The poly(A) tail of mRNAs: bodyguard in eukaryotes, scavenger in bacteria. Cell *111*, 611-613.

Dunlap, J. (1998). An end in the beginning. Science *280*, 1548-1549.

Dushay, M. S., Konopka, R. J., Orr, D., Greenacre, M. L., Kyriacou, C. P., Rosbash, M., and Hall, J. C. (1990). Phenotypic and genetic analysis of *Clock*, a new circadian rhythm mutant in *Drosophila melanogaster*. Genetics *125*, 557-578.

Dushay, M. S., Rosbash, M., and Hall, J. C. (1989). The *disconnected* visual system mutations in *Drosophila melanogaster* drastically disrupt circadian rhythms. J Biol Rhythms *4*, 1-27.

Edery, I., Rutila, J. E., and Rosbash, M. (1994). Phase shifting of the circadian clock by induction of the *Drosophila* PERIOD protein. Science *263*, 237-240.

Emery, P., So, W. V., Kaneko, M., Hall, J. C., and Rosbash, M. (1998). CRY, a *Drosophila* clock and light-regulated *cryptochrome*, is a major contributor to circadian rhythm resetting and photosensitivity. Cell *95*, 669-679.

Emery, P., Stanewsky, R., Hall, J. C., and Rosbash, M. (2000a). A unique circadian-rhythm photoreceptor. Nature *404*, 456-457.

Emery, P., Stanewsky, R., Helfrich-Forster, C., Emery-Le, M., Hall, J. C., and Rosbash, M. (2000b). *Drosophila* CRY is a deep brain circadian photoreceptor. Neuron *26*, 493-504.

Ewer, J., Frisch, B., Hamblen-Coyle, M. J., Rosbash, M., and Hall, J. C. (1992). Expression of the *period* clock gene within different cell types in the brain of *Drosophila* adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. J Neurosci *12*, 3321-3349.

Field, M. D., Maywood, E. S., O'Brien, J. A., Weaver, D. R., Reppert, S. M., and Hastings, M. H. (2000). Analysis of clock proteins in mouse SCN demonstrates phylogenetic divergence of the circadian clockwork and resetting mechanisms. Neuron *25*, 437-447.

Foulkes, N. S., Borjigin, J., Snyder, S. H., and Sassone-Corsi, P. (1996). Transcriptional control of circadian hormone synthesis via the CREM feedback loop. Proc Natl Acad Sci U S A *93*, 14140-14145.

Francis, A.J.P. & Coleman, G.J. (1988) The effect of ambient temperature cycles upon circadian running and drinking activity in male and female laboratory rats. Physiol. Behav. 43, 471-477.

Freedman, M. S., Lucas, R. J., Soni, B., von Schantz, M., Munoz, M., David-Gray, Z., and Foster, R. (1999). Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors. Science *284*, 502-504.

Frisch, B., Hardin, P. E., Hamblen-Coyle, M. J., Rosbash, M., and Hall, J. C. (1994). A promoterless period gene mediates behavioral rhythmicity and cyclical per expression in a restricted subset of the *Drosophila* nervous system. Neuron *12*, 555-570.

Gekakis, N., Saez, L., Delahaye-Brown, A. M., Myers, M. P., Sehgal, A., Young, M. W., and Weitz, C. J. (1995). Isolation of TIMELESS by PER protein interaction: defective interaction between TIMELESS protein and long-period mutant PER^{L}. Science *270*, 811-815.

Giebultowicz, J. M. (2000). Molecular mechanism and cellular distribution of insect circadian clocks. Annu Rev Entomol *45*, 769-793.

Giebultowicz, J. M. (2001). Peripheral clocks and their role in circadian timing: insights from insects. Philos Trans R Soc Lond B Biol Sci *356*, 1791-1799.

Giebultowicz, J. M., and Hege, D. M. (1997). Circadian clock in Malpighian tubules. Nature *386*, 664.

Ginty, D. D., Kornhauser, J. M., Thompson, M. A., Bading, H., Mayo, K. E., Takahashi, J. S., and Greenberg, M. E. (1993). Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. Science *260*, 238-241.

Gloor, G. B., Preston, C. R., Johnson-Schlitz, D. M., Nassif, N. A., Phillis, R. W., Benz, W. K., Robertson, H. M., and Engels, W. R. (1993). Type I repressors of P element mobility. Genetics *135*, 81-95.

Glossop, N. R., Houl, J. H., Zheng, H., Ng, F. S., Dudek, S. M., and Hardin, P. E. (2003). VRILLE feeds back to control circadian transcription of Clock in the *Drosophila* circadian oscillator. Neuron *37*, 249-261.

Glossop, N. R., Lyons, L. C., and Hardin, P. E. (1999). Interlocked feedback loops within the *Drosophila* circadian oscillator. Science *286*, 766-768.
Golden, A., and Cohen-Fix, O. (2003). Getting (chromosomes) loaded--a new role for TIMELESS. Dev Cell *5*, 7-9.

Gooley, J. J., Lu, J., Chou, T. C., Scammell, T. E., and Saper, C. B. (2001). Melanopsin in cells of origin of the retinohypothalamic tract. Nat Neurosci *4*, 1165.

Gotter, A. L., Manganaro, T., Weaver, D. R., Kolakowski, L. F., Jr., Possidente, B., Sriram, S., MacLaughlin, D. T., and Reppert, S. M. (2000). A time-less function for mouse timeless. Nat Neurosci *3*, 755-756.

Grima, B., Lamouroux, A., Chelot, E., Papin, C., Limbourg-Bouchon, B., and Rouyer, F. (2002). The F-box protein SLIMB controls the levels of clock proteins PERIOD and TIMELESS. Nature *420*, 178-182.

Gruwez, G., Hoste, C., Lints, C. V., and Lints, F. A. (1971). Oviposition rhythm in *Drosophila melanogaster* and its alteration by a change in the photoperiodicity. Experientia *27*, 1414-1416.

Hall, J. C. (1997). Circadian pacemakers blowing hot and cold--but they're clocks, not thermometers. Cell *90*, 9-12.

Hannibal, J., and Fahrenkrug, J. (2002). Melanopsin: a novel photopigment involved in the photoentrainment of the brain's biological clock? Ann Med *34*, 401-407.

Hao, H., Allen, D. L., and Hardin, P. E. (1997). A circadian enhancer mediates PER-dependent mRNA cycling in *Drosophila melanogaster*. Mol Cell Biol *17*, 3687-3693.

Hardin, P. E., Hall, J. C., and Rosbash, M. (1990). Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. Nature *343*, 536-540.

Hardin, P. E., Hall, J. C., and Rosbash, M. (1992a). Behavioral and molecular analyses suggest that circadian output is disrupted by *disconnected* mutants in *D.melanogaster*. EMBO J *11*, 1-6.

Hardin, P. E., Hall, J. C., and Rosbash, M. (1992b). Circadian oscillations in *period* gene mRNA levels are transcriptionally regulated. Proc Natl Acad Sci U S A *89*, 11711-11715.

Hastings, M. H., Field, M. D., Maywood, E. S., Weaver, D. R., and Reppert, S. M. (1999). Differential regulation of mPER1 and mTIM proteins in the mouse suprachiasmatic nuclei: new insights into a core clock mechanism. J Neurosci *19*, RC11.

Hattar, S., Liao, H. W., Takao, M., Berson, D. M., and Yau, K. W. (2002). Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science *295*, 1065-1070.

Hattar, S., Lucas, R. J., Mrosovsky, N., Thompson, S., Douglas, R. H., Hankins, M. W., Lem, J., Biel, M., Hofmann, F., Foster, R. G., and Yau, K. W. (2003). Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice. Nature *424*, 75-81.

Helfrich-Forster, C. (1995). The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster*. Proc Natl Acad Sci U S A *92*, 612-616.

Helfrich-Forster, C. (2000). Differential control of morning and evening components in the activity rhythm of *Drosophila melanogaster*--sex-specific differences suggest a different quality of activity. J Biol Rhythms *15*, 135-154.

Helfrich-Forster, C., Edwards, T., Yasuyama, K., Wisotzki, B., Schneuwly, S., Stanewsky, R., Meinertzhagen, I. A., and Hofbauer, A. (2002). The extraretinal eyelet of *Drosophila*: development, ultrastructure, and putative circadian function. J Neurosci *22*, 9255-9266.

Helfrich-Forster, C., Tauber, M., Park, J. H., Muhlig-Versen, M., Schneuwly, S., and Hofbauer, A. (2000). Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in *Drosophila melanogaster*. J Neurosci *20*, 3339-3353.

Helfrich-Forster, C., Winter, C., Hofbauer, A., Hall, J. C., and Stanewsky, R. (2001). The circadian clock of fruit flies is blind after elimination of all known photoreceptors. Neuron *30*, 249-261.

Hennessy, J. M. (1999) Functional analysis of a *Drosophila* clock gene, PhD, University of Leicester, Leicester.

Huang, Z. J., Curtin, K. D., and Rosbash, M. (1995). PER protein interactions and temperature compensation of a circadian clock in *Drosophila*. Science *267*, 1169-1172.

Huang, Z. J., Edery, I., and Rosbash, M. (1993). PAS is a dimerization domain common to *Drosophila period* and several transcription factors. Nature *364*, 259-262.

Hunter-Ensor, M., Ousley, A., and Sehgal, A. (1996). Regulation of the *Drosophila* protein TIMELESS suggests a mechanism for resetting the circadian clock by light. Cell *84*, 677-685.

Inoue, S., Shimoda, M., Nishinokubi, I., Siomi, M. C., Okamura, M., Nakamura, A., Kobayashi, S., Ishida, N., and Siomi, H. (2002). A role for the *Drosophila* fragile X-related gene in circadian output. Curr Biol *12*, 1331-1335.

Ishikawa, T., Matsumoto, A., Kato, T., Jr., Togashi, S., Ryo, H., Ikenaga, M., Todo, T., Ueda, R., and Tanimura, T. (1999). DCRY is a *Drosophila* photoreceptor protein implicated in light entrainment of circadian rhythm. Genes Cells *4*, 57-65.

Ivanchenko, M., Stanewsky, R., and Giebultowicz, J. M. (2001). Circadian photoreception in *Drosophila*: functions of *cryptochrome* in peripheral and central clocks. J Biol Rhythms *16*, 205-215.

Kay, S. A. (1997). PAS, present, and future: clues to the origins of circadian clocks. Science *276*, 753-754.

Kim, E. Y., Bae, K., Ng, F. S., Glossop, N. R., Hardin, P. E., and Edery, I. (2002). *Drosophila* CLOCK protein is under posttranscriptional control and influences light-induced activity. Neuron *34*, 69-81.

Kloss, B., Price, J. L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C. S., and Young, M. W. (1998). The *Drosophila* clock gene *double-time* encodes a protein closely related to *human casein kinase Iε*. Cell *94*, 97-107.

Kloss, B., Rothenfluh, A., Young, M. W., and Saez, L. (2001). Phosphorylation of PERIOD is influenced by cycling physical associations of DOUBLETIME, PERIOD, and TIMELESS in the *Drosophila* clock. Neuron *30*, 699-706.

Ko, H. W., Jiang, J., and Edery, I. (2002). Role for SLIMB in the degradation of *Drosophila* PERIOD protein phosphorylated by DOUBLETIME. Nature *20*, 20.

Krishnan, B., Levine, J. D., Lynch, M. K., Dowse, H. B., Funes, P., Hall, J. C., Hardin, P. E., and Dryer, S. E. (2001). A new role for *cryptochrome* in a *Drosophila* circadian oscillator. Nature *411*, 313-317.

Kume, K., Zylka, M. J., Sriram, S., Shearman, L. P., Weaver, D. R., Jin, X., Maywood, E. S., Hastings, M. H., and Reppert, S. M. (1999). mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell *98*, 193-205.

Kyriacou, C. P., and Hall, J. C. (1980). Circadian rhythm mutations in *Drosophila melanogaster* affect short-term fluctuations in the male's courtship song. Proc Natl Acad Sci U S A 77, 6729-6733.

Kyriacou, C. P., and Hall, J. C. (1989). Spectral analysis of Drosophila courtship song rhythms. Animal Behaviour 37, 850-859.

Lee, C., Bae, K., and Edery, I. (1999). PER and TIM inhibit the DNA binding activity of a *Drosophila* CLOCK- CYC/dBMAL1 heterodimer without disrupting formation of the heterodimer: a basis for circadian transcription. Mol Cell Biol *19*, 5316-5325.

Lee, C., Etchegaray, J. P., Cagampang, F. R., Loudon, A. S., and Reppert, S. M. (2001). Posttranslational mechanisms regulate the mammalian circadian clock. Cell *107*, 855-867.

Lee, C. G., Bae, K. H., and Edery, I. (1998). The *Drosophila* CLOCK protein undergoes daily rhythms in abundance, phosphorylation, and interactions with the PER-TIM complex. Neuron *21*, 857-867.

Lee, C. G., Parikh, V., Itsukaichi, T., Bae, K., and Edery, I. (1996). Resetting the *Drosophila* clock by photic regulation of PER and a PER- TIM complex. Science *271*, 1740-1744.

Levine, J. D., Funes, P., Dowse, H. B., and Hall, J. C. (2002). Resetting the circadian clock by social experience in *Drosophila melanogaster*. Science *298*, 2010-2012.

Lin, F. J., Song, W., Meyer-Bernstein, E., Naidoo, N., and Sehgal, A. (2001). Photic signaling by cryptochrome in the *Drosophila* circadian system. Mol Cell Biol *21*, 7287-7294.

Lin, J. M., Kilman, V. L., Keegan, K., Paddock, B., Emery-Le, M., Rosbash, M., and Allada, R. (2002a). A role for *casein kinase 2α* in the *Drosophila* circadian clock. Nature *24*, 24.

Lin, R. F., Chou, H. M., and Huang, T. C. (1999). Priority of light/dark entrainment over temperature in setting the circadian rhythms of the prokaryote Synechococcus RF-1. Planta *209*, 202-206.

Lin, Y., Han, M., Shimada, B., Wang, L., Gibler, T. M., Amarakone, A., Awad, T. A., Stormo, G. D., Van Gelder, R. N., and Taghert, P. H. (2002b). Influence of the *period*-dependent circadian clock on diurnal, circadian, and aperiodic gene expression in *Drosophila melanogaster*. Proc Natl Acad Sci U S A *99*, 9562-9567.

Liu, X., Lorenz, L., Yu, Q. N., Hall, J. C., and Rosbash, M. (1988). Spatial and temporal expression of the *period* gene in *Drosophila melanogaster*. Genes Dev *2*, 228-238.

Lowrey, P. L., Shimomura, K., Antoch, M. P., Yamazaki, S., Zemenides, P. D., Ralph, M. R., Menaker, M., and Takahashi, J. S. (2000). Positional syntenic cloning and functional characterization of the mammalian circadian mutation *tau*. Science *288*, 483-492.

Lucas, R. J., Freedman, M. S., Munoz, M., Garcia-Fernandez, J. M., and Foster, R. G. (1999). Regulation of the mammalian pineal by non-rod, non-cone, ocular photoreceptors. Science *284*, 505-507.

Majercak, J., Kalderon, D., and Edery, I. (1997). *Drosophila melanogaster* deficient in protein kinase a manifests behavior-specific arrhythmia but normal clock function. Mol Cell Biol *17*, 5915-5922.

Majercak, J., Sidote, D., Hardin, P. E., and Edery, I. (1999). How a circadian clock adapts to seasonal decreases in temperature and day length. Neuron *24*, 219-230.

Malpel, S., Klarsfeld, A., and Rouyer, F. (2002). Larval optic nerve and adult extra-retinal photoreceptors sequentially associate with clock neurons during *Drosophila* brain development. Development *129*, 1443-1453.

Martinek, S., Inonog, S., Manoukian, A. S., and Young, M. W. (2001). A role for the segment polarity gene *shaggy*/GSK-3 in the *Drosophila* circadian clock. Cell *105*, 769-779.

Matsumoto, A., Tomioka, K., Chiba, Y., and Tanimura, T. (1999). timrit Lengthens circadian period in a temperature-dependent manner through suppression of PERIOD protein cycling and nuclear localization. Mol Cell Biol *19*, 4343-4354.

McDonald, M. J., and Rosbash, M. (2001). Microarray analysis and organization of circadian gene expression in *Drosophila*. Cell *107*, 567-578.

McKay, R. R., Chen, D. M., Miller, K., Kim, S., Stark, W. S., and Shortridge, R. D. (1995). Phospholipase C rescues visual defect in *norpA* mutant of *Drosophila melanogaster*. J Biol Chem *270*, 13271-13276.

McNeil, G. P., Zhang, X. L., Genova, G., and Jackson, F. R. (1998). A molecular rhythm mediating circadian clock output in *Drosophila*. Neuron *20*, 297-303.

Menaker, M. (1959) Endogenous rhythms of body temperature in hibernating bats. Nature 184, 1251-1252.

Menaker, M. & Wisner, S. (1983) Temperature-compensated circadian clock in the pineal of *Anolis*. Proc. Natl. Acad. Sci. USA 80, 6119-6121.

Miyamoto, Y., and Sancar, A. (1998). Vitamin B2-based blue-light photoreceptors in the retinohypothalamic tract as the photoactive pigments for setting the circadian clock in mammals. Proc Natl Acad Sci U S A *95*, 6097-6102.

Moses, K., Ellis, M. C., and Rubin, G. M. (1989). The *glass* gene encodes a zinc-finger protein required by *Drosophila* photoreceptor cells. Nature *340*, 531-536.

Murata, T., Matsumoto, A., Tomioka, K., and Chiba, Y. (1995). *ritsu*: a rhythm mutant from a natural population of *Drosophila melanogaster*. J Neurogenet *9*, 239-249.

Myers, M. P., Wager-Smith, K., Rothenfluh-Hilfiker, A., and Young, M. W. (1996). Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock. Science *271*, 1736-1740.

Myers, M. P., Wager-Smith, K., Wesley, C. S., Young, M. W., and Sehgal, A. (1995). Positional cloning and sequence analysis of the *Drosophila* clock gene, *timeless*. Science *270*, 805-808.

Naidoo, N., and Sehgal, A. (1999). Light induced degradation of the clock protein, TIMELESS. Faseb Journal *13*, A1484.

Naidoo, N., Song, W., Hunter-Ensor, M., and Sehgal, A. (1999). A role for the proteasome in the light response of the TIMELESS clock protein. Science *285*, 1737-1741.

Newby, L. M., and Jackson, F. R. (1993). A new biological rhythm mutant of *Drosophila melanogaster* that identifies a gene with an essential embryonic function. Genetics *135*, 1077-1090.

Newby, L. M., and Jackson, F. R. (1996). Regulation of a specific circadian clock output pathway by *lark*, a putative RNA-binding protein with repressor activity. J Neurobiol *31*, 117-128.

Ouyang, Y., Andersson, C. R., Kondo, T., Gloden, S. S., and Johnson, C. H. (1998). Resonating circadian clocks enhance fitness in *cyanobacteria*. Proc Natl Acad Sci USA *95*, 8660-8664.

Panda, S., Sato, T. K., Castrucci, A. M., Rollag, M. D., DeGrip, W. J., Hogenesch, J. B., Provencio, I., and Kay, S. A. (2002). Melanopsin (Opn4) requirement for normal light-induced circadian phase shifting. Science *298*, 2213-2216.

Park, J. H., Helfrich-Forster, C., Lee, G., Liu, L., Rosbash, M., and Hall, J. C. (2000a). Differential regulation of circadian pacemaker output by seperate clock genes in *Drosophila*. Proc Natl Acad Sci U.S.A. *97*, 3608-3613.

Park, S. K., Sedore, S. A., Cronmiller, C., and Hirsh, J. (2000b). Type II cAMP-dependent protein kinase-deficient *Drosophila* are viable but show developmental, circadian, and drug response phenotypes. J Biol Chem *275*, 20588-20596.

Pearn, M. T., Randall, L. L., Shortridge, R. D., Burg, M. G., and Pak, W. L. (1996). Molecular, biochemical, and electrophysiological characterization of *Drosophila norpA* mutants. J Biol Chem *271*, 4937-4945.

Peixoto, A. A., Campesan, S., Costa, R., and Kyriacou, C. P. (1993). Molecular Evolution of a Repetitive Region Within the Per Gene of *Drosophila*. Molecular Biology and Evolution *10*, 127-139.

Peixoto, A. A., Costa, R., Wheeler, D. A., Hall, J. C., and Kyriacou, C. P. (1992). Evolution of the Threonine-Glycine Repeat Region of the Period Gene in the *Melanogaster* Species Subgroup of *Drosophila*. J Mol Evol *35*, 411-419.

Peixoto, A. A., Hennessy, J. M., Townson, I., Hasan, G., Rosbash, M., Costa, R., and Kyriacou, C. P. (1998). Molecular coevolution within a *Drosophila* clock gene. Proc Natl Acad Sci U.S.A. *95*, 4475-4480.

Petersen, G., Hall, J. C., and Rosbash, M. (1988). The *period* gene of *Drosophila* carries species-specific behavioural instructions. EMBO J *7*, 3939-3947.

Piccin, A., Couchman, M., Clayton, J. D., Chalmers, D., Costa, R., and Kyriacou, C. P. (2000). The clock gene period of the housefly, *Musca domestica*, rescues behavioral rhythmicity in *Drosophila melanogaster*. Evidence for intermolecular coevolution? Genetics *154*, 747-758.

Pittendrigh, C.S. (1954) On temperature independence in the clock-system controlling emergence time in Drosophila. Proc. Natl. Acad. Sci. USA 40, 1018-1029.

Pittendrigh, C. S., Caldarola, P. C., and Cosbey, E. S. (1973). A differential effect of heavy water on temperature-dependent and temperature-compensated aspects of circadian system of *Drosophila pseudoobscura*. Proc Natl Acad Sci U S A *70*, 2037-2041.

Plautz, J. D., Kaneko, M., Hall, J. C., and Kay, S. A. (1997). Independent photoreceptive circadian clocks throughout *Drosophila*. Science *278*, 1632-1635.

Price, J. L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B., and Young, M. W. (1998). *doubletime* is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. Cell *94*, 83-95.

Price, J. L., Dembinska, M. E., Young, M. W., and Rosbash, M. (1995). Suppression of PERIOD protein abundance and circadian cycling by the *Drosophila* clock mutation *timeless*. EMBO J *14*, 4044-4049.

Proudfoot, N. J., Furger, A., and Dye, M. J. (2002). Integrating mRNA processing with transcription. Cell *108*, 501-512.

Qiu, J., and Hardin, P. E. (1996). *per* mRNA cycling is locked to lights-off under photoperiodic conditions that support circadian feedback loop function. Mol Cell Biol *16*, 4182-4188.

Quesada, V., Macknight, R., Dean, C., and Simpson, G. G. (2003). Autoregulation of FCA pre-mRNA processing controls *Arabidopsis* flowering time. EMBO J *22*, 3142-3152.

Ralph, M. R., and Menaker, M. (1988). A mutation of the circadian system in golden hamsters. Science *241*, 1225-1227.

Recht, L. D., Lew, R. A., and Schwartz, W. J. (1995). Baseball teams beaten by jet lag. Nature *377*, 583.

Reed, R., and Hurt, E. (2002). A conserved mRNA export machinery coupled to pre-mRNA splicing. Cell *108*, 523-531.

Renn, S. C., Park, J. H., Rosbash, M., Hall, J. C., and Taghert, P. H. (1999). A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. Cell *99*, 791-802.

Reppert, S. M., Tsai, T., Roca, A. L., and Sauman, I. (1994). Cloning of a structural and functional homolog of the circadian clock gene *period* from the giant silkmoth *Antheraea pernyi*. Neuron *13*, 1167-1176.

Roberts, D. H., Lehar, J., and Dreher, J. W. (1987). Time series with CLEAN.I. Derivation of a spectrum. Astronomy Journal 93, 968-989.

Rosato, E., Codd, V., Mazzotta, G., Piccin, A., Zordan, M., Costa, R., and Kyriacou, C. P. (2001). Light-dependent interaction between *Drosophila* CRY and the clock protein PER mediated by the carboxy terminus of CRY. Curr Biol *11*, 909-917.

Rosato, E., and Kyriacou, C. P. (2001). Flies, clocks and evolution. Philos Trans R Soc Lond B Biol Sci *356*, 1769-1778.

Rothenfluh, A., Abodeely, M., Price, J. L., and Young, M. W. (2000a). Isolation and analysis of six *timeless* alleles that cause short- or long-period circadian rhythms in *Drosophila*. Genetics *156*, 665-675.

Rothenfluh, A., Abodeely, M., and Young, M. W. (2000b). Short-period mutations of *per* affect a *double-time*-dependent step in the *Drosophila* circadian clock. Curr Biol *10*, 1399-1402.

Rothenfluh, A., Young, M. W., and Saez, L. (2000c). A TIMELESS-independent function for PERIOD proteins in the *Drosophila* clock. Neuron *26*, 505-514.

Ruby, N. F., Brennan, T. J., Xie, X., Cao, V., Franken, P., Heller, H. C., and O'Hara, B. F. (2002). Role of melanopsin in circadian responses to light. Science *298*, 2211-2213.

Rutila, J. E., Maltseva, O., and Rosbash, M. (1998a). The tim^{SL} mutant affects a restricted portion of the *Drosophila melanogaster* circadian cycle. J Biol Rhythms *13*, 380-392.

Rutila, J. E., Suri, V., Le, M., So, W. V., Rosbash, M., and Hall, J. C. (1998b). CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. Cell *93*, 805-814.

Rutila, J. E., Zeng, H., Le, M., Curtin, K. D., Hall, J. C., and Rosbash, M. (1996). The tim^{SL} mutant of the *Drosophila* rhythm gene *timeless* manifests allele-specific interactions with period gene mutants. Neuron *17*, 921-929.

Saez, L., and Young, M. W. (1996). Regulation of nuclear entry of the *Drosophila* clock proteins PERIOD and TIMELESS. Neuron *17*, 911-920.

Sakai, T., and Ishida, N. (2001). Circadian rhythms of female mating activity governed by clock genes in *Drosophila*. Proc Natl Acad Sci U S A *98*, 9221-9225.

Sauman, I., and Reppert, S. M. (1996). Circadian clock neurons in the silkmoth *Antheraea pernyi*: novel mechanisms of PERIOD protein regulation. Neuron *17*, 889-900.

Sawyer, L. A., Hennessy, J. M., Peixoto, A. A., Rosato, E., Parkinson, H., Costa, R., and Kyriacou, C. P. (1997). Natural variation in a *Drosophila* clock gene and temperature compensation. Science *278*, 2117-2120.

Schneuwly, S., Burg, M. G., Lending, C., Perdew, M. H., and Pak, W. L. (1991). Properties of photoreceptor-specific phospholipase C encoded by the *norpA* gene of *Drosophila melanogaster*. J Biol Chem *266*, 24314-24319.

Sehgal, A., Price, J. L., Man, B., and Young, M. W. (1994). Loss of circadian behavioral rhythms and *per* RNA oscillations in the *Drosophila* mutant *timeless*. Science *263*, 1603-1606.

Sehgal, A., Rothenfluh-Hilfiker, A., Hunter-Ensor, M., Chen, Y., Myers, M. P., and Young, M. W. (1995). Rhythmic expression of *timeless*: a basis for promoting circadian cycles in *period* gene autoregulation. Science *270*, 808-810.

Selby, C. P., Thompson, C., Schmitz, T. M., Van Gelder, R. N., and Sancar, A. (2000). Functional redundancy of *cryptochromes* and classical photoreceptors for non-visual ocular photoreception in mice. Proc Natl Acad Sci U S A *97*, 14697-14702.

SenGupta, D. J., Zhang, B., Kraemer, B., Pochart, P., Fields, S., and Wickens, M. (1996). A three-hybrid system to detect RNA-protein interactions in vivo. Proc Natl Acad Sci U S A *93*, 8496-8501.

Shafer, O. T., Rosbash, M., and Truman, J. W. (2002). Sequential nuclear accumulation of the clock proteins PERIOD and TIMELESS in the pacemaker neurons of *Drosophila melanogaster*. J Neurosci *22*, 5946-5954.

Shearman, L. P., Sriram, S., Weaver, D. R., Maywood, E. S., Chaves, I., Zheng, B., Kume, K., Lee, C. C., van der Horst, G. T., Hastings, M. H., and Reppert, S. M. (2000). Interacting molecular loops in the mammalian circadian clock. Science *288*, 1013-1019.

Sidote, D., Majercak, J., Parikh, V., and Edery, I. (1998). Differential effects of light and heat on the *Drosophila* circadian clock proteins PER and TIM. Mol Cell Biol *18*, 2004-2013.

Simpson, G. G., Dijkwel, P. P., Quesada, V., Henderson, I., and Dean, C. (2003). FY is an RNA 3' end-processing factor that interacts with FCA to control the *Arabidopsis* floral transition. Cell *113*, 777-787.

So, W. V., and Rosbash, M. (1997). Post-transcriptional regulation contributes to *Drosophila clock* gene mRNA cycling. EMBO J *16*, 7146-7155.

Stanewsky, R. (2002). Clock mechanisms in *Drosophila*. Cell Tissue Res *309*, 11-26.

Stanewsky, R. (2003). Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals. J Neurobiol *54*, 111-147.

Stanewsky, R., Jamison, C. F., Plautz, J. D., Kay, S. A., and Hall, J. C. (1997). Multiple circadian-regulated elements contribute to cycling period gene expression in *Drosophila*. EMBO J *16*, 5006-5018.

Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., Kay, S. A., Rosbash, M., and Hall, J. C. (1998). The *cry*^{b} mutation identifies *cryptochrome* as a circadian photoreceptor in *Drosophila*. Cell *95*, 681-692.

Stanewsky, R., Lynch, K. S., Brandes, C., and Hall, J. C. (2002). Mapping of elements involved in regulating normal temporal *period* and *timeless* RNA expression patterns in *Drosophila melanogaster*. J Biol Rhythms *17*, 293-306.

Stehle, J. H., Foulkes, N. S., Molina, C. A., Simonneaux, V., Pevet, P., and Sassone-Corsi, P. (1993). Adrenergic signals direct rhythmic expression of transcriptional repressor CREM in the pineal gland. Nature *365*, 314-320.

Sugano, S., Andronis, C., Green, R. M., Wang, Z. Y., and Tobin, E. M. (1998). Protein kinase CK2 interacts with and phosphorylates the *Arabidopsis* circadian clock-associated 1 protein. Proc Natl Acad Sci U S A *95*, 11020-11025.

Sugano, S., Andronis, C., Ong, M. S., Green, R. M., and Tobin, E. M. (1999). The protein kinase CK2 is involved in regulation of circadian rhythms in *Arabidopsis*. Proc Natl Acad Sci U S A *96*, 12362-12366.

Suri, V., Hall, J. C., and Rosbash, M. (2000). Two novel *doubletime* mutants alter circadian properties and eliminate the delay between RNA and protein in *Drosophila*. J Neurosci *20*, 7547-7555.

Suri, V., Lanjuin, A., and Rosbash, M. (1999). TIMELESS-dependent positive and negative autoregulation in the *Drosophila* circadian clock. EMBO J *18*, 675-686.

Suri, V., Qian, Z., Hall, J. C., and Rosbash, M. (1998). Evidence that the TIM light response is relevant to light-induced phase shifts in *Drosophila melanogaster*. Neuron *21*, 225-234.

Tamaru, T., Isojima, Y., Yamada, T., Okada, M., Nagai, K., and Takamatsu, K. (2000). Light and glutamate-induced degradation of the circadian oscillating protein BMAL1 during the mammalian clock resetting. J Neurosci *20*, 7525-7530.

Tauber, E., Roe, H., Costa, R., Hennessy, J. M., and Kyriacou, C. P. (2003). Temporal mating isolation driven by a behavioral gene in *Drosophila*. Curr Biol *13*, 140-145.

Toh, K., Jones, C., He, Y., Eide, E., Hinz, W., Virshup, D., Ptacek, L., and Fu, Y.-H. (2000). An h*Per2* Phosphorylation Site mutation in Familial advanced Sleep Phase Syndrome. Science *291*, 1040.

Toma, D. P., Bloch, G., Moore, D., and Robinson, G. E. (2000). Changes in *period* mRNA levels in the brain and division of labor in honey bee colonies. Proc Natl Acad Sci U S A *97*, 6914-6919.

van der Horst, G. T., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S., Takao, M., de Wit, J., Verkerk, A., Eker, A. P., van Leenen, D., *et al.* (1999). Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature *398*, 627-630.

Vodovar, N., Clayton, J. D., Costa, R., Odell, M., and Kyriacou, C. P. (2002). The *Drosophila* clock protein TIMELESS is a member of the Arm/HEAT family. Curr Biol *12*, R610-611.

Vosshall, L. B., Price, J. L., Sehgal, A., Saez, L., and Young, M. W. (1994). Block in nuclear localization of PERIOD protein by a second clock mutation, *timeless*. Science *263*, 1606-1609.

Vosshall, L. B., and Young, M. W. (1995). Circadian rhythms in *Drosophila* can be driven by *period* expression in a restricted group of central brain cells. Neuron *15*, 345-360.

Wager-Smith, K., and Kay, S. A. (2000). Circadian rhythm genetics: from flies to mice to humans. Nat Genet *26*, 23-27.

Wang, R. L., and Hey, J. (1996). The speciation history of *Drosophila pseudoobscura* and close relatives: inferences from DNA sequence variation at the *period* locus. Genetics *144*, 1113-1126.

Weaver, D. R. (1998). The suprachiasmatic nucleus: a 25-year retrospective. J Biol Rhythms *13*, 100-112.

Wheeler, D. A., Hamblen-Coyle, M. J., Dushay, M. S., and Hall, J. C. (1993). Behavior in light-dark cycles of *Drosophila* mutants that are arrhythmic, blind, or both. J Biol Rhythms *8*, 67-94.

Williams, J. A., Su, H. S., Bernards, A., Field, J., and Sehgal, A. (2001). A circadian output in *Drosophila* mediated by *neurofibromatosis-1* and *Ras/MAPK*. Science *293*, 2251-2256.

Yagita, K., Yamaguchi, S., Tamanini, F., van Der Horst, G. T., Hoeijmakers, J. H., Yasui, A., Loros, J. J., Dunlap, J. C., and Okamura, H. (2000). Dimerization and nuclear entry of mPER proteins in mammalian cells. Genes Dev *14*, 1353-1363.

Yang, Z., and Sehgal, A. (2001). Role of molecular oscillations in generating behavioral rhythms in *Drosophila*. Neuron *29*, 453-467.

Yang, Z. H., Emerson, M., Su, H. S., and Sehgal, A. (1998). Response of the timeless protein to light correlates with behavioral entrainment and suggests a non-visual pathway for circadian photoreception. Neuron *21*, 215-223.

Yu, Q., Colot, H. V., Kyriacou, C. P., Hall, J. C., and Rosbash, M. (1987a). Behaviour modification by *in vitro* mutagenesis of a variable region within the period gene of *Drosophila*. Nature *326*, 765-769.

Yu, Q., Jacquier, A. C., Citri, Y., Hamblen, M., Hall, J. C., and Rosbash, M. (1987b). Molecular mapping of point mutations in the period gene that stop or speed up biological clocks in *Drosophila melanogaster*. Proc Natl Acad Sci U S A *84*, 784-788.

Zeng, H. K., Qian, Z. W., Myers, M. P., and Rosbash, M. (1996). A light-entrainment mechanism for the *Drosophila* circadian clock. Nature *380*, 129-135.

Zerr, D. M., Hall, J. C., Rosbash, M., and Siwicki, K. K. (1990). Circadian fluctuations of PERIOD protein immunoreactivity in the CNS and the visual system of *Drosophila*. J Neurosci *10*, 2749-2762.

Zheng, B., Larkin, D. W., Albrecht, U., Sun, Z. S., Sage, M., Eichele, G., Lee, C. C., and Bradley, A. (1999). The mPER2 gene encodes a functional component of the mammalian circadian clock. Nature *400*, 169-173.

Zheng, X. Z., Zhang, Y. P., Zhu, D. L., and Geng, Z. C. (1999c). The period gene: high conservation of the region coding for Thr-Gly dipeptides in the *Drosophila nasuta* species subgroup. J Mol Evol *49*, 406-410.

Zilian, O., Frei, E., Burke, R., Brentrup, D., Gutjahr, T., Bryant, P. J., and Noll, M. (1999). *double-time* is identical to *discs overgrown*, which is required for cell survival, proliferation and growth arrest in *Drosophila* imaginal discs. Development *126*, 5409-5420.

Zimmerman, W. F., Pittendrigh, C. S., and Pavlidis, T. (1968). Temperature compensation of the circadian oscillation in *Drosophila pseudoobscura* and its entrainment by temperature cycles. J Insect Physiol *14*, 669-684.

Appendix to Chapter 3

All statistical data contained within the appendices was produced through ANOVA analysis of variance except Table 3.1 (T-test). Significant p values are indicated in red.

3.2 (p. 57-58)

1. All Time points	**SS**	**df**	**MS**	**F**	**p**
Temperature	7711	1	7711	28.79	0.000000
Time	1271764	47	27059	101.04	0.000000
Temperature*Time	320136	47	6811	25.43	0.000000
Error	2014396	7522	268		

2. Morning Peak (11-15)	**SS**	**df**	**MS**	**F**	**p**
Temperature	1002.9	1	1002.9	2.936	0.086989
Time	24265.1	4	6066.3	17.759	0.000000
Temperature* Time	12005.9	4	3001.5	8.787	0.000001
Error	288637.7	845	341.6		

3. Day activity (20-29)	**SS**	**df**	**MS**	**F**	**p**
Temperature	105510.9	1	105510.947	2.603	0.000000
Time	22653.7	9	2517.1	11.274	0.000000
Temperature*Time	8848.3	9	983.1	4.404	0.000010
Error	46491.2	1552	223.3		

4. Evening peak (30-38)	**SS**	**df**	**MS**	**F**	**p**
Temperature	23866	1	23866	58.646	0.000000
Time	324622	8	40578	99.711	0.000000
Temperature*Time	119052	8	14882	36.568	0.000000
Error	571772	1405	407		

3.3

Splicing	**SS**	**df**	**MS**	**F**	**p**
Temperature	0.671114	1	0.671114	156.542	0.000000
Time	0.027897	5	0.005579	1.301	0.274744
Temperature*Time	0.033227	5	0.006645	1.550	0.187295
Error	0.270088	63	0.004287		

3.4

1. *per*^{01}, all Times	**SS**	**df**	**MS**	**F**	**p**
Temperature	325	1	325	1.207	0.272133
Time	289101	47	6151	22.858	0.000000
Temperature*Time	18228	47	388	1.441	0.027377
Error	503749	1872	269		

2. *tim*^{01}, all Times	**SS**	**df**	**MS**	**F**	**p**
Temperature	319.9	1	319.9	2.147	0.143012
Time	342381.5	47	7284.7	48.908	0.000000
Temperature*Time	11621.1	47	247.3	1.660	0.003584
Error	228782.7	1536	148.9		

3. per^{01}, tim^{01}, all Times	SS	df	MS	F	p
Genotype	5440	1	5440	25.307	0.000001
Temperature	2	1	2	0.008	0.930091
Time	605641	47	12886	59.950	0.000000
Genotype*Temperature	642	1	642	2.989	0.083943
Genotype*Time	31633	47	673	3.131	0.000000
Temperature*Time	19551	47	416	1.935	0.000148
Genotype*Temp*Time	9581	47	204	0.948	0.573524
Error	732532	3408	215		

4. All Genotypes, Time Bins 12-14	SS	df	MS	F	p
Genotype	52683.1	2	26341.6	77.104	0.000000
Temperature	3504.1	1	3504.1	10.257	0.001422
Time	135509.6	2	67754.8	198.324	0.000000
Genotype*Temperature	3151.1	2	1575.5	4.612	0.010230
Genotype*Time	51358.7	4	12839.7	37.583	0.000000
Temperature*Time	6123.0	2	3061.5	8.961	0.000143
Genotype*Temp*Time	1687.5	4	421.9	1.235	0.294604
Error	245979.2	720	341.6		

5. Canton-S, Bins 12-14	SS	df	MS	F	p
Temperature	1624.3	1	1624.3	5.103	0.024343
Time	16606.9	2	8303.5	26.088	0.000000
Temperature*Time	6293.9	2	3147.0	9.887	0.000062
Error	148002.0	465	318.3		

6. per^{0}, Bins 12-14	SS	df	MS	F	p
Temperature	4674.3	1	4674.3	11.7718	0.000832
Time	42438.9	2	21219.5	53.4394	0.000000
Temperature*Time	3173.5	2	1586.7	3.9960	0.020953
Error	46457.8	117	397.1		

7. tim^{0}, Bins 12-14	SS	df	MS	F	p
Temperature	292.4	1	292.4	1.340	0.249924
Time	78877.3	2	39438.6	180.735	0.000000
Temperature*Time	1734.8	2	867.4	3.975	0.021948
Error	20948.4	96	218.2		

8. per^{01}, tim^{01}, 18°C	SS	df	MS	F	p
Genotype	287.3	1	287.3	0.8934	0.346926
Time	66525.8	2	33262.9	103.4514	0.000000
Genotype*Time	1695.1	2	847.6	2.6360	0.076828
Error	30867.0	96	321.5		

9. per^{01}, tim^{01}, Canton-S 18°C	SS	df	MS	F	p
Genotype	34168.8	2	17084.4	45.968	0.000000
Time	83638.0	2	41819.0	112.520	0.000000
Genotype*Time	21921.0	4	5480.2	14.745	0.000000
Error	134911.9	363	371.7		

10. per^{01}, tim^{01} 29°C	SS	df	MS	F	p
Genotype	4651.4	1	4651.4	14.8938	0.000187
Time	54908.7	2	27454.3	87.9099	0.000000
Genotype*Time	6117.2	2	3058.6	9.7938	0.000117
Error	36539.2	117	312.3		

11. *per*^{01}, Canton-S, 29°C

	SS	df	MS	F	p
Genotype	3798.3	1	3798.3	11.5812	0.000757
Time	15273.2	2	7636.6	23.2844	0.000000
Genotype*Time	6974.6	2	3487.3	10.6330	0.000035
Error	98391.2	300	328.0		

12. *tim*^{01}, Canton-S, 29°C

	SS	df	MS	F	p
Genotype	21252.5	1	21252.5	72.3818	0.000000
Time	45146.8	2	22573.4	76.8805	0.000000
Genotype*Time	30556.5	2	15278.2	52.0346	0.000000
Error	87204.2	297	293.6		

3.5

1. All genotypes	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.06902	2	0.03451	8.183	0.000432
Temperature	0.64024	1	0.64024	151.819	0.000000
Time	0.10667	5	0.02133	5.059	0.000267
Genotype*Temperature	0.13363	2	0.06682	15.844	0.000001
Genotype*Time	0.04221	10	0.00422	1.001	0.445652
Temperature*Time	0.10388	5	0.02078	4.927	0.000343
Genotype*Temp*Time	0.06771	10	0.00677	1.606	0.110554
Error	0.60305	143	0.00422		

2. *per*^{01}	**SS**	**df**	**MS**	**F**	**p**
Temperature	0.099085	1	0.099085	23.805	0.000012
Time	0.090607	5	0.018121	4.354	0.002393
Temperature*Time	0.063127	5	0.012625	3.033	0.018535
Error	0.199796	48	0.004162		

3. *tim*^{01}	**SS**	**df**	**MS**	**F**	**p**
Temperature	0.090878	1	0.090878	21.279	0.000065
Time	0.032780	5	0.006556	1.535	0.207782
Temperature*Time	0.062341	5	0.012468	2.919	0.028415
Error	0.132395	31	0.004271		

4. All Genotypes, 18°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.01268	2	0.00634	1.471	0.235304
Time	0.03162	5	0.00632	1.466	0.208809
Genotype*Time	0.07085	10	0.00708	1.643	0.107376
Error	0.38377	89	0.00431		

5. All Genotypes, 29°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.153532	2	0.076766	18.904	0.000001
Time	0.163268	5	0.032654	8.041	0.000010
Genotype*Time	0.046517	10	0.004652	1.146	0.347280
Error	0.219281	54	0.004061		

6. *per*^{0} and *tim*^{0}, 29°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.006062	1	0.006062	1.1643	0.287950
Time	0.148632	5	0.029726	5.7095	0.000594
Genotype*Time	0.007785	5	0.001557	0.2990	0.910093
Error	0.182228	35	0.005207		

3.6

1. *per*^{0}, Canton-S	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.00094	1	0.00094	0.218	0.641848
Light regime	0.15115	1	0.15115	34.854	0.000000
Time	0.02149	5	0.00430	0.991	0.427747
Genotype*Light regime	0.00702	1	0.00702	1.620	0.206455
Genotype*Time	0.02969	5	0.00594	1.369	0.243545
Light regime*Time	0.02219	5	0.00444	1.023	0.408862
Genotype* Light regime*Time	0.03703	5	0.00741	1.708	0.140997
Error	0.38595	89	0.00434		

2. Canton-S 12:12 and 6:18	**SS**	**df**	**MS**	**F**	**p**
Light Regime	0.06027	1	0.06027	12.736	0.000744
Time	0.03039	5	0.00608	1.284	0.283589
Light regime*Time	0.02963	5	0.00593	1.252	0.297441
Error	0.26500	56	0.00473		

3. *per*^{0} 12:12 and 6:18	**SS**	**df**	**MS**	**F**	**p**
Light Regime	0.088053	1	0.088053	24.340	0.000022
Time	0.016269	5	0.003254	0.899	0.493061
Light Regime*Time	0.026270	5	0.005254	1.452	0.23190
Error	0.119380	33	0.003618		

4. *per*^{0}, Canton-S, 6:18	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.004343	1	0.004343	1.296	0.271728
Time	0.021040	5	0.004208	1.256	0.329985
Genotype* Time	0.031916	5	0.006383	1.905	0.149624
Error	0.053619	16	0.003351		

3.7

1. Canton-S, LD and DD, 18°C	**SS**	**df**	**MS**	**F**	**p**
Light Regime	0.13764	1	0.13764	28.735	0.000002
Time	0.08150	5	0.01630	3.403	0.009178
Light Regime*Time	0.05434	5	0.01087	2.269	0.059389
Error	0.27781	58	0.00479		

2. Canton-S, LD and DD, 29°C	**SS**	**df**	**MS**	**F**	**p**
Light Regime	0.050486	1	0.050486	27.5197	0.000099
Time	0.005899	4	0.001475	0.8039	0.541487
Light Regime*Time	0.015371	4	0.003843	2.0947	0.132223
Error	0.027518	15	0.001835		

3. *per*^{0}, LD and DD, 18°C	**SS**	**df**	**MS**	**F**	**p**
Light Regime	0.000749	1	0.000749	0.255	0.616215
Time	0.064704	5	0.012941	4.412	0.002798
Light Regime*Time	0.022704	5	0.004541	1.548	0.197582
Error	0.114388	39	0.002933		

4. *per*^{0}, LD and DD, 29°C	**SS**	**df**	**MS**	**F**	**p**
Light Regime	0.008002	1	0.008002	2.3398	0.140356
Circadian Time	0.040114	4	0.010028	2.9325	0.043852
Light Regime*Time	0.022154	4	0.005538	1.6196	0.204813
Error	0.075234	22	0.003420		

5. Canton-S and *per*^{0}, DD 18°C

	SS	df	MS	F	p
Genotype	0.130412	1	0.130412	52.285	0.000000
Time	0.046342	5	0.009268	3.716	0.012422
Genotype* Time	0.069115	5	0.013823	5.542	0.001568
Error	0.059862	24	0.002494		

6. Canton-S and *per*^{0}, DD 29°C

	SS	df	MS	F	p
Genotype	0.001712	1	0.001712	2.879	0.140656
Time	0.004913	3	0.001638	2.755	0.134466
Genotype* Time	0.005582	3	0.001861	3.129	0.108919
Error	0.003567	6	0.000595		

3.8

1.	SS	df	MS	F	p
Genotype	21.35	4	5.34	9.8	0.000000
Temperature	0.48	1	0.48	0.9	0.349146
Genotype*Temperature	2.52	4	0.63	1.2	0.331734
Error	109.18	200	0.55		

2. 17 Thr-Gly	SS	df	MS	F	p
Temperature	0.18	1	0.18	0.34	0.560397
Error	27.13	51	0.53		

3. 20 Thr-Gly	SS	df	MS	F	p
Temperature	0.12	1	0.12	0.61	0.442511
Error	4.68	24	0.19		

4. 21 Thr-Gly	SS	df	MS	F	p
Temperature	0.33	1	0.33	0.64	0.427835
Error	24.76	48	0.52		

5. 23 Thr-Gly	SS	df	MS	F	p
Temperature	1.67	1	1.67	2.60	0.119325
Error	16.04	25	0.64		

6. Canton-S	SS	df	MS	F	p
Temperature	0.35	1	0.35	0.49	0.486252
Error	36.57	52	0.70		

7. 18°C	SS	df	MS	F	p
Genotype	16.58	4	4.14	9.2	0.000001
Error	63.63	142	0.45		

8. 29°C	SS	df	MS	F	p
Genotype	10.85	4	2.71	3.45	0.013389
Error	45.55	58	0.79		

3.9

1. 17 Thr-Gly	SS	df	MS	F	p
Temperature	8542	1	8542	22.723	0.000002
Time	103524	8	12941	34.423	0.000000
Temperature*Time	44589	8	5574	14.827	0.000000
Error	250365	666	376		

2. 20 Thr-Gly	SS	df	MS	F	p
Temperature	2983.8	1	2983.8	12.901	0.000368
Time	121735.1	8	15216.96	5.792	0.000000
Temperature*Time	92488.5	8	11561.14	9.986	0.000000
Error	95752.6	414	231.3		

3. 21 Thr-Gly	SS	df	MS	F	p
Temperature	8069.8	1	8069.8	27.131	0.000000
Time	70315.0	8	8789.4	29.550	0.000000
Temperature*Time	31588.8	8	3948.6	13.275	0.000000
Error	214156.3	720	297.4		

4. 23 Thr-Gly	SS	df	MS	F	p
Temperature	2182	1	2182	5.146	0.023648
Time	55707	8	6963	16.421	0.000000
Temperature*Time	35712	8	4464	10.527	0.000000
Error	259520	612	424		

Time Bins 18°C = 32-37; 29°C = 30-38

5. 18°C, 17, 21 Canton-S	SS	df	MS	F	p
Genotype	470	2	235	0.590	0.554554
Time	49997	5	9999	25.112	0.000000
Genotype*Time	5664	10	566	1.423	0.164719
Error	433238	1088	398		

6. 18°C, 17, 21, 23 Canton-S	SS	df	MS	F	p
Genotype	3541	3	1180	3.086	0.026403
Time	39920	5	7984	20.876	0.000000
Genotype*Time	10531	15	70	2.836	0.025758
Error	98723	1304	382		

7. 20, 23	SS	df	MS	F	p
Genotype	1278.5	1	1278.5	3.924	0.048410
Time	24749.6	5	4949.9	15.191	0.000000
Genotype*Time	13134.1	5	2626.8	8.062	0.000000
Error	111440.2	342	325.8		

8. 18°C, 17, 21, 20 Canton-S	SS	df	MS	F	p
Genotype	472	3	157	0.399	0.753862
Time	67953	5	13591	34.431	0.000000
Genotype*Time	19876	15	1325	3.357	0.000014
Error	479194	1214	395		

9. 29°C 17, 21, Canton-S	SS	df	MS	F	p
Genotype	1491	2	745	1.923	0.146601
Time	223123	8	27890	71.967	0.000000
Genotype*Time	6606	16	413	1.065	0.384051
Error	449939	1161	388		

10. 29°C All genotypes	SS	df	MS	F	p
Genotype	6447	4	1612	4.125	0.002493
Time	407615	8	50952	130.411	0.000000
Genotype*Time	35647	32	1114	2.851	0.000000
Error	654037	1674	391		

11. 29°C, 20, 23	SS	df	MS	F	p
Genotype	1528.6	1	1528.6	3.842	0.050519
Time	194104.2	8	24263.0	60.985	0.000000
Genotype*Time	16467.9	8	2058.5	5.174	0.000003
Error	204098.6	513	397.9		

3.10

1. 17 Thr-Gly	**SS**	**df**	**MS**	**F**	**p**
Temperature	0.278191	1	0.278191	52.1616	0.000000
Time	0.013390	5	0.002678	0.5021	0.772533
Temperature*Time	0.041816	5	0.008363	1.5681	0.194667
Error	0.186664	35	0.005333		

2. 20 Thr-Gly	**SS**	**df**	**MS**	**F**	**p**
Temperature	0.104383	1	0.104383	33.122	0.000003
Time	0.061028	5	0.012206	3.873	0.008244
Temperature*Time	0.061084	5	0.012217	3.877	0.008206
Error	0.091394	29	0.003152		

3. 21 Thr-Gly	**SS**	**df**	**MS**	**F**	**p**
Temperature	0.397622	1	0.397622	49.1099	0.000000
Time	0.073120	5	0.014624	1.8062	0.134287
Temperature*Time	0.042599	5	0.008520	1.0523	0.401349
Error	0.315766	39	0.008097		

4. 23 Thr-Gly	**SS**	**df**	**MS**	**F**	**p**
Temperature	0.115391	1	0.115391	16.7624	0.000229
Time	0.040849	5	0.008170	1.1868	0.334922
Temperature*Time	0.022751	5	0.004550	0.6610	0.655268
Error	0.247820	36	0.006884		

5. 17, 21, 23 18°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.01392	2	0.00696	0.732	0.485017
Time	0.05705	5	0.01141	1.200	0.319948
Genotype *Time	0.11961	10	0.01196	1.258	0.274243
Error	0.57999	61	0.00951		

6. 17, 20, 21, 23 18°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.02539	3	0.00846	1.028	0.384658
Time	0.12590	5	0.02518	3.059	0.014080
Genotype*Time	0.18152	15	0.01210	1.470	0.136762
Error	0.65848	80	0.00823		

7. 17, 21, 23, Canton-S 18°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.10328	3	0.03443	4.178	0.007709
Time	0.05589	5	0.01118	1.357	0.246548
Genotype*Time	0.14529	15	0.00969	1.175	0.301996
Error	0.88174	107	0.00824		

8. 20, Canton-S 18°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.01263	1	0.01263	2.160	0.146512
Time	0.11618	5	0.02324	3.972	0.003323
Genotype*Time	0.06317	5	0.01263	2.160	0.069367
Error	0.38025	65	0.00585		

9. All except 20 Thr-Gly, 18°C, Night only	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.06885	3	0.02295	2.741	0.051521
Time	0.00578	2	0.00289	0.345	0.709639
Genotype*Time	0.05709	6	0.00952	1.136	0.353219
Error	0.47725	57	0.00837		

10. All variants

18°C, Night only	SS	df	MS	F	p
Genotype	0.09582	4	0.02396	3.160	0.019416
Time	0.01369	2	0.00684	0.903	0.410357
Genotype*Time	0.06584	8	0.00823	1.086	0.384164
Error	0.50028	66	0.00758		

11. 17, 20 29°C	SS	df	MS	F	p
Genotype	0.055682	1	0.055682	33.006	0.000006
Time	0.024854	5	0.004971	2.946	0.031705
Genotype*Time	0.021327	5	0.004265	2.528	0.055271
Error	0.042176	25	0.001687		

12. 17, 21 29°C	SS	df	MS	F	p
Genotype	0.011740	1	0.011740	4.8202	0.034849
Time	0.027650	5	0.005530	2.2705	0.068786
Genotype*Time	0.011666	5	0.002333	0.9579	0.456642
Error	0.085245	35	0.002436		

13. 17, 23 29°C	SS	df	MS	F	p
Genotype	0.060336	1	0.060336	15.4379	0.000464
Time	0.046706	5	0.009341	2.3901	0.061338
Genotype*Time	0.004710	5	0.000942	0.2410	0.941049
Error	0.117248	30	0.003908		

14. 17, Canton-S 29°C	SS	df	MS	F	p
Genotype	0.002276	1	0.002276	1.177	0.285986
Time	0.053845	5	0.010769	5.571	0.000842
Genotype*Time	0.010229	5	0.002046	1.058	0.401460
Error	0.061859	32	0.001933		

15. 20, 21 29°C	SS	df	MS	F	p
Genotype	0.119580	1	0.119580	52.088	0.000000
Time	0.008001	5	0.001600	0.697	0.629834
Genotype*Time	0.024926	5	0.004985	2.172	0.083890
Error	0.068872	30	0.002296		

16. 20, 23 29°C	SS	df	MS	F	p
Genotype	0.000073	1	0.000073	0.0182	0.893892
Time	0.031209	5	0.006242	1.5469	0.211495
Genotype*Time	0.013019	5	0.002604	0.6453	0.667514
Error	0.100875	25	0.004035		

17. 20, Canton-S 29°C	SS	df	MS	F	p
Genotype	0.036410	1	0.036410	21.613	0.000078
Time	0.020400	5	0.004080	2.422	0.061503
Genotype*Time	0.033407	5	0.006681	3.966	0.007951
Error	0.045486	27	0.001685		

18. 21, 23 29°C	SS	df	MS	F	p
Genotype	0.134387	1	0.134387	32.6762	0.000002
Time	0.021074	5	0.004215	1.0248	0.418165
Genotype*Time	0.018468	5	0.003694	0.8981	0.493209
Error	0.143944	35	0.004113		

19. 21, Canton-S 29°C	SS	df	MS	F	p
Genotype	0.024938	1	0.024938	10.4195	0.002613
Time	0.034029	5	0.006806	2.8436	0.028561
Genotype*Time	0.016425	5	0.003285	1.3726	0.256887
Error	0.088555	37	0.002393		

20. 23, Canton-S 29°C	SS	df	MS	F	p
Genotype	0.038716	1	0.038716	10.2764	0.003051
Time	0.052805	5	0.010561	2.8032	0.032878
Genotype*Time	0.009754	5	0.001951	0.5178	0.760831
Error	0.120558	32	0.003767		

	free-running period (hours)		proportion spliced *per* mRNA - ZT 0-20		proportion spliced *per* mRNA - ZT 12-20		difference in spliced *per* mRNA levels, ZT 0-20	difference in spliced *per* mRNA levels, ZT 12-20	free-running period difference
	18	29	18	29	18	29			
17	23.83538	23.96857	0.381411	0.2263132	0.3779337	0.2468246	0.1550975	0.1311091	-0.13319
20	22.89526	22.74286	0.413559	0.3104391	0.4786844	0.3028181	0.1031198	0.1758663	0.1524
21	23.98154	23.78545	0.375441	0.192047	0.403122	0.1953969	0.1833944	0.2077251	0.19609
23	23.82688	23.32091	0.407984	0.3068808	0.4470778	0.3087943	0.1011027	0.1382835	0.50597
Canton-S	23.83176	23.9975	0.447971	0.2434532	0.4589369	0.2528741	0.2045181	0.2060628	-0.16574
	18C, all	18C, night	29C, all	29C, night	Difference, all	Difference, Night			
Correlation	-0.241579	-0.648968	-0.805804	-0.706072036	-0.678737525	-0.240147593	all n.s.		

Table 3.1 Period lengths and splicing levels in natural *per* variants

Appendix to Chapter 4

4.1

1. *cry*^{b}, all Time points	SS	df	MS	F	p
Temperature	107.4	1	107.4	0.553	0.457043
Time	508605.4	47	10821.4	55.740	0.000000
Temperature*Time	50265.8	47	1069.5	5.509	0.000000
Error	680273.8	3504	194.1		

2. *cry*^{b}, Morning Peak	SS	df	MS	F	p
Temperature	609.98	1	609.98	2.6017	0.107616
Time	5849.73	4	1462.43	6.2375	0.000073
Temperature*Time	196.63	4	49.16	0.2097	0.933005
Error	85576.98	365	234.46		

3. *cry*^{b}, Evening Peak	SS	df	MS	F	p
Temperature	6580.6	1	6580.6	18.702	0.000018
Time	92054.7	8	11506.8	32.702	0.000000
Temperature*Time	29540.7	8	3692.6	10.494	0.000000
Error	231180.7	657	351.9		

4. *cry*^{b}, Time bins 20-29	SS	df	MS	F	p
Temperature	8287.9	1	8287.9	40.4337	0.000000
Time	21954.4	9	2439.4	11.9008	0.000000
Temperature*Time	2608.1	9	289.8	1.4138	0.177893
Error	149632.2	730	205.0		

5. *cry*^{b}, Canton-S, all Time points	SS	df	MS	F	p
Genotype	28042	1	28042	114.742	0.000000
Temperature	2563	1	2563	10.488	0.001205
Time	1236227	47	26303	107.625	0.000000
Genotype*Temperature	1047	1	1047	4.285	0.038476
Genotype*Time	120242	47	2558	10.468	0.000000
Temperature*Time	139517	47	2968	12.146	0.000000
Genotype*Temperature*Time	81752	47	1739	7.117	0.000000
Error	2694670	11026	244		

7. *cry*^{b}, Canton-S, Morning Peak 18°C	SS	df	MS	F	p
Genotype	11492.2	1	11492.20	34.1420	0.000000
Time	11956.3	4	2989.06	8.8802	0.000001
Genotype*Time	3162.6	4	790.66	2.3490	0.053624
Error	148104.1	440	336.60		

8. *cry*^{b}, Canton-S, Evening Peak 18°C	SS	df	MS	F	p
Genotype	3118.3	1	3118.3	7.343	0.006874
Time	87362.8	8	10920.4	25.717	0.000000
Genotype*Time	20235.8	8	2529.5	5.957	0.000000
Error	340560.3	802	424.6		

9. *cry*^{b}, Canton-S, Morning Peak 29°C	SS	df	MS	F	p
Genotype	51569.9	1	51569.9	201.2518	0.000000
Time	8304.0	4	2076.0	8.1016	0.000002
Genotype*Time	1359.5	4	339.9	1.3263	0.258550
Error	179371.8	700	256.2		

10. *cry*^{b}, Canton-S, Evening Peak 29°C

	SS	df	MS	F	p
Genotype	36522	1	36522	99.521	0.00
Time	303415	8	37927	103.349	0.00
Genotype*Time	142042	8	17755	48.382	0.00
Error	462392	1260	367		

4.2

1. *cry*^{b}	**SS**	**df**	**MS**	**F**	**p**
Temperature	0.191985	1	0.191985	47.412	0.000000
Time	0.103654	5	0.020731	5.120	0.001147
Temperature* Time	0.064322	5	0.012864	3.177	0.017432
Error	0.149822	37	0.004049		

2. *cry*^{b}, *per*^{01}, *tim*^{01}, Canton-S

	SS	df	MS	F	p
Genotype	0.16867	3	0.05622	13.442	0.000000
Temperature	0.83040	1	0.83040	198.535	0.000000
Time	0.18701	5	0.03740	8.942	0.000000
Genotype *Temperature	0.13404	3	0.04468	10.682	0.000002
Genotype *Time	0.07547	15	0.00503	1.203	0.273018
Temperature*Time	0.10173	5	0.02035	4.864	0.000337
Genotype *Temp*Time	0.12689	15	0.00846	2.023	0.015950
Error	0.75287	180	0.00418		

3. All Genotypes, 18°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.06118	3	0.02039	4.741	0.003760
Time	0.04652	5	0.00930	2.163	0.063165
Genotype*Time	0.13582	15	0.00905	2.105	0.014374
Error	0.48176	112	0.00430		

***4.* All Genotypes, 29°C**	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.193419	3	0.064473	16.171	0.000000
Time	0.219338	5	0.043868	11.003	0.000000
Genotype*Time	0.078488	15	0.005233	1.312	0.219443
Error	0.271118	68	0.003987		

5. *per*^{0}, *tim*^{0}, *cry*^{b}, 29°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.010333	2	0.005167	1.082	0.347010
Time	0.215592	5	0.043118	9.027	0.000004
Genotype*Time	0.031357	10	0.003136	0.656	0.758160
Error	0.234065	49	0.004777		

4.3

1. *per*^{01} *cry*^{b} all Time points	**SS**	**df**	**MS**	**F**	**p**
Temperature	1545	1	1545.3	4.857	0.027591
Time	434642	47	9247.7	29.070	0.000000
Temperature*Time	36677	47	780.4	2.453	0.000000
Error	1129967	3552	318.1		

2. *per*^{0} *cry*^{b}, Morning Peak	**SS**	**df**	**MS**	**F**	**p**
Temperature	782.2	1	782.2	2.2392	0.135405
Time	88497.4	4	22124.4	63.3315	0.000000
Temperature*Time	2143.9	4	536.0	1.5342	0.191592
Error	129256.6	370	349.3		

3. per^{0} cry^{b}, Evening Peak	SS	df	MS	F	p
Temperature	4480.3	1	4480.3	8.9417	0.002890
Time	51307.0	8	6413.4	12.7996	0.000000
Temperature*Time	4662.9	8	582.9	1.1633	0.318970
Error	333707.2	666	501.1		

4. per^{01} cry^{b}, per^{0} all Time points	SS	df	MS	F	p
Genotype	0.17027	1	0.17027	20.256	0.000020
Temperature	0.20669	1	0.20669	24.589	0.000003
Time	0.09660	5	0.01932	2.298	0.051626
Genotype *Temperature	0.00092	1	0.00092	0.109	0.741755
Genotype *Time	0.04615	5	0.00923	1.098	0.367216
Temperature*Time	0.05487	5	0.01097	1.305	0.268862
Genotype *Temperature*Time	0.01545	5	0.00309	0.368	0.869454
Error	0.74812	89	0.00841		

5. per^{01} cry^{b}, per^{01}, Morning Peak, 18°C	SS	df	MS	F	p
Genotype	19323.4	1	19323.4	61.6968	0.000000
Time	71333.8	4	17833.4	56.9394	0.000000
Genotype*Time	3147.7	4	786.9	2.5125	0.043041
Error	61074.1	195	313.2		

*6. per^{01} cry^{b}, per^{01}, Morning Peak, 29°C	SS	df	MS	F	p
Genotype	1397.5	1	1397.5	3.1148	0.078958
Time	56950.1	2	28475.1	63.4666	0.000000
Genotype*Time	1744.9	2	872.4	1.9445	0.145484
Error	99602.9	222	448.7		

7. per^{0} cry^{b}, per^{0}, 18°C Evening Peak	SS	df	MS	F	p
Genotype	714.9	1	714.9	1.9816	0.160112
Time	28282.0	8	3535.3	9.7989	0.000000
Genotype *Time	5964.3	8	745.5	2.0665	0.038394
Error	126633.7	351	360.8		

8. per^{0} cry^{b}, per^{0}, 29°C Evening Peak	SS	df	MS	F	p
Genotype	39924	1	39924	97.569	0.00
Time	150584	8	18823	46.001	0.00
Genotype*Time	80885	8	10111	24.709	0.00
Error	419823	1026	409		

9. per^{01} cry^{b}, cry^{b} all Time points	SS	df	MS	F	p
Genotype	0.06907	1	0.06907	7.716	0.006851
Temperature	0.29300	1	0.29300	32.736	0.000000
Time	0.11444	5	0.02289	2.557	0.033930
Genotype *Temperature	0.00540	1	0.00540	0.604	0.439514
Genotype *Time	0.04706	5	0.00941	1.052	0.393710
Temperature*Time	0.04034	5	0.00807	0.901	0.484594
Genotype *Temp*Time	0.03455	5	0.00691	0.772	0.572720
Error	0.69814	78	0.00895		

10. per^0 cry^b, cry^b, Morning Peak, 18°C

	SS	df	MS	F	p
Genotype	1699.39	1	1699.39	4.7360	0.030959
Time	18066.38	4	4516.59	12.5873	0.000000
Genotype*Time	4894.62	4	1223.66	3.4102	0.010407
Error	59205.76	165	358.82		

11. per^0 cry^b, cry^b, Morning Peak, 29°C

	SS	df	MS	F	p
Genotype	23851.4	1	23851.4	87.3578	0.00
Time	79996.3	4	19999.1	73.2483	0.00
Genotype *Time	28804.8	4	7201.2	26.3750	0.00
Error	155627.8	570	273.0		

12. per^0 cry^b, cry^b, 18°C Evening Peak

	SS	df	MS	F	p
Genotype	7703.5	1	7703.5	15.7718	0.000090
Time	50915.3	8	6364.4	13.0302	0.000000
Genotype *Time	2496.4	8	312.0	0.6389	0.744842
Error	145065.2	297	488.4		

13. per^0 cry^b, cry^b, 29°C Evening Peak

	SS	df	MS	F	p
Genotype	39924	1	39924	97.569	0.00
Time	150584	8	18823	46.001	0.00
Genotype*Time	80885	8	10111	24.709	0.00
Error	419823	1026	409		

4.4

1. per^{01} cry^b, per^{01}, tim^{01}, cry^b, Canton-S

	SS	df	MS	F	p
Genotype	0.50738	4	0.12684	21.544	0.000000
Temperature	0.91964	1	0.91964	156.195	0.000000
Time	0.20225	5	0.04045	6.870	0.000006
Genotype *Temperature	0.14600	4	0.03650	6.199	0.000096
Genotype *Time	0.11453	20	0.00573	0.973	0.496500
Temperature*Time	0.10974	5	0.02195	3.728	0.002927
Genotype *Temperature*Time	0.13329	20	0.00666	1.132	0.318324
Error	1.30119	221	0.00589		

2. per^{01} cry^b	SS	df	MS	F	p
Temperature	0.107735	1	0.107735	8.0558	0.007023
Time	0.057274	5	0.011455	0.8565	0.518316
Temperature*Time	0.014309	5	0.002862	0.2140	0.954614
Error	0.548320	41	0.013374		

3. per^0 cry^b, Canton-S, 18°C	SS	df	MS	F	p
Genotype	0.07486	1	0.07486	10.439	0.002068
Time	0.01926	5	0.00385	0.537	0.747320
Genotype*Time	0.01985	5	0.00397	0.554	0.734877
Error	0.40160	56	0.00717		

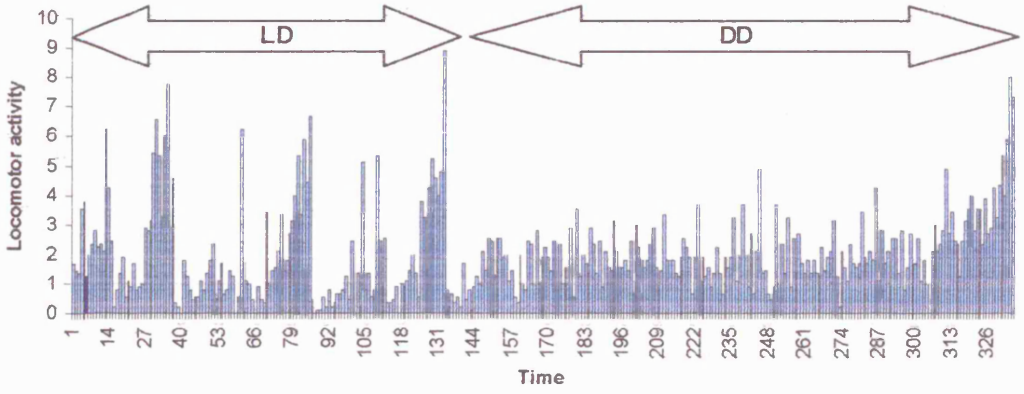
4. per^0 cry^b, cry^b, 18°C	SS	df	MS	F	p
Genotype	0.01646	1	0.01646	2.106	0.155883
Time	0.02525	5	0.00505	0.646	0.666334
Genotype*Time	0.06095	5	0.01219	1.559	0.197987
Error	0.26578	34	0.00782		

5. per^{01} cry^{b}, cry^{b}, per^{01}, tim^{01}, 29°C

	SS	df	MS	F	p
Genotype	0.16124	3	0.05375	6.909	0.000344
Time	0.26525	5	0.05305	6.819	0.000025
Genotype*Time	0.04911	15	0.00327	0.421	0.968695
Error	0.61459	79	0.00778		

6. per^{01} cry^{b}, Canton-S, 29°C

	SS	df	MS	F	p
Genotype	0.474141	1	0.474141	55.6378	0.000000
Time	0.073148	5	0.014630	1.7167	0.148402
Genotype*Time	0.024357	5	0.004871	0.5716	0.721323
Error	0.417575	49	0.008522		



Appendix 4.5 per^{01} cry^{b} in LD and DD

Appendix to Chapter 5

5.1

1. *norpA*, Canton-S, all Time points

	SS	df	MS	F	p
Genotype	102163	1	102163	383.35	0.000000
Temperature	39274	1	39274	147.37	0.000000
Time	572126	47	12173	45.68	0.000000
Genotype*Temperature	71880	1	71880	269.72	0.000000
Genotype*Time	107410	47	2285	8.58	0.000000
Temperature*Time	241577	47	5140	19.29	0.000000
Genotype*Temperature*Time	33108	47	704	2.64	0.000000
Error	2369968	8893	266		

2. *norpA*, cry^b all Time points	SS	df	MS	F	p
Genotype	152396	1	152396	717.220	0.00
Temperature	36487	1	36487	171.720	0.00
Time	519166	47	11046	51.986	0.00
Genotype*Temperature	41640	1	41640	195.971	0.00
Genotype*Time	81938	47	1743	8.205	0.00
Temperature*Time	103575	47	2204	10.371	0.00
Genotype*Temperature*Time	60935	47	1296	6.102	0.00
Error	1035845	4875	212		

3. *norpA*, all Time points	SS	df	MS	F	p
Temperature	64596	1	64596	249.069	0.00
Time	162880	47	3466	13.362	0.00
Temperature*Time	102861	47	2189	8.438	0.00
Error	355571	1371	259		

4. *norpA*, Morning Peak	SS	df	MS	F	p
Temperature	21514.4	1	21514.4	70.3744	0.000000
Time	5634.6	4	1408.6	4.6077	0.001567
Temperature*Time	5178.7	4	1294.7	4.2349	0.002849
Error	44328.5	145	305.7		

5. *norpA*, Evening Peak	SS	df	MS	F	p
Temperature	628.1	1	628.1	1.726	0.190094
Time	46274.4	8	5784.3	15.893	0.000000
Temperature*Time	17347.8	8	2168.5	5.958	0.000001
Error	94990.5	261	363.9		

6. *norpA*, Canton-S Morning Peak 18°C

	SS	df	MS	F	p
Genotype	261.3	1	261.3	0.8338	0.361671
Time	20805.7	4	5201.4	16.5966	0.000000
Genotype*Time	977.4	4	244.4	0.7797	0.538815
Error	136329.9	435	313.4		

7. *norpA*, per^0 Morning Peak, 29°C

	SS	df	MS	F	p
Genotype	6137.9	1	6137.9	19.7837	0.000021
Time	12349.9	2	6174.9	19.9032	0.000000
Genotype*Time	2873.4	2	1436.7	4.6308	0.011705
Error	34437.6	111	310.2		

8. *norpA*, Canton-S Evening Peak, 18°C

	SS	df	MS	F	p
Genotype	1281.0	1	1281.0	3.086	0.079335
Time	92229.4	8	11528.7	27.776	0.000000
Genotype*Time	33611.2	8	4201.4	10.122	0.000000
Error	329139.0	793	415.1		

9. *norpA*, Canton-S Evening Peak, 29°C

	SS	df	MS	F	p
Genotype	7771.5	1	7771.5	20.095	0.000008
Time	59057.8	8	7382.2	19.088	0.000000
Genotype*Time	63470.1	8	7933.8	20.514	0.000000
Error	337623.6	873	386.7		

10. *norpA*, cry^b Evening Peak, 18°C

	SS	df	MS	F	p
Genotype	203.3	1	203.3	0.4456	0.505138
Time	66502.0	8	8312.7	18.2180	0.000000
Genotype*Time	2853.3	8	356.7	0.7816	0.619295
Error	102665.8	225	456.3		

11. *norpA*, cry^b Evening Peak, 29°C

	SS	df	MS	F	p
Genotype	1234.9	1	1234.9	3.829	0.050774
Time	100962.6	8	12620.3	39.131	0.000000
Genotype*Time	21601.5	8	2700.2	8.372	0.000000
Error	223505.4	693	322.5		

5.2

1. *norpA* cry^b, all Time points

	SS	df	MS	F	p
Temperature	1227.8	1	1227.8	5.2269	0.022341
Time	292441.5	47	6222.2	26.4876	0.000000
Temperature*Time	21847.6	47	464.8	1.9788	0.000097
Error	484851.0	2064	234.9		

2. *norpA*, *norpA* cry^b, all Time points

	SS	df	MS	F	p
Genotype	290581	1	290581	1187.670	0.000000
Temperature	47457	1	47457	193.969	0.000000
Time	345161	47	7344	30.016	0.000000
Genotype*Temperature	29945	1	29945	122.394	0.000000
Genotype*Time	87285	47	1857	7.591	0.000000
Temperature*Time	97174	47	2068	8.450	0.000000
Genotype*Temperature*Time	42785	47	910	3.721	0.000000
Error	840422	3435	245		

3. *norpA* cry^b, Morning Peak	**SS**	**df**	**MS**	**F**	**p**
Genotype	90.60	1	90.60	0.46333	0.496805
Time	5062.12	4	1265.53	6.47172	0.000062
Genotype*Time	3137.21	4	784.30	4.01080	0.003696
Error	42042.75	215	195.55		

4. *norpA* cry^b, Evening Peak	**SS**	**df**	**MS**	**F**	**p**
Temperature	68.6	1	68.6	0.1101	0.740243
Time	88652.2	8	11081.5	17.7871	0.000000
Temperature*Time	9142.6	8	1142.8	1.8344	0.069300
Error	241104.0	387	623.0		

5. *norpA cry^b*, *cry^b* Morning Peak 18°C

	SS	df	MS	F	p
Genotype	757.12	1	757.12	2.21301	0.138951
Time	7644.00	4	1911.00	5.58575	0.000322
Genotype*Time	528.03	4	132.01	0.38585	0.818515
Error	51318.08	150	342.12		

6. *norpA cryb*, *cry^b* Morning Peak, 29°C

	SS	df	MS	F	p
Genotype	542.22	1	542.22	3.0557	0.081169
Time	3075.44	4	768.86	4.3329	0.001906
Genotype*Time	1650.03	4	412.51	2.3247	0.055807
Error	76301.66	430	177.45		

7. *norpA cryb*, *norpA* Evening Peak, 18°C

	SS	df	MS	F	p
Genotype	5625.5	1	5625.5	10.7739	0.001170
Time	44767.5	8	5595.9	10.7172	0.000000
Genotype *Time	33437.0	8	4179.6	8.0047	0.000000
Error	136279.5	261	522.1		

8. *norpA cryb*, *norpA* Evening Peak, 29°C

	SS	df	MS	F	p
Genotype	16377.5	1	16377.5	31.7197	0.000000
Time	59325.5	8	7415.7	14.3626	0.000000
Genotype *Time	17605.4	8	2200.7	4.2623	0.000063
Error	199815.1	387	516.3		

9. *norpA cryb*, *cry^b* Evening Peak, 18°C

	SS	df	MS	F	p
Genotype	3745.1	1	3745.1	6.8462	0.009384
Time	41186.0	8	5148.3	9.4111	0.000000
Genotype *Time	22824.5	8	2853.1	5.2155	0.000004
Error	147700.8	270	547.0		

10. *norpA cryb*, *cry^b* Evening Peak, 29°C

	SS	df	MS	F	p
Genotype	43784.3	1	43784.3	104.408	0.000000
Time	193010.0	8	24126.3	57.531	0.000000
Genotype *Time	24197.4	8	3024.7	7.213	0.000000
Error	324583.9	774	419.4		

5.3

1. *norpA*, *norpA cry^b*	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.04787	1	0.04787	7.428	0.008107
Temperature	0.37792	1	0.37792	58.647	0.000000
Time	0.02597	5	0.00519	0.806	0.549167
Genotype*Temperature	0.00159	1	0.00159	0.247	0.621081
Genotype*Time	0.00323	5	0.00065	0.100	0.991725
Temperature*Time	0.01163	5	0.00233	0.361	0.873554
Genotype*Temperature*Time	0.01925	5	0.00385	0.598	0.701882
Error	0.45108	70	0.00644		

2. *norpA*	**SS**	**df**	**MS**	**F**	**p**
Temperature	0.16658	1	0.16658	31.949	0.000002
Time	0.01419	5	0.00284	0.544	0.741325
Temperature*Time	0.01227	5	0.00245	0.471	0.795451
Error	0.18770	36	0.00521		

3. *norpA cry^b*	SS	df	MS	F	p
Temperature	0.21257	1	0.21257	27.440	0.000008
Time	0.01590	5	0.00318	0.410	0.838120
Temperature*Time	0.01845	5	0.00369	0.476	0.791260
Error	0.26338	34	0.00775		

4. *norpA vs. norpA cry^b*, 18°C	SS	df	MS	F	p
Genotype	0.03408	1	0.03408	5.183	0.028853
Time	.01390	5	0.00278	0.423	0.829688
Genotype*Time	0.01163	5	0.00233	0.354	0.876443
Error	0.23670	36	0.00658		

5. *norpA* vs. *norpA cry^b*, 29°C	SS	df	MS	F	p
Genotype	0.01571	1	0.01571	2.492	0.123659
Time	0.02374	5	0.00475	0.753	0.589788
Genotype*Time	0.01110	5	0.00222	0.352	0.877202
Error	0.21438	34	0.00631		

6. *norpA, norpA cry^b*, Canton-S	SS	df	MS	F	p
Genotype	2.88308	2	1.44154	267.566	0.000000
Temperature	0.90671	1	0.90671	168.296	0.000000
Time	0.03844	5	0.00769	1.427	0.218585
Genotype*Temperature	0.06137	2	0.03068	5.695	0.004228
Genotype*Time	0.02097	10	0.00210	0.389	0.949587
Temperature*Time	0.01748	5	0.00350	0.649	0.662866
Genotype*Temperature*Time	0.04296	10	0.00430	0.797	0.631319
Error	0.72194	134	0.00539		

7. *norpA cry^b* vs. Canton-S, 18°C	SS	df	MS	F	p
Genotype	1.07924	1	1.07924	182.953	0.000000
Time	0.00525	5	0.00105	0.178	0.969910
Genotype*Time	0.01056	5	0.00211	0.358	0.875198
Error	0.36574	62	0.00590		

8. *norpA, norpA cry^b, cry^b*	SS	df	MS	F	p
Genotype	0.66736	2	0.33368	37.061	0.000000
Temperature	0.47702	1	0.47702	52.981	0.000000
Time	0.03741	5	0.00748	0.831	0.530347
Genotype *Temperature	0.00811	2	0.00406	0.450	0.638505
Genotype *Time	0.04926	10	0.00493	0.547	0.853069
Temperature*Time	0.00511	5	0.00102	0.114	0.989171
Genotype *Temperature*Time	0.03986	10	0.00399	0.443	0.922227
Error	0.99940	111	0.00900		

9. *norpA cry^b*, *per^0 cry^b*, 18°C	SS	df	MS	F	p
Genotype	0.33274	1	0.33274	31.084	0.000006
Time	0.02139	5	0.00428	0.400	0.844810
Genotype*Time	0.01034	5	0.00207	0.193	0.962585
Error	0.29973	28	0.01070		

10. *norpA cry^b*, *per^0 cry^b*, 29°C	SS	df	MS	F	p
Genotype	0.29544	1	0.29544	27.122	0.000004
Time	0.02276	5	0.00455	0.418	0.833991
Genotype*Time	0.05614	5	0.01123	1.031	0.410645
Error	0.51197	47	0.01089		

5.4

1. *glass*, all Time points	**SS**	**df**	**MS**	**F**	**p**
Temperature	22949.4	1	22949.4	61.551	0.000000
Time	103658.5	47	2205.5	5.915	0.000000
Temperature*Time	19070.1	47	405.7	1.088	0.317105
Error	679709.0	1823	372.9		

2. *glass* Morning Peak	**SS**	**df**	**MS**	**F**	**p**
Temperature	548.94	1	548.94	1.4979	0.222503
Time	1561.82	4	390.46	1.0655	0.374891
Temperature*Time	1128.97	4	282.24	0.7702	0.545838
Error	69627.28	190	366.46		

3. *glass* Evening Peak	**SS**	**df**	**MS**	**F**	**p**
Temperature	81.6	1	81.6	0.1680	0.682179
Time	22536.5	8	2817.1	5.7980	0.000001
Temperature*Time	1345.5	8	168.2	0.3462	0.947274
Error	166167.0	342	485.9		

4. *glass*, Canton-S, all Time points

	SS	**df**	**MS**	**F**	**p**
Genotype	42580	1	42580	147.697	0.000000
Temperature	12754	1	12754	44.238	0.000000
Time	424173	47	9025	31.305	0.000000
Genotype*Temperature	29669	1	29669	102.911	0.000000
Genotype*Time	48973	47	1042	3.614	0.000000
Temperature*Time	74225	47	1579	5.478	0.000000
Genotype*Temperature*Time	32743	47	697	2.416	0.000000
Error	2694105	9345	288		

5. *glass*, Canton-S: Morning Peak

	SS	**df**	**MS**	**F**	**p**
Genotype	1001.1	1	1001.1	3.1010	0.078559
Temperature	485.8	1	485.8	1.5048	0.220236
Time	993.7	4	248.4	0.7695	0.545122
Genotype*Temperature	485.7	1	485.7	1.5046	0.220262
Genotype*Time	7730.4	4	1932.6	5.9866	0.000093
Temperature*Time	3463.0	4	865.8	2.6818	0.030417
Genotype*Temperature*Time	1831.8	4	457.9	1.4186	0.225727
Error	311526.2	965	322.8		

6. *glass*, Canton-S: Evening Peak, 18°C

	SS	**df**	**MS**	**F**	**p**
Genotype	2.1	1	2.1	0.0051	0.943365
Time	33534.1	8	4191.8	10.0453	0.000000
Genotype *Time	10284.1	8	1285.5	3.0806	0.002011
Error	304618.7	730	417.3		

7. *glass*, Canton-S: Evening Peak, 29°C

	SS	**df**	**MS**	**F**	**p**
Genotype	10759	1	10759	25.250	0.000001
Time	170174	8	21272	49.925	0.000000
Genotype *Time	21578	8	2697	6.330	0.000000
Error	433320	1017	426		

8. *glass, norpA cry^b* all Time points

	SS	df	MS	F	p
Genotype	177126	1	177126.1	591.201	0.000000
Temperature	20938	1	20938.0	69.886	0.000000
Time	301608	47	6417.2	21.419	0.000000
Genotype*Temperature	11027	1	11027.1	36.806	0.000000
Genotype*Time	26812	47	570.5	1.904	0.000210
Temperature*Time	24410	47	519.4	1.733	0.001471
Genotype*Temperature*Time	15512	47	330.1	1.102	0.294303
Error	1164560	3887	299.6		

9. *glass, norpA cry^b*: Evening Peak 18°C

	SS	df	MS	F	p
Genotype	6720.7	1	6720.7	11.9069	0.000683
Time	27601.5	8	3450.2	6.1126	0.000000
Genotype*Time	3571.2	8	446.4	0.7909	0.611241
Error	111759.2	198	564.4		

10. *glass, norpA cry^b*: Evening Peak 29°C

	SS	df	MS	F	p
Genotype	20786.9	1	20786.9	37.352	0.000000
Time	89616.4	8	11202.0	20.129	0.000000
Genotype *Time	12876.5	8	1609.6	2.892	0.003665
Error	295511.8	531	556.5		

11. *glass, norpA cry^b* bins 32-37 only

	SS	df	MS	F	p
Genotype	4465.7	1	4465.7	6.9134	0.008928
Time	34246.3	5	6849.3	10.6034	0.000000
Genotype *Time	4991.2	5	998.2	1.5454	0.175017
Error	228665.5	354	645.9		

5.5

1. *glass*	**SS**	**df**	**MS**	**F**	**p**
Temperature	60200	1	60200	145.953	0.000000
Time	62092	47	1321	3.203	0.000000
Temperature*Time	72075	47	1534	3.718	0.000000
Error	1148297	2784	412		

2. *glass, glass cry^b*	**SS**	**df**	**MS**	**F**	**p**
Genotype	2623	1	2623	6.611	0.010163
Temperature	64765	1	64765	163.222	0.000000
Time	145791	47	3102	7.818	0.000000
Genotype*Temperature	52	1	52	0.131	0.717279
Genotype*Time	40415	47	860	2.167	0.000008
Temperature*Time	27173	47	578	1.457	0.022783
Genotype*Temperature*Time	37888	47	806	2.032	0.000043
Error	1828006	4607	397		

3. *glass, glass cry^b* Morning Peak

	SS	df	MS	F	p
Genotype	52.0	1	52.0	0.1354	0.713089
Temperature	724.4	1	724.4	1.8855	0.170350
Time	2737.0	4	684.2	1.7810	0.131394
Genotype*Temperature	183.9	1	183.9	0.4786	0.489401
Genotype*Time	832.9	4	208.2	0.5420	0.704980
Temperature*Time	1762.4	4	440.6	1.1468	0.333742
Genotype*Temperature*Time	321.2	4	80.3	0.2090	0.933417
Error	184409.8	480	384.2		

4. *glass, glass cry^b* Evening Peak

	SS	df	MS	F	p
Genotype	18205.0	1	18205.0	38.902	0.000000
Temperature	15828.9	1	15828.9	33.825	0.000000
Time	27565.6	8	3445.7	7.363	0.000000
Genotype *Temperature	19999.4	1	19999.4	42.736	0.000000
Genotype *Time	11653.3	8	1456.7	3.113	0.001792
Temperature*Time	2792.9	8	349.1	0.746	0.650792
Genotype *Temperature*Time	2369.1	8	296.1	0.633	0.750586
Error	404326.0	864	468.0		

5. *glass cry^b* Evening Peak	**SS**	**df**	**MS**	**F**	**p**
Temperature	70304.5	1	70304.5	154.094	0.000000
Time	11010.0	8	1376.2	3.016	0.002548
Temperature*Time	6210.8	8	776.4	1.702	0.095304
Error	238159.0	522	456.2		

6. *glass cry^b, per^0* Evening Peak, 18°C

	SS	df	MS	F	p
Genotype	12501.4	1	12501.4	35.4845	0.000000
Time	15567.8	8	1946.0	5.5235	0.000001
Genotype *Time	2438.7	8	304.8	0.8653	0.545848
Error	155367.1	441	352.3		

7. *glass cry^b, cry^b* Evening Peak, 29°C

	SS	df	MS	F	p
Genotype	1788	1	1788	4.792	0.028890
Time	8309	8	10387	27.836	0.000000
Genotype *Time	61007	8	7626	20.436	0.000000
Error	295541	792	373		

8. *glass, glass cry^b* Evening Peak, 29°C

	SS	df	MS	F	p
Genotype	43.4	1	43.4	0.089	0.765146
Time	34363.1	8	4295.4	8.850	0.000000
Genotype *Time	5335.1	8	666.9	1.374	0.204981
Error	266469.0	549	485.4		

5.6

1. *glass, glass cry^b*	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.00690	1	0.00690	1.658	0.203420
Temperature	0.98712	1	0.98712	237.274	0.000000
Time	0.00678	5	0.00136	0.326	0.895042
Genotype *Temperature	0.00360	1	0.00360	0.865	0.356662
Genotype *Time	0.00975	5	0.00195	0.469	0.797967
Temperature*Time	0.00533	5	0.00107	0.256	0.934894
Genotype *Temperature*Time	0.03688	5	0.00738	1.773	0.134414
Error	0.22049	53	0.00416		

2. *glass, glass cry^b*, Canton-S, 18°C

	SS	df	MS	F	p
Genotype	0.29664	2	0.14832	28.624	0.000000
Time	0.00421	5	0.00084	0.163	0.975405
Genotype *Time	0.03466	10	0.00347	0.669	0.749264
Error	0.37308	72	0.00518		

3. *glass, glass cry^b, cry^b*, 18°C

	SS	df	MS	F	p
Genotype	0.10137	2	0.05068	10.681	0.000137
Time	0.01634	5	0.00327	0.689	0.634269
Genotype *Time	0.08414	10	0.00841	1.773	0.090370
Error	0.23726	50	0.00475		

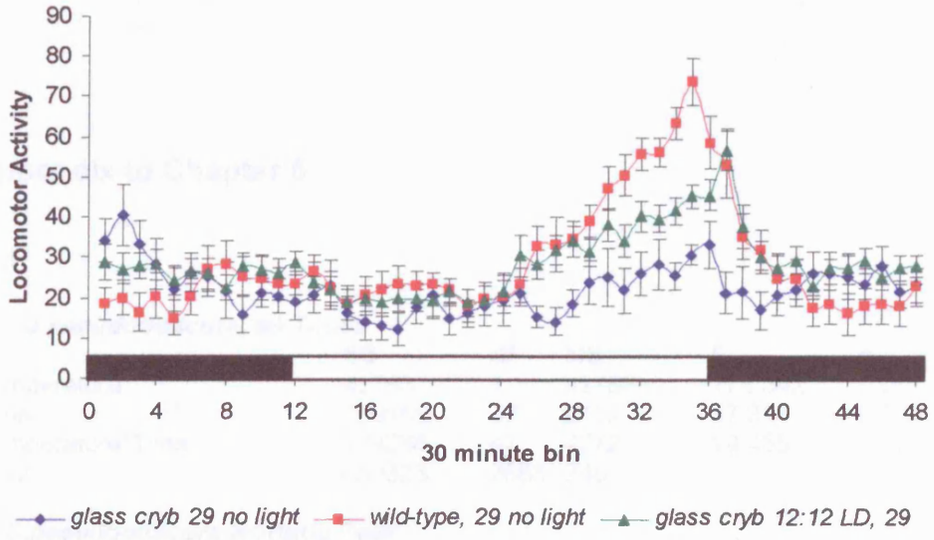
4. *glass, glass cry^b, cry^b*, 29°C

	SS	df	MS	F	p
Genotype	0.014161	2	0.007080	2.128	0.132305
Time	0.029241	5	0.005848	1.758	0.143760
Genotype *Time	0.089902	10	0.008990	2.703	0.012419
Error	0.133060	40	0.003327		

5. *glass, glass cry^b*, Canton-S, 29°C

	SS	df	MS	F	p
Genotype	0.149140	2	0.074570	28.371	0.000000
Time	0.013934	5	0.002787	1.060	0.394888
Genotype *Time	0.056977	10	0.005698	2.168	0.037991
Error	0.118276	45	0.002628		

6. *glass, glass cry^b, norpA*	SS	df	MS	F	p
Genotype	0.62082	2	0.31041	67.680	0.000000
Temperature	1.11659	1	1.11659	243.456	0.000000
Time	0.00380	5	0.00076	0.166	0.974514
Genotype *Temperature	0.08700	2	0.04350	9.484	0.000185
Genotype *Time	0.02816	10	0.00282	0.614	0.798200
Temperature*Time	0.00165	5	0.00033	0.072	0.996245
Genotype *Temperature*Time	0.05172	10	0.00517	1.128	0.350916
Error	0.40819	89	0.00459		



Appendix 5.7: Locomotor activity of *glass*^{60j} *cry*^{b} shielded from the light

glass^{60j} *cry*^{b}, no light (blue), wild-type, no light (red) and *glass*^{60j} *cry*^{b} in LD 12:12 (green). all at 29°C. Error bars represent SEM.

Appendix to Chapter 6

6.3

1. *D.pseudoobscura*, all Times

	SS	df	MS	F	p
Temperature	42763	1	42763	174.342	0.00
Time	430165	47	9152	37.314	0.00
Temperature*Time	224288	47	4772	19.455	0.00
Error	659323	2688	245		

2. *D.pseudoobscura* Morning Peak

	SS	df	MS	F	p
Temperature	5161.4	1	5161.4	11.7515	0.000699
Time	72824.0	4	18206.0	41.4516	0.000000
Temperature*Time	12026.8	4	3006.7	6.8457	0.000029
Error	122979.1	280	439.2		

3. *D.pseudoobscura* Evening peak

	SS	df	MS	F	p
Temperature	150280.1	1	150280.1	933.712	0.000000
Time	11641.4	8	1455.2	9.041	0.000000
Temperature*Time	12592.2	8	1574.0	9.780	0.000000
Error	81118.3	504	160.9		

4. *D.pseudoobscura, D.melanogaster*, all Times

	SS	df	MS	F	p
Genotype	45061	1	45061	172.07	0.00
Temperature	49951	1	49951	190.75	0.00
Time	88237	4	47	1877471.69	0.00
Genotype*Temperature	19256	1	19256	73.53	0.00
Genotype*Time	370347	47	7880	30.09	0.00
Temperature*Time	364992	47	7766	29.65	0.00
Genotype*Temperature*Time	128786	47	2740	10.46	0.00
Error	2673719	10210	262		

Morning Activity Peak
D.pseudoobscura, D.melanogaster

5. 18°C	SS	df	MS	F	p
Genotype	845.6	1	845.6	2.5930	0.108201
Time	30217.0	3	10072.3	30.8860	0.000000
Genotype*Time	111.4	3	37.1	0.1138	0.951974
Error	118705.0	364	326.1		

6. 29°C	SS	df	MS	F	p
Genotype	26798.8	1	26798.8	74.503	0.000000
Time	85223.1	4	21305.8	59.232	0.000000
Genotype*Time	74186.6	4	18546.7	51.562	0.000000
Error	215819.8	600	359.7		

Evening activity peak
D.pseudoobscura, D.melanogaster

7. 18°C	SS	df	MS	F	p
Genotype	371.4	1	371.4	0.919	0.338079
Time	72226.3	8	9028.3	22.331	0.000000
Genotype*Time	25229.2	8	3153.6	7.801	0.000000
Error	335152.8	829	404.3		

8. 29°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	183406.0	1	183406.0	623.403	0.000000
Time	95231.6	8	11903.9	40.462	0.000000
Genotype*Time	74155.7	8	9269.5	31.507	0.000000
Error	317737.5	1080	294.2		

6.5

1. *D.pseudoobscura*	**SS**	**df**	**MS**	**F**	**p**
Temperature	0.00145	1	0.00145	0.117	0.734265
Time	0.12685	5	0.02537	2.051	0.093247
Temperature*Time	0.01411	5	0.00282	0.228	0.947935
Error	0.46998	38	0.01237		

2. *D.melanogaster, D.pseudoobscura*

	SS	**df**	**MS**	**F**	**p**
Genotype	5.68584	1	5.68584	775.964	0.000000
Temperature	0.31577	1	0.31577	43.094	0.000000
Time	0.10104	5	0.02021	2.758	0.022331
Genotype*Temperature	0.25420	1	0.25420	34.691	0.000000
Genotype*Time	0.07585	5	0.01517	2.070	0.075249
Temperature*Time	0.02053	5	0.00411	0.560	0.730029
Genotype*Temperature*Time	0.02319	5	0.00464	0.633	0.674914
Error	0.74007	101	0.00733		

6.6

1. *D.pseudoobscura*	**SS**	**df**	**MS**	**F**	**p**
Temperature	0.27835	1	0.27835	14.0492	0.000607
Time	1.46128	5	0.29226	14.7510	0.000000
Temperature*Time	0.25740	5	0.05148	2.5983	0.041214
Error	0.73307	37	0.01981		

2. *D.melanogaster*	**SS**	**df**	**MS**	**F**	**p**
Temperature	0.087687	1	0.087687	9.1774	0.005345
Time	0.573408	5	0.114682	12.0027	0.000004
Temperature*Time	0.438429	5	0.087686	9.1773	0.000034
Error	0.257976	27	0.009555		

3. *D.pseudoobscura, D.melanogaster*

18°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.442303	1	0.442303	24.4610	0.000029
Time	1.005645	5	0.201129	11.1232	0.000005
Genotype*Time	0.458140	5	0.091628	5.0674	0.001854
Error	0.524378	29	0.018082		

4. 29°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	0.261694	1	0.261694	19.6272	0.000088
Time	0.760734	5	0.152147	11.4111	0.000001
Genotype*Time	0.138609	5	0.027722	2.0791	0.091455
Error	0.466665	35	0.013333		

6.7

	SS	**df**	**MS**	**F**	**p**
Temperature	2.1313	1	2.1313	3.9793	0.061429
Time	2.4499	5	0.4900	0.9149	0.493508
Temperature*Time	2.9590	5	0.5918	1.1050	0.392091
Error	9.6405	18	0.5356		

6.9

1. *mps3*, All Times	**SS**	**df**	**MS**	**F**	**p**
Temperature	73987.1	1	73987.1	299.882	0.00
Time	444261.9	47	9452.4	38.312	0.00
Temperature*Time	59186.7	47	1259.3	5.104	0.00
Error	864509.2	3504	246.7		

2. *mps3* morning peak	**SS**	**df**	**MS**	**F**	**p**
Temperature	25338.2	1	25338.2	67.1693	0.000000
Time Bin	71637.1	4	17909.3	47.4759	0.000000
Temperature*Time	11698.3	4	2924.6	7.7528	0.000005
Error	137688.7	365	377.2		

3. *mps3* evening peak	**SS**	**df**	**MS**	**F**	**p**
Temperature	44749.1	1	44749.1	107.789	0.000000
Time Bin	45598.8	8	5699.8	13.729	0.000000
Temperature*Time	15095.8	8	1887.0	4.545	0.000021
Error	272756.9	657	415.2		

4. *mps3*, *per*^{01} all Times	**SS**	**df**	**MS**	**F**	**p**
Genotype	75865	1	75865	298.078	0.000000
Temperature	21691	1	21691	85.224	0.000000
Time	637589	47	13566	53.301	0.000000
Genotype*Temperature	31064	1	31064	122.055	0.000000
Genotype*Time	50367	47	1072	4.211	0.000000
Temperature*Time	47405	47	1009	3.963	0.000000
Genotype*Temperature*Time	18024	47	383	1.507	0.014410
Error	1368259	5376	255		

5. *mps3*, *D.melanogaster*, all Times	**SS**	**df**	**MS**	**F**	**p**
Genotype	26526	1	26526	101.59	0.00
Temperature	75017	1	75017	287.31	0.00
Time	1314239	47	27963	107.09	0.00
Genotype*Temperature	30415	1	30415	116.49	0.00
Genotype*Time	106867	47	2274	8.71	0.00
Temperature*Time	223626	47	4758	18.22	0.00
Genotype*Temperature*Time	62959	47	1340	5.13	0.00
Error	2878905	11026	261		

6. *mps3*, *D.pseudoobscura*, all Times	**SS**	**df**	**MS**	**F**	**p**
Genotype	97789	1	97789	397.361	0.000000
Temperature	109866	1	109866	446.434	0.000000
Time	757633	47	16120	65.502	0.000000
Genotype*Temperature	76	1	76	0.307	0.579398
Genotype*Time	113720	47	2420	9.832	0.000000
Temperature*Time	205914	47	4381	17.803	0.000000
Genotype*Temperature*Time	113564	47	2416	9.818	0.000000
Error	1523832	6192	246		

morning peak

7. *mps3*, *per*^{01} 18°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	1262.4	1	1262.4	3.4997	0.062463
Time	108342.9	4	27085.7	75.0886	0.000000
Genotype*Time	1936.8	4	484.2	1.3423	0.254514
Error	97393.5	270	360.7		

8. *mps3*, *per*^{01} 29°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	9302.5	1	9302.5	25.0722	0.000001
Time	33110.0	4	8277.5	22.3097	0.000000
Genotype*Time	4884.6	4	1221.2	3.2913	0.011677
Error	107597.8	290	371.0		

9. *mps3*, *D.melanogaster* 29°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	10765.5	1	10765.5	31.7382	0.000000
Time	13250.4	4	3312.6	9.7660	0.000000
Genotype*Time	6903.7	4	1725.9	5.0883	0.000490
Error	200126.1	590	339.2		

evening peak, 30-38

10. *mps3*, *D.melanogaster* 18°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	8084	1	8084	19.005	0.000014
Time	177697	8	22212	52.219	0.000000
Genotype*Time	8401	8	1050	2.469	0.011891
Error	425361	1000	425		

11. *mps3*, *D.pseudoobscura* 18°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	6276.3	1	6276.3	14.889	0.000130
Time	43720.7	8	5465.1	12.965	0.000000
Genotype*Time	7827.2	8	978.4	2.321	0.018942
Error	193480.7	459	421.5		

12. mps3, *D.melanogaster* 29°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	53689.2	1	53689.2	133.014	0.000000
Time	84569.1	5	16913.8	41.904	0.000000
Genotype*Time	30759.7	5	6151.9	15.241	0.000000
Error	285773.5	708	403.6		

13. mps3, *D.pseudoobscura* 29°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	33086.9	1	33086.9	144.2343	0.000000
Time	5903.6	5	1180.7	5.1471	0.000131
Genotype*Time	2019.6	5	403.9	1.7608	0.119449
Error	107357.6	468	229.4		

evening peak 35-38

14. *mps3*, *D.melanogaster* 18°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	7243.6	1	7243.6	17.199	0.000040
Time	152726.5	3	50908.8	120.877	0.000000
Genotype*Time	5057.0	3	1685.7	4.002	0.007869
Error	188680.2	448	421.2		

15. *mps3*, *D.pseudoobscura* 18°C	**SS**	**df**	**MS**	**F**	**p**
Genotype	283.6	1	283.6	0.7257	0.395277
Time	31496.4	3	10498.8	26.8647	0.000000
Genotype*Time	2070.9	3	690.3	1.7664	0.154764
Error	79723.8	204	390.8		

6.11

1. *mps3*	SS	df	MS	F	p
Temperature	0.00541	1	0.00541	0.467	0.501363
Time	0.02269	5	0.00454	0.392	0.849458
Temperature* Time	0.01690	5	0.00338	0.292	0.912653
Error	0.26654	23	0.01159		

2. *mps3, D.pseudoobscura*	SS	df	MS	F	p
Genotype	0.09217	1	0.09217	7.633	0.007563
Temperature	0.00647	1	0.00647	0.536	0.466874
Time	0.10767	5	0.02153	1.783	0.129657
Genotype*Temperature	0.00093	1	0.00093	0.077	0.781859
Genotype*Time	0.04011	5	0.00802	0.664	0.651881
Temperature*Time	0.02661	5	0.00532	0.441	0.818347
Genotype*Temperature*Time	0.00478	5	0.00096	0.079	0.995205
Error	0.73652	61	0.01207		

3. *mps3, D.melanogaster*	SS	df	MS	F	p
Genotype	6.30655	1	6.30655	1010.696	0.000000
Temperature	0.30059	1	0.30059	48.173	0.000000
Time	0.01959	5	0.00392	0.628	0.678897
Genotype*Temperature	0.18516	1	0.18516	29.674	0.000000
Genotype*Time	0.02899	5	0.00580	0.929	0.466232
Temperature*Time	0.03404	5	0.00681	1.091	0.371216
Genotype*Temperature*Time	0.01156	5	0.00231	0.371	0.867494
Error	0.53662	86	0.00624		

Appendix to Chapter 7

7.2

1. Morning Activity Peak (11-15)

	SS	df	MS	F	p
Temperature	2369.4	1	2369.40	10.6471	0.001166
Time	17307.2	4	4326.80	19.4428	0.000000
Temperature*Time	767.5	4	191.87	0.8622	0.486376
Error	131298.7	590	222.54		

2. Evening Activity Peak (30-38)

	SS	df	MS	F	p
Temperature	15861	1	15861	31.567	0.000000
Time	205754	8	25719	51.186	0.000000
Temperature*Time	10311	8	1289	2.565	0.008983
Error	533618	1062	502		

3. Upswing (20-28)	**SS**	**df**	**MS**	**F**	**p**
Temperature	38897.1	1	38897.1	136.9837	0.000000
Time	28275.2	8	3534.4	12.4471	0.000000
Temperature*Time	1254.2	8	156.8	0.5521	0.817373
Error	301559.5	1062	284.0		

4. DD all Time points	**SS**	**df**	**MS**	**F**	**p**
Temperature	5010.6	1	5010.6	13.902	0.000200
Time	122416.5	47	2604.6	7.226	0.000000
Temperature*Time	22441.3	47	477.5	1.325	0.071139
Error	536313.9	1488	360.4		

7.3

1. Morning Peak (11-15) 18°C	**SS**	**df**	**MS**	**F**	**p**
Species	51459.2	1	51459.2	152.6353	0.000000
Time	28099.1	4	7024.8	20.8365	0.000000
Species*Time	574.8	4	143.7	0.4262	0.789782
Error	75908.9	1115	337.1		

2. Day (20-29) 18°C	**SS**	**df**	**MS**	**F**	**p**
Species	768.6	1	768.6	2.152	0.142664
Time	50029.7	9	5558.9	15.562	0.000000
Species*Time	2791.1	9	310.1	0.868	0.553283
Error	457936.6	1282	357.2		

3. Evening Peak (30-38) 18°C	**SS**	**df**	**MS**	**F**	**p**
Species	20734	1	20734	46.950	0.000000
Time	216296	8	27037	61.223	0.000000
Species*Time	19499	8	2437	5.519	0.000001
Error	513156	1162	442		

4. Morning Peak (11-15) 29°C	**SS**	**df**	**MS**	**F**	**p**
Species	79976.1	1	79976.1	316.9922	0.000000
Time	7665.8	4	1916.5	7.5961	0.000005
Species*Time	1968.0	4	492.0	1.9501	0.100398
Error	182915.0	725	252.3		

5. Day (20-29) 29°C	SS	df	MS	F	p
Species	2465.7	1	2465.7	14.2701	0.000165
Time	23382.0	9	2598.0	15.0355	0.000000
Species*Time	5708.1	9	634.2	3.6706	0.000148
Error	250546.7	1450	172.8		

6. Evening Peak (30-38) 29°C	SS	df	MS	F	p
Species	17824	1	17824	39.275	0.000000
Time	252587	8	31573	69.572	0.000000
Species*Time	150087	8	18761	41.340	0.000000
Error	592234	1305	454		

7. DD, 18°C	SS	df	MS	F	p
Species	186708	1	186708	598.471	0.000000
Time	119373	47	2540	8.141	0.000000
Species*Time	70574	47	1502	4.813	0.000000
Error	584017	1872	312		

8. DD, 29°C	SS	df	MS	F	p
Species	122899.8	1	122899.8	442.664	0.000000
Time	131355.8	47	2794.8	10.066	0.000000
Species*Time	163614.6	47	3481.2	12.539	0.000000
Error	533063.0	1920	277.6		