

UNIVERSITY OF LEICESTER

# Genetic Analyses of Circadian and Seasonal Phenotypes in *Drosophila*

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Submitted for Degree of PhD

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# Abstract

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## Genetic Analyses of Circadian and Seasonal Phenotypes

When an organism migrates from one area to another it comes into contact with many boundaries to its survival and fitness. The fruitfly *Drosophila melanogaster* has migrated from Africa, into Europe and colonised much of the rest of the world. The subject of this thesis is to better understand how *Drosophila* has adapted to survive the temperate climates in Europe. The variability of temperature and light from one season to the next makes adaptation of the circadian clock and life history strategy all the more important. *Drosophila* appear to have adjusted to the new conditions by exhibiting diapause in low temperatures when the nights are long and by altering several other characteristics of its circadian clock that may be related to diapause. One of these is a novel European single nucleotide polymorphism in the *timeless* gene that allows flies to maintain a more robust diapause than flies carrying the ancestral allele. This variant exists in all European populations and winter simulation experiments reveal that it maintains its diapause for longer than the ancestral variant. These experiments also supported the possibility that *Drosophila* diapause can be maintained for much longer periods than previous studies have indicated.

Experiments with ancestral African *D. melanogaster* lines alongside several closely related species indicates that diapause may not be a recent adaptation, but an ancient response to stressful conditions that has adapted in Europe to be more sensitive to low temperatures and short photoperiods. I also discover that a splice variant of the *period* gene has a dramatic, further cementing the controversial relationship between clock genes and diapause. In addition I have performed a study of putative functional polymorphisms in this untranslated region around this splice site from European populations. Finally, a study of putative clock genes reported in Chapter 3 provides a cautionary tale as to the dangers of using RNAi.

## Acknowledgements

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I'm not very good with words. A shocking revelation at the very start of a PhD thesis I know, but there are two words that I've used a lot last few years. Thank You. There are a great many people who have heard these two words more than a few times from me over the course of my PhD, and each one can take some credit for what I've achieved, no matter how large or small they see their involvement to have been. I would like to thank everyone that has made me feel welcome and reminded me that I deserve to be here when I've occasionally doubted myself.

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The rest of lab 124 have chipped in a great deal too. Pretty much everyone in lab 124 has helped me in some way at some point, whether it's feedback, reagents, techniques or a distraction there is a group of people who have been vital to my survival in Leicester. Of those involved in my project Ed was the first (chapter 3). Ed oversaw my first project and if it weren't for the flies he provided me with, I wouldn't have tasted failure early on and as a scientist, a feeling most scientists should get used to at an early stage. Aside from that, Ed has been on top of the latest techniques and offered plenty of guidance in areas where I've needed it, particularly his computer/bioinformatics input. The other person responsible for my first year is João who taught me a lot of the basics about diapause and dissection, areas in which Valeria and Mirko have also offered guidance. Giorgio has been involved in a separate project, unrelated to my own, but the working dynamic between the two of us has been important and he's been helpful in supplying me with reagents I'm low on and teaching me one or two techniques. We didn't just share lab space, but showers and a bedroom at a conference. The rest of lab 124 have offered feedback in my meetings, as well as lunchtime discussion and evening outings to the pub, where we naturally only discuss work and nobody ever has one too many.

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My partner Helen has been performing the vital job of keeping me stable for the last two years and supporting my thesis writing period by reminding me to take regular breaks and

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Finally, I would like to dedicate this thesis to the memory of two important people that I have lost in the last four years. The first is my school science teacher Mrs Linda Smith who nurtured my scientific curiosity and taught me all of the extra stuff I didn't need to know for the curriculum to keep me interested, constantly reminding me that science is not a dull classroom subject, but it is the world around us. The other person is my Grandfather Leo who so dutifully cared for and repeatedly watched the same five Whinnie the Pooh episodes with all fourteen of his grandchildren spanning over the 25 years that they were young enough to allow him to pretend it wasn't for his benefit. His incessant encouragement has helped his family become the people they wanted to be from engineers to teachers, magicians, musicians, photographers and artists. It is never easy to sum up in words just how important the people who guide you through life we can but hope that they know just how much their input was appreciated while they were alive.

Thank you all!

# List of Commonly Used Abbreviations

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UAS – Upstream activator Sequence	CC – <i>Corpora Cardiaca</i>
ZT – Zeitgeber Time	TG- Thoracic Gland
h- Hour	PTG – Prothoracic Gland
RNAi – RNA interference	ILP – Insulin-Like Peptides
LD – Light:Dark	PI – <i>Pars Intercerebralis</i>
DD – Constant Dark	InR – Insulin Receptor
bHLH – basic-helix-loop-helix	PI3K – Phosphatidylinositol-3-Kinase
PAS – Period Arnt Sim	PIP – Phosphatidylinositol
LN – Lateral Neurones	DAM – <i>Drosophila Activity Monitor</i>
DN – Dorsal Neurones	PBS – Phosphate Buffered Saline
UTR – Untranslated Region	SB – Squishing Buffer
SNP – Single Nucleotide Polymorphism	PCR – Polymerase Chain Reaction
Wt – Wildtype	RT-PCR – Reverse Transcriptase PCR
CA – <i>Corpora Allata</i>	Kb - Kilobase
JH – Juvenile Hormone	

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# 1 General Introduction

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## 1.1 *Drosophila melanogaster* as a model for genetics research

First described in 1830 by Johann Wilhelm Meigen, the fruit fly – *Drosophila melanogaster* – has become one of the most common animal model systems for studying evolution, genetics, development and behaviour. To this end, it was the first insect to have its genome published in 2000 (Adams et al., 2000) but prior to this, nearly a century of research into *Drosophila* had already been published. The first well documented experiment with *Drosophila* was undertaken by Morgan in 1910. In this work he discussed the heredity of a gene for white eyes and explained some important basics of sex linked inheritance of traits (Morgan, 1910). Morgan used a number of other observable characteristics of fruit flies to form the very basis of the mechanical concept of chromosomes carrying genetic information from parent to offspring (Morgan et al., 1915). This is arguably one of the most significant discoveries that formed the basis of modern genetics research and cemented *Drosophila* as a model organism, also winning Morgan the Nobel Prize for physiology or medicine in 1933.

Since Morgan's discoveries, historical advances using the fruit fly model include Muller's (1927) discovery that X-rays can artificially induce mutations, leading to ground-breaking research in mutagenesis, as well as a better understanding of the dangers of radiation (Muller, 1927, Muller and Altenburg, 1930). It also led to the use of forward genetics as a tool to understand the roles of genes in behaviour, disease and development. This information has been used to study a vast array of genes in *Drosophila* and other organisms. Like Morgan before him, Muller was awarded the 1946 Nobel Prize in physiology or medicine for his work. Edward Lewis, Christiane Nüsslein-Volhard and Eric Wieschaus were

awarded a Nobel Prize in 1995 for their discovery of genes which influence development (Nüsslein-Volhard, 1991). More recently, a Nobel was shared by Jules Hoffman in 2011 for his research in innate immunity. Hoffman's work in *Drosophila* and his discovery of *Toll* (Lemaitre et al., 1996) inspired the corresponding discovery in mammals and a greater understanding of its role in human disease. The awarding of four separate Nobel awards for research in *Drosophila* is a strong indicator of its importance as a research model

The use of *Drosophila* as a model is facilitated by the development of many genetic and molecular tools, for example the UAS/GAL4 misexpression system is now in common usage. GAL4 is a constitutively expressed transcription factor discovered in the yeast *Saccharomyces cerevisiae* responsible for positively regulating a number of genes in the presence of galactose. GAL4 is not induced by galactose, but the GAL4 negative factor - GAL80 – and is inhibited by its presence (Laughon and Gesteland, 1982). Brand and Perrimon (1993) took advantage of GAL4 to develop a method of misexpression of genes in fruit flies by creating two lines. One would carry a vector with GAL4 which can be expressed in specific tissues by using a gene specific promoter. A second line can be produced by attaching the upstream activator sequence (UAS) for GAL4 to a cloned gene of interest and transforming into flies. Crossing the GAL4 and UAS carrying lines together results in GAL4 driven expression in UAS-YFG (that is, your favourite gene) in specific cells or tissues. A common example of GAL4 mediated misexpression is the use of *reaper* and *head involution defective (hid)* which can be misexpressed to ablate specific cells using suitable GAL4 lines (Zhou et al., 1997). Molecular markers such as green fluorescent proteins (GFP) from jellyfish are also useful as to illuminate particular tissues by using a UAS/GFP line (Yeh et al., 1995). Further development of this tool saw the creation of lines carrying RNAi vectors,

allowing over expression of short interfering RNAs to target a gene of interest for downregulation (Misquitta and Paterson, 1999). These tools are unsurpassed for their flexibility in manipulating fly genes for the study of genes in physiology, behaviour and development.

## **1.2 Circadian clock in *Drosophila melanogaster***

The 24h rotation of the earth about its axis leads to predictable cycles of temperature, humidity and light which generates powerful selection pressures on plants and animals to seek out the best time to undertake their biological functions. For this reason, circadian rhythms have evolved to anticipate these changes and give organisms the best chance of survival by avoiding predators, maximising the visible light for successful foraging, etcetera

Circadian rhythms (from the Latin ‘*circa diem*’ meaning ‘approximately a day’) are most simply defined as a pattern of behaviours or physiological events that happen repeatedly every ~24h. Into this definition, there are two additional stipulations. Firstly, the rhythm must be entrainable by cues that indicate the day length, but secondly, they must be able to operate in the absence of these cues or Zeitgebers (from the German meaning “time giver”) (Pittendrigh, 1960). Beneath this simply defined output however is a deeply complicated genetic mechanism which ensures the constant running of these rhythms.

The circadian clock has been well characterised in *Drosophila*, indeed, the earliest experiments to uncover genes involved in the mechanism were performed in the fruit fly. Konopka and Benzer (1971) induced three mutations in flies and observed that one mutant expressed a fast 19h locomotor rhythm (termed *per*<sup>s</sup>), another a slow 28h cycle (*per*<sup>l</sup>) and a third that was arrhythmic (*per*<sup>0</sup>). All three phenotypes were mapped to one gene, subsequently termed *period* (*per*). The *per* gene was not only of the first behavioural gene

to be identified by mutation, but it was also the first to be molecularly cloned (Smith and Konopka, 1982, Bargiello and Young, 1984, Reddy et al., 1984, Zehring et al., 1984). Further analysis of the classic three *per* mutations indicated that they were all caused by single nucleotide changes, with *per*<sup>0</sup> coding for a premature stop codon (Baylies et al., 1987). The development of anti-PER antibodies allowed Siwicki and colleagues (1988) to conclude that PER was expressed in a diverse range of tissues but it is most prominent in the eyes, optic lobes and central brain regions. PER expression also cycles in abundance between day and night, later supported by evidence of mRNA cycling over a 24h period (Hardin et al., 1992, Hardin et al., 1990). The *per* DNA sequence is highly variable even among Drosophilids as revealed by the sequences in *D. virilis* and *D. pseudoobscura* (Colot et al., 1988). Nevertheless the same antibody identified a PER-like antigen in the molluscs *Aplysia* and *Bulla* (Siwicki et al., 1989). Subsequently, *per* homologues were discovered in mice suggesting a conserved clock mechanism in higher eukaryotes (Tei et al., 1997, Sun et al., 1997).

Sehgal and colleagues (1994) induced mutations in the second and third chromosomes and screened for abnormal locomotor and eclosion rhythms, and identified the *timeless* (*tim*) mutant. These mutants expressed quasi-normal locomotor activity in light-dark (LD) cycles of 12:12 but became arrhythmic in constant darkness (DD). Further analyses indicated that TIM is important for aiding PER's nuclear localisation (Vosshall et al., 1994). This supported the early concept of the transcriptional feedback loop (Hardin et al., 1990) in which PER (now with TIM) would translocate into the nucleus and prevent their own transcription (Reppert and Sauman, 1995).

How PER and TIM inhibit their own transcription was unclear before the discovery of E-boxes in the *per* and *tim* promoters – short sequences of CACGTG that act to regulate the expression of genes *via* basic helix-loop-helix (bHLH) transcription factors (Hao et al., 1997). The gene *Clock* (*Clk*), first cloned in mice after a mouse mutagenesis programme had identified mutant with a long rhythm (Vitaterna et al., 1994, Antoch et al., 1997) was a good candidate (King et al., 1997) as it encoded a bHLH domain and a PAS (Period Arnt SIM) domain similar to that found in PER. The *Clk* orthologue was subsequently identified in *Drosophila* by mutagenesis when a mutant termed *Clik<sup>rk</sup>* that exhibited arrhythmic locomotor activity was identified (Allada et al., 1998). The influence of *Clik* within the circadian mechanism was inferred from its influence on the expression of *per* which is misregulated in *Clik<sup>rk</sup>* homozygotes (Allada et al., 2001). Another arrhythmic fly mutant with low *per* and *tim* transcription was termed *cycle* (*cyc*) (Rutila et al., 1998). The homologue of this PAS domain containing gene was almost simultaneously identified in mice where it was termed *Bmal1* (Gekakis et al., 1998) and in *Drosophila* it is sometimes referred to as *dBmal1* (Darlington et al., 1998). Darlington and colleagues (1998) observed, through yeast experiments, that fly CLK and CYC bind each other, but also are only capable of binding the E-box sequences when coexpressed. In order for this feedback loop to operate PER and TIM would negatively regulate themselves by interfering with the transcription factors that bound to their E-boxes, so CLK-CYC immediately became the best candidates for representing these positive factors in the circadian feedback loop.

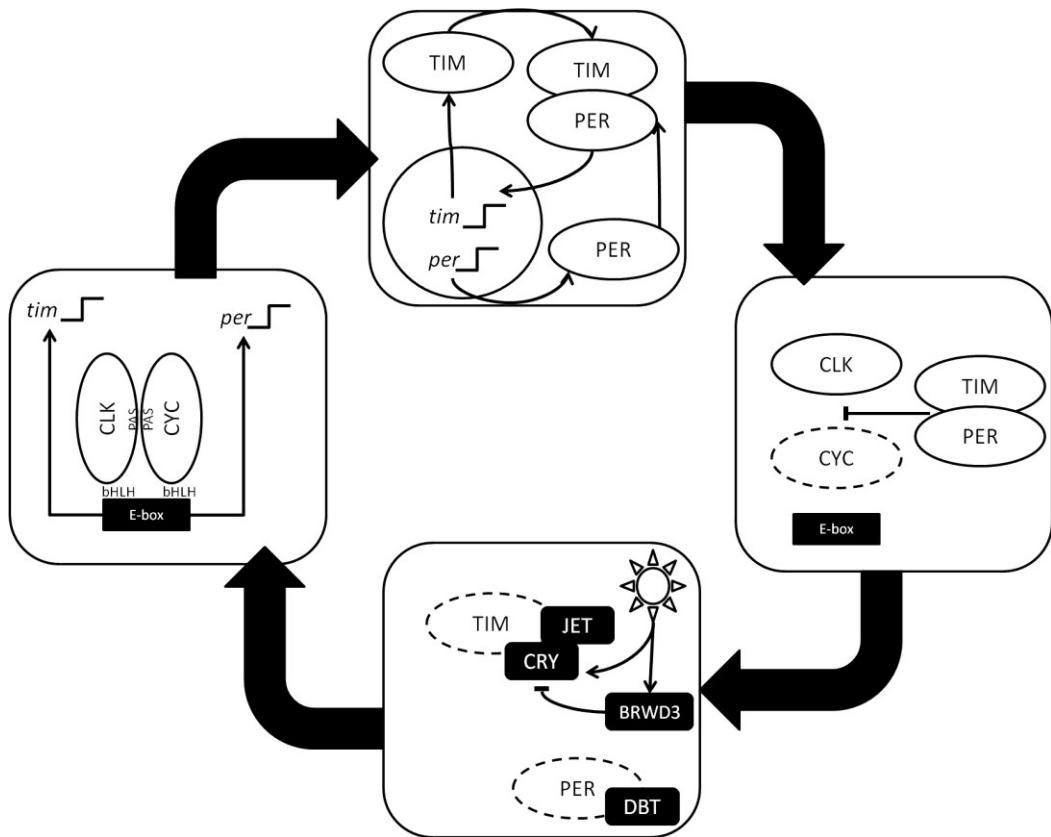
Furthermore, PER/TIM would need to be degraded to permit CLK/CYC dimerisation and then *per/tim* activation. The mechanism for this degradation involves DOUBLE-TIME (DBT) – a casein kinase 1 which phosphorylates PER (Price et al., 1998). Mutants for *dbt* have similar

effects to mutations in *per* with slow and fast rhythm mutations, though null mutations were non-viable. The 4-6h delay between *per* mRNA and protein cycle involves the phosphorylation of PER by DBT early at night which delays PER accumulation until stabilised by dimerisation with TIM (Kloss et al., 1998, Price et al., 1998). However, DBT also plays a role late at night/dawn when TIM, which protects PER as a heterodimer, is degraded in light, leaving PER once again, as a monomer susceptible to DBT-mediated degradation.

The degradation of TIM relies indirectly on light (Hunter-Ensor et al., 1996) and on the blue light photoreceptor CRYPTOCHROME (CRY). A mutant form of CRY in *Drosophila* revealed how environmental light interacted with the clock. Termed *cry<sup>b</sup>*, the primary mutation resulted in an amino acid substitution in a region which is highly conserved within the class of photolyases (Stanewsky et al., 1998). The mutants were shown to inhibit normal *per* and *tim* cycling in LD and DD conditions (reflecting the effect of *cry* mutants in the periphery, the eyes, which make up most of the clock expressing cells in the head), but these flies also failed to respond normally to light pulse experiments which would ordinarily reset the fly's clock to a different phase (Stanewsky et al., 1998). The *cry* mRNA transcripts cycle robustly over 24h in LD, but CRY does not cycle in DD, merely accumulating in darkness. The rhythm of CRY in LD is posttranscriptional because light causes the degradation of CRY (Emery et al., 1998). Ceriani and colleagues (1999) showed that CRY is capable of binding TIM or TIM/PER heterodimer, but this binding was only possible in light and does not occur in the dark.

Another unusual behaviour observed in *cry<sup>b</sup>* mutants was the persistence of circadian behaviours in constant intense light (LL), where typically flies would exhibit a period lengthening effect or arrhythmic behaviour depending on the intensity of the light (Emery et al., 2000). Direct degradation of TIM by CRY however does not occur. CRY undergoes a

conformational change in light which allows it to bind TIM, leaving TIM prone to degradation by phosphorylation (Emery et al., 1998, Emery et al., 2000, Naidoo et al., 1999), aided by another protein, JETLAG (JET) which is responsible for TIM ubiquitination and degradation *via* the proteasome pathway (Koh et al., 2006, Peschel et al., 2006, Van Gelder, 2006). JET is particularly important for circadian resetting in light and mutants for this gene – termed *jet<sup>R</sup>* and *jet<sup>C</sup>* – were shown to exhibit normal behavioural rhythms under LL conditions and respond poorly to a change in the timing of light in the morning (Koh et al., 2006). It has recently been suggested that CRY is also degraded in light through ubiquitination as a consequence of binding the protein Bromodomain and WD repeat domain containing 3 (BRWD3), also known as *ramshackle* and knockdown of this in S2 cells shows attenuation of CRY degradation (Ozturk et al., 2013). The culmination of all this research outlined a simple model for the circadian clock outlined in Figure 1-1.



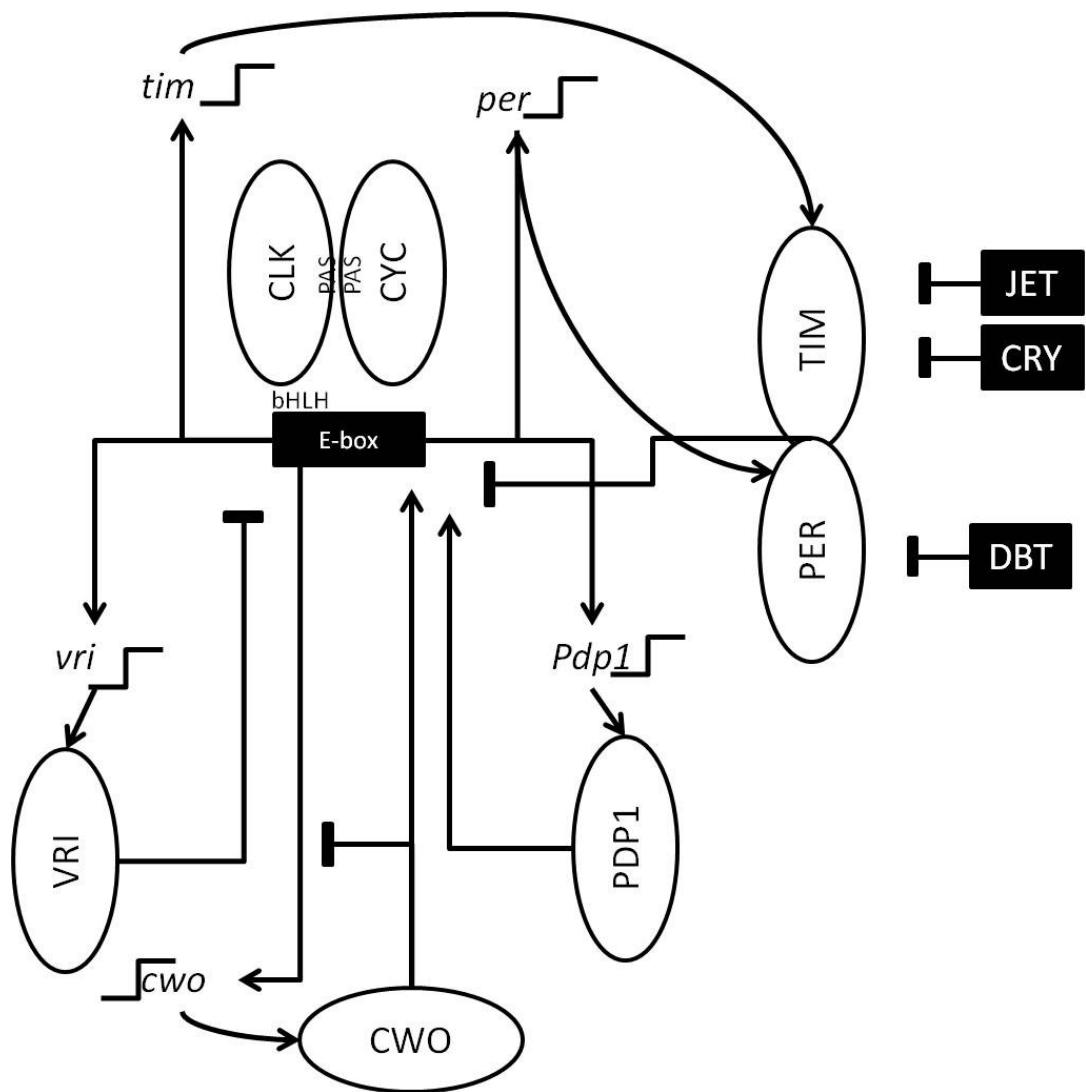
**Figure 1-1 Basic concept of the molecular circadian clock in *Drosophila*.** CLK and CYC bind the E-boxes of *per* and *tim* increasing their levels of transcription. In the cytoplasm *tim* and *per* are translated into proteins where they dimerise and translocate into the nucleus to inhibit CLK/CYC dimerisation, blocking *tim/per* transcription. DBT leads to degradation of PER in the early night, generating the delay between *per* mRNA and protein cycles. At dawn (ZT0), CRY and JET lead to degradation of TIM, leaving PER vulnerable to DBT phosphorylation/degradation. In the absence of the main negative regulator, PER, consequently CLK and CYC restart the cycle *per/tim* transcription-translation cycle and the clock has completed a cycle.

The clock is not a single feedback loop as indicated (Figure 1-1), but consists of several loops feeding into each other. The second loop regulates the expression of CLK, shown to be rhythmic in *Drosophila*, (Allada, 2003, Cyran et al., 2003, Blau and Young, 1999). Like other clock genes, *vrielle* (*vri*) mutations generate locomotor phenotypes with flies carrying only one functional copy exhibit short rhythms while constitutive expression of *vri* results in long or arrhythmic locomotor behaviours (Blau and Young, 1999) indicating a direct role within the clock. *vri* mRNA cycles over 24h in phase with *per* and *tim* and antiphase to *Cik* and also contains the CLK binding E-box (Blau and Young, 1999). *Cik*<sup>Jrk</sup> and *cyc*<sup>0</sup> mutations also led to

low levels of *vri*, indicating regulation by the central clock, while *tim<sup>0</sup>* and *per<sup>01</sup>* mutants resulted in high levels of VRI, indicating a possibility that VRI is negatively regulated by the TIM/PER complex. VRI contains a DNA binding domain, suggesting the possibility that it may regulate transcription of other genes, in this case, *Clk*.

Further to *vri*, another gene – this one containing a PAR (proline and acidic rich) domain – gene was shown to be involved in this secondary loop, termed *Pdp1* (*Par domain protein 1*) (Cyran et al., 2003). Viable mutants for *Pdp1* lengthened the period of locomotor behaviour, eliminating cycling of PER and TIM and maintaining low levels of both, similar to the phenotype found in *Clk<sup>Jrk</sup>* mutants. The presence of three binding sites for the PAR domain in the *Clk* promoter region strongly implied that *Pdp1* is a positive regulator for *Clk*. The discovery of this secondary feedback with PDP1 and VRI acting as positive and negative regulators respectively of *Clk* explained how *Clk* was able to cycle in a rhythmic fashion.

An additional loop was also discovered involving the gene *clockwork orange* (*cwo*) which also has an E-box, meaning its expression may be regulated by CLK/CYC complexes. CWO also carries a similar bHLH domain to CLK and displays an ability to bind the E-boxes of *per*, *tim* and itself (Lim et al., 2007, Richier et al., 2008). Mutants for *cwo* affect levels of *cwo* mRNA, but *tim*, *per*, *vri* and *Pdp1* transcripts were all decreased, implying that while CWO is responsible for repressing its own transcription, it is also responsible for activating transcription of other E-box containing genes (Richier et al., 2008) This forms a tertiary loop within the core clock mechanism (Figure 1-2).

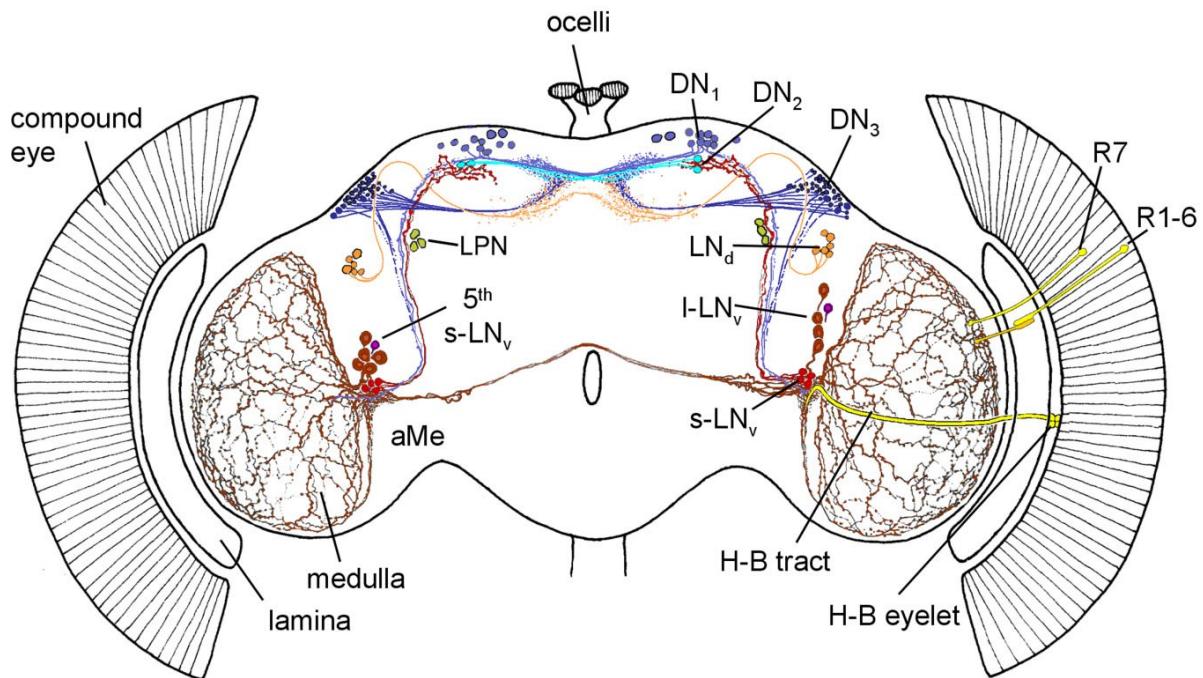


**Figure 1-2 Interaction map of the circadian clock.** A more complete image of how the circadian clock operates on a molecular level in *Drosophila*. The diagram shows the primary loop with CLK/CYC activating TIM/PER which repress CLK/CYC and the loop reset by DBT, JET and CRY. The secondary loop shows VRI and PDP1 feeding back to negatively and positively influence CLK expression and a tertiary loop involves CWO repressing itself while helping to overexpress other E-box clock genes.

For the circadian system to influence behaviour, a neurological output is required.

Experiments using a *perGAL4* driver to express GFP by Plautz and colleagues (1997), was intended to discover what cells drove the clock in *Drosophila*. The findings indicated that most tissues in the *Drosophila* body express *per*, but also showed cycling which was largely maintained when tissues were kept in culture, suggesting that many of the cells will maintain a rhythm independently. In spite of this finding, a central “master” behavioural

clock was identified in the *Drosophila* brain, consisting of approximately 150 cells (Figure 1-3). The cells were classified into two groups, the lateral (LN<sub>s</sub>) and dorsal (DN<sub>s</sub>) neurones and further categorised as dorsal/lateral neurones (LN<sub>d</sub>s), the large and small ventral lateral neurones I-LN<sub>v</sub>s and s-LN<sub>v</sub>s and three sets of dorsal neurones termed DN<sub>1</sub>s, DN<sub>2</sub>s and DN<sub>3</sub>s (Helfrich-Förster et al., 2007).



**Figure 1-3 Diagram of the *Drosophila* brain displaying the major clock cells and arborisations. As well as the major classes outlined, the R1-6, R7/8 and Hoffbauer-Buchner (H-B) cells of the compound eye act as light input. (Helfrich-Förster et al., 2007)**

Communication between these cells is largely down to the neuropeptide Pigment Dispersing Factor (PDF) which helps clock cells to maintain synchrony, while driving the clock in cells which are not independently rhythmic (Peng et al., 2003). Loss of synchrony between the pacemaker neurones results in abolition of DD locomotor rhythms in *Pdf*<sup>01</sup> mutants (Renn et al., 1999) similar to the effect of ablating PDF expressing cells among the LN<sub>v</sub>s (Stoleru et al., 2004). The different roles played by clock neurones are evident from the influence of the s-

$\text{LN}_{\text{V}}$ s on morning activity and the  $\text{LN}_{\text{d}}$ s and  $\text{DN}_1$ s on evening activity (Stoleru et al., 2004). The output of the clock has been observed, largely in locomotor activity, but how the clock can influence changes in behaviour is not so clear. Im and Taghert (2010) stained cells for the PDF receptor and indicated that, as expected, the receptor is found in all of the clock cells, staining particularly strongly in the  $\text{LN}_{\text{d}}$ s, 5<sup>th</sup> s- $\text{LN}_{\text{V}}$  and  $\text{DN}_1$ s, all of which project to PDF secretory cells. They also indicated that the receptor was expressed in a large number of non-clock cells and cells in the visual system, indicating a putative output mechanism where the clock oscillates in central pacemaker cells which then communicate *via* PDF signalling with various neurones to elicit behavioural responses in a circadian manner. The output is not just neurological however, McDonald and Rosbash (McDonald and Rosbash, 2001) identified 137 genes with diverse roles by microarray with many of them involved in immunity, neuropeptides and pheromone and odorant binding proteins, all of which can be involved in influencing physiology and behaviour over the course of a day.

## **1.3 Seasonal Fitness Adaptations**

### **1.3.1 Seasonal Adaptations in Insects: Diapause**

The tilt of the earth's axis also produces variation in behaviours elicited in one complete orbit around the Sun, giving rise to the earth's seasons. As these are more extreme in temperate and arctic regions, plants and animals have been forced to adapt to survive the hot summers and cold winters. The typical example of seasonal variation in animals is the observation of hibernation in mammals – a state where animals lower their body temperature and metabolism and reduce activity to survive during food shortages. Hibernation is a phenomenon which has been documented since Aristotle (c. 384-322 B.C.)

and early experiments performed by renowned naturalist Conrad Gessner in the mid 16<sup>th</sup> century in his *Historiae animalium* volume *Quadrepedes vivipares* (Rasmussen, 1916).

Diapause is a less well known adaptation for maximising an organism's survival and reproductive potential that involves a halt in development until conditions improve. It is important to separate the study of hibernation and diapause, as both states have been observed in insects. The term diapause was first coined by Wheeler in 1893 to describe a state of embryonic development in locusts, bearing little resemblance to the current use of the term (Wheeler, 1893). He used the Greek term *διάπανσις* meaning to "rest in between" to describe a rest phase in development in the movement of an embryo within the egg. Wheeler's use of the term was incidental and the word was later used to discuss a somewhat different phenomenon. Henneguy (1904) described a developmental arrest in insects that could be induced under various stressful conditions, listing electrocution, temperature, high pressure, acid and physical damage as methods of arresting development temporarily. Shelford (1929) however, used a more discrete definition to describe a dormancy, induced by unfavourable conditions and broken in a favourable environment, but the key element of Shelford's definition was to separate diapause from hibernation. He described diapause as "*a condition in which no further activity or progress can be induced*", adding the observation that chemical, physical or environmental conditions must be altered to end this state and resume development. This distinguished diapause from hibernation which he defined as "*a state of inactivity without feeding*".

Furthermore, diapause can be distinguished from other stress avoidance strategies in insects. Presenting in a similar manner to diapause is quiescence, where an organism may cease development at any life cycle stage in response to stressful conditions including

drought, high temperatures or cold (Tauber and Tauber, 1986). Unlike diapause, which may be maintained by indicators of stressful conditions such as photoperiod, quiescence is a short response to stress and immediately reversed when the conditions become more hospitable. This is a common case in mosquitoes which will often undergo quiescence in response to drought or shorter cooler periods experience in the tropics (McMeniman and O'Neill, 2010). Oligopause has a longer effect than quiescence, often lasting long periods such as the entire winter, however, it is still a more simple response to conditions rather than the more complicated diapause response (Leather, 1995). Ladybirds are shown to overwinter in oligopause, living off fat reserves during this period, however, unlike in diapause, they may feed for periods during the winter to restock (Roy and Majerus, 2010). Australian populations of the monarch butterfly *Danaus plexippus* also undergo oligopause, exhibiting dormancy for long periods at low temperatures (James, 1982). Some insects have evolved strategies to avoid the stressful conditions of winter altogether, typically migrating to more tolerable climes. *D. plexippus* for example, migrates from North America to areas in Mexico and California as short days indicate an approaching winter, which is more tolerable further south (Froy et al., 2003). Greater still is the migration of the milkweed bug, *Oncopeltus fasciatus*, migrating between Brazil and Canada and undergoing as many as four generations on the northerly migration (Saunders, 2010).

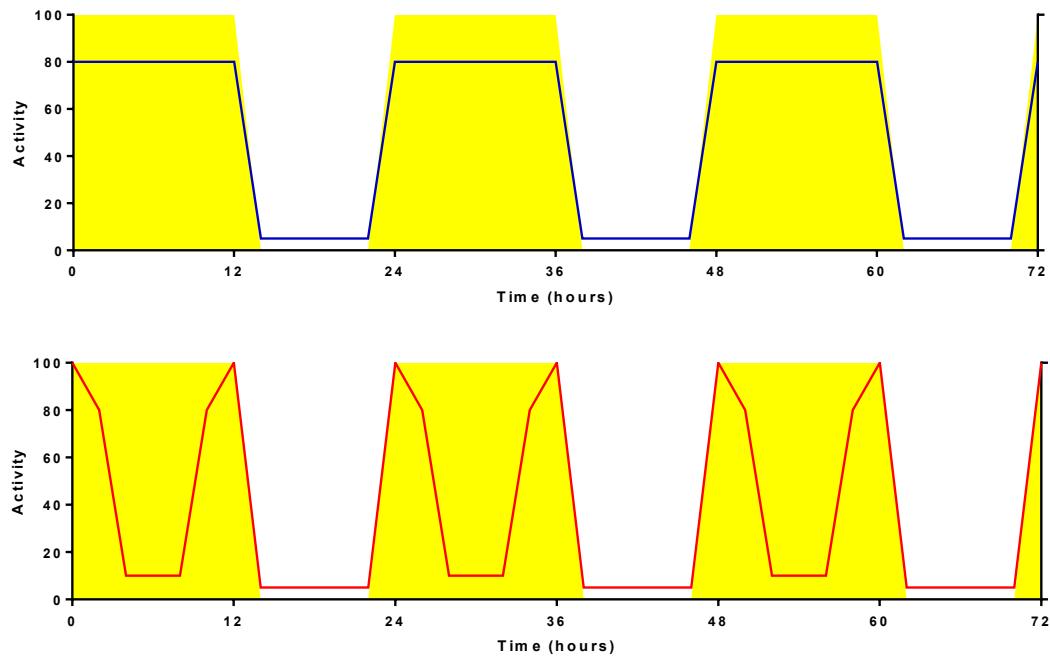
The majority of models for diapause research are arthropods with Lepidoptera taking a prominent position, particularly the silkworm *Bombyx mori*, because of its commercial value in producing silk. Silkworms exhibit a summer diapause, maturing as far as the pupal stage and then halting development until the appropriate cues to emerge in a cooler environment are met (Lees, 1955). Kogure (1933) provided the first evidence that photoperiod could

influence the diapause of silkworms, but since then, a plethora of other models have been confirmed to exhibit photoperiod induced diapause including the pitcher plant mosquito (Bradshaw and Lounibos, 1972), the European Corn Borer (Beck, 1962) *Nasonia vitripennis* (Saunders, 1965) and among many others *D. melanogaster* (Saunders et al., 1989).

Although typically studied in arthropod models, it is important to note that diapause is known to exist in a number of vertebrates. A commonly used example is the annual Killifish which inhabit temporary pools. They lay eggs in the mud which protects them from drying out and the arrival of rain stimulates hatching (Hand and Podrabsky, 2000). Chameleons have also been shown to exhibit an embryonic diapause, responding to temperature and moisture cues (Andrews and Donoghue, 2004) and turtles, which enter a preovipositional arrest as well as an embryonic arrest (Booth, 2002). Even mammals have been observed to exhibit diapause, the classical example being the roe deer which elicits an embryonic arrest over winter (Aitken, 1975) and it has been implied that almost all phyla within the animal kingdom contain at least one species which exhibits diapause with a range of different cues influencing development (Hand and Podrabsky, 2000).

As well as diapause, a seasonal response has been observed in *D. melanogaster*, responding to changes in day length and temperature that is associated with splicing in the 3' untranslated region (UTR) of *per*. Three isoforms were originally identified with variant A containing seven introns without exhibiting a splicing event, variant B excises a short sequence in the 3' UTR forming an eighth intron and variant C included the last three introns to create a large exon (Citri et al., 1987). Variant C was subsequently shown to be artefactual, but the A and B variants have been studied in greater detail to understand their effects on behaviour (Cheng et al., 1998). Majercak (2004) showed evidence that *perA* has

higher expression during warmer conditions where splicing of the eighth intron (*Drosophila melanogaster period* intron 8 or *dmp18*) occurs at lower frequencies. *dmp18* is more readily spliced out at cooler temperatures and this has been linked with siesta behaviour (Figure 1-4) seen at higher temperatures to avoid dessication during the day, but is abolished under lower temperatures to maximise the use of daylight.



**Figure 1-4 Siesta behaviours in *Drosophila*.** At low temperatures (blue), *Drosophila* maintain activity throughout the day to maximise the light usage. In higher temperatures (red), *Drosophila* are active at dawn and dusk, but avoid afternoon heat by performing a siesta

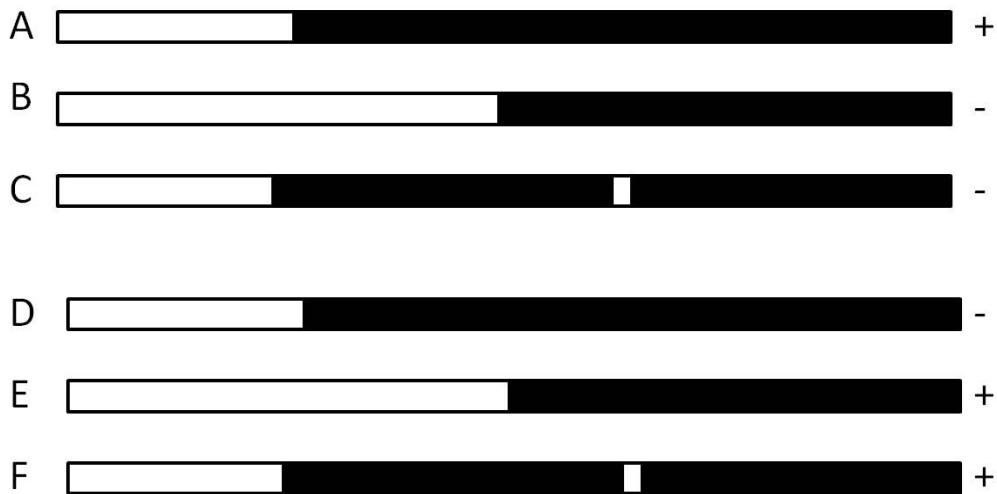
Enhanced splicing, results in more rapid accumulation of *per* mRNA transcripts (Low et al., 2008, Majercak et al., 1999) which is likely responsible for the alterations in siesta behaviour. The findings suggest that the splicing event is important for seasonal behaviours, which raises the question of whether and how this may be involved in diapause. Other evidence for a similar event has been described in closely related species, *D. yakuba*, *D.*

*simulans* and *D. santomea* - though the splicing in *D. yakuba* is modulated by daylength (Low et al., 2008).

### **1.3.2 Models of photoperiodic measurement**

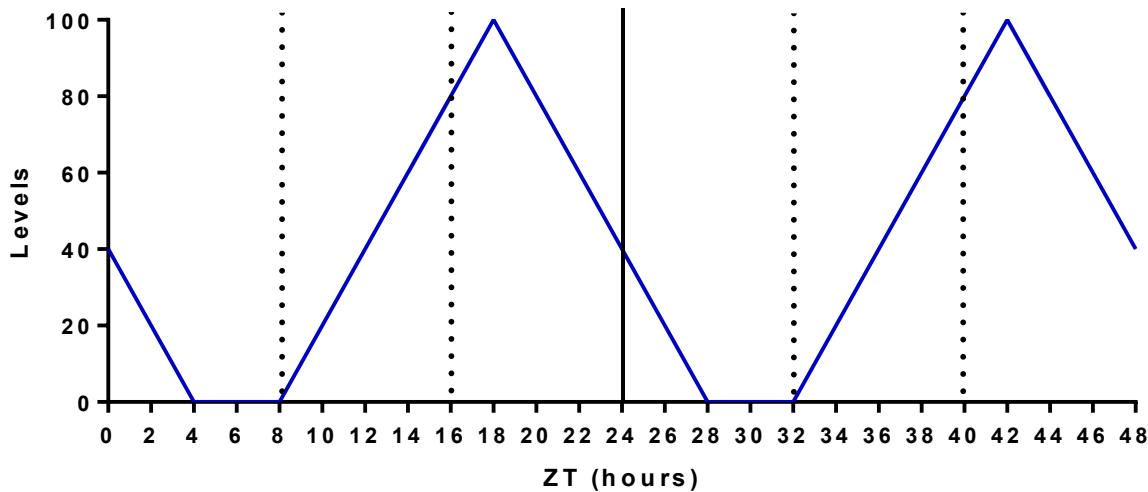
#### The External Coincidence Model

The idea that the clock has a role to play in photoperiodic timing is far from new. Erwin Bünning conceptualised and produced a model to explain the role of photoperiodic measurements (Bünning, 1936). It was later elaborated by Colin Pittendrigh and colleagues (Pittendrigh and Minis, 1964, Pittendrigh, 1972). This model, like many of the models for photoperiodism was based on plant flowering behaviours, but has been applied in many ways to photoperiodism in animals. Under this hypothesis, the subject is susceptible to light at a specific time of day, in the late night or early morning. If the organism were illuminated during this time, a long day response is initiated. Bünsow (1960) developed a protocol to test this hypothesis which involved illuminating a plant under short day conditions and treating some with a light pulse in the middle of the night. In these experiments (Figure 1-5) illumination in the middle of the triggers a long day response, which makes long day flowering plants flower and inhibits flowering in short day flowering plants



**Figure 1-5 Bünsow protocol applied to short day and long day flowering plants.**  
**Interrupting the long night results in long day behaviours.** A-C shows a short day flowering plant, flowering in short days, but not in long days or days with a night interruption. In contrast, D-F shows a long day flowering plant flowering in long day or night interruption, but not in short day (Adapted from (Bünning, 1973))

As a concept, this model would rely on a single oscillating molecule that is sensitive to light to act as a 'counter'. Figure 1-6 indicates how this particular molecule might operate as it oscillates through a 24h cycle, larger amounts of it accumulate during the night. When light falls on this molecule, if there is a significant amount of it, a signal is generated, informing downstream processes that it is a long day. Using the Bünsow method, stimulating the molecule with a light pulse during dark phases, when large amounts of the molecule have accumulated would trigger a long day response

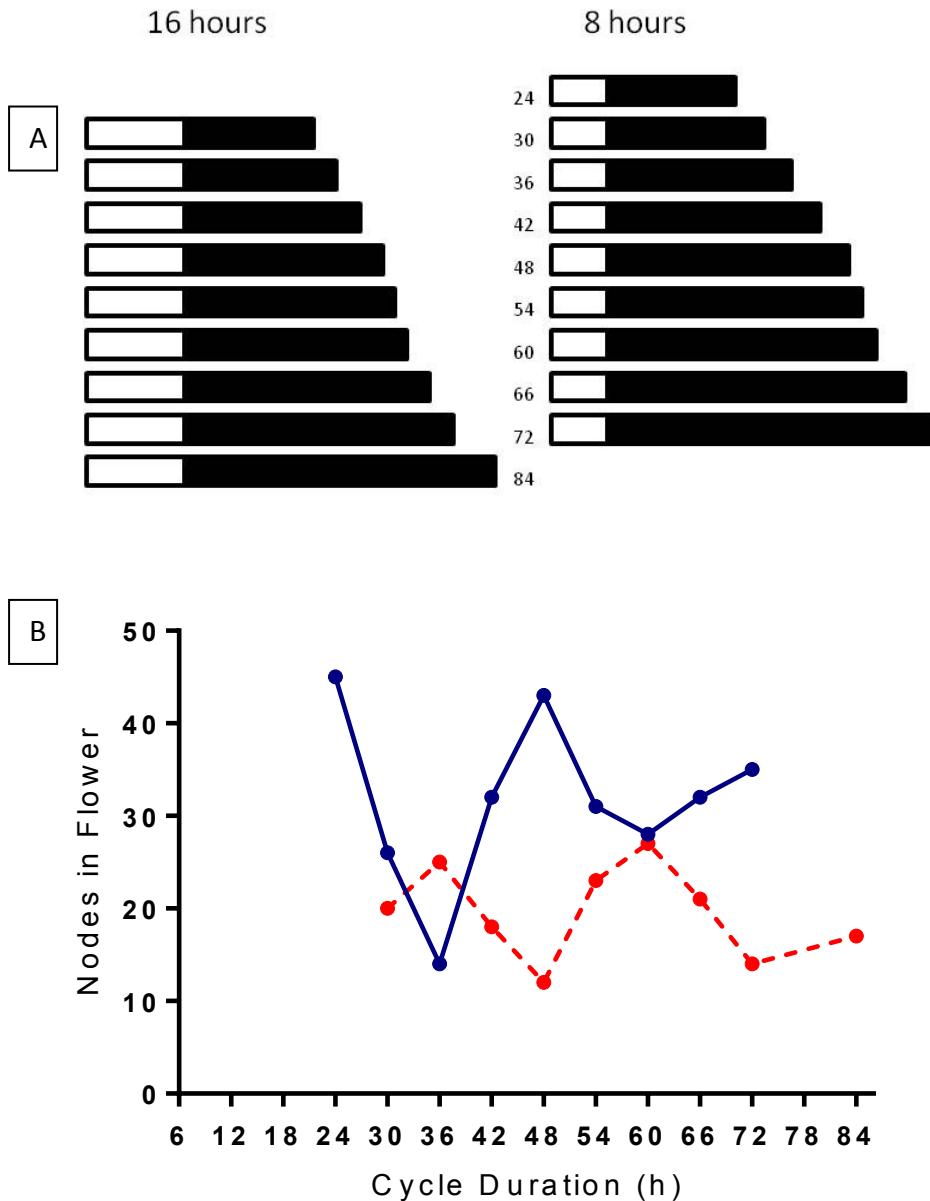


**Figure 1-6 Concept diagram for the external coincidence model. An oscillating light sensitive molecule is expressed rhythmically over a 24h period. Larger amounts of it under light can trigger a long day response. At high levels (LD16:8) the counter can signal a long day where the low levels in LD 8:16 mean that the counter's influence is minimal**

### The Internal Coincidence Model

The internal coincidence model is a more complicated system as it involves two oscillators as opposed to the single oscillator in the external model. The model was proposed by Pittendrigh (Pittendrigh, 1972) based on the findings of Nanda and Hamner's experiments in soybeans (Nanda and Hamner, 1958). As Nanda and Hamner describe the hypothesis, it is reliant upon the theory that the organism undergoes a series of reactions in the light (A), and a different series of reactions in the dark (B). The result of the interactions between these reactions is the stimulus (C), or as Pittendrigh suggests a dawn and a dusk oscillator. The Nanda-Hamner protocol involves subjecting an organism to a range of photoperiods and artificial day lengths. Their subjects were kept in 8, 16 or 32h photophases and the scotophase lengthened by 6h for each experiment (Figure 1-7A). The 8h photophase subjects received total day lengths of 24-72h, the 16h photophase 30-84h and the 32h

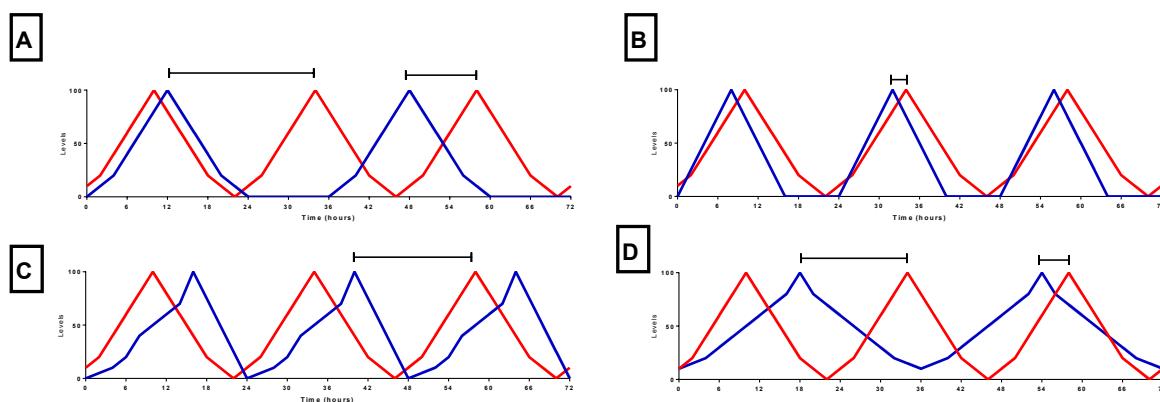
photophase 48-96h. The results indicated that the photoperiodic response was strongest in cycles of 24h (i.e. 24, 48 and 72h) and weakened when the light/dark oscillators were unsynchronised, as in 36 and 60h cycles (12h out of phase), eliciting the opposite photoperiodic response. For example (Figure 1-7B), the short day flowering plants would flower in an 8h photophase in a 24h day. Peaks in flowering were observed when the total day length was a multiple of 24h, i.e. 24h, 48h and 72h. Troughs in flowering were observed when overall day length was the most out of phase with a 24h day, i.e. 36h or 60h. The opposite effect was observed when a long photophase (16h) was used, with troughs of flowering in multiples of 24h and peaks when out of phase.



**Figure 1-7 Nanda-Hamner experiments in Soybean flowering. A – experimental procedure.** An 8h or 16h photophase is paired with an increasing length of scotophase in different experiments. **B – 8h photoperiod experiments (blue line),** show that in a 24h cycle a short day response is elicited. At the peak out of phase (36h and 60h) the flowering response is like that of a long day. Contrarily, 16h photoperiod responses (red line) show the lowest levels of flowering when in phase, as in a long day (reproduced from data in Nanda and Hamner (1958)). Differences in peaks are likely due to lower temperature in the 16h experiments

The importance of the Nanda-Hamner experiments was that they indicated that there must be two separate systems in play to measure day length, one measuring the length of the photoperiod and the other circadian regulated, or influenced by other zeitgebers (Figure

1-8). Conceptually two proteins are cycling in a daily rhythm and their interaction is responsible for inhibiting or eliciting the seasonal response. In this example of a short day, a circadian protein “A” peaks at ZT0 daily and a light induced protein “B” peaks at ZT8, 16h earlier. In a long day, LD 16:8, the difference in peak times is only 8h leading to greater accumulation of A and B at the same time, triggering a long day response. In a Nanda-Hamner experiment, because of the change in phase in a 36h experiment, relative to the 24h experiment, B is expressed in some cycles within 4h of the peak of A leading to very high accumulation of both representing an extreme long day, similar to a cycle of LD 20:4. It is also possible that an oscillator, driven by light is set to longer phases. In Nanda-Hamner experiments, the phase of this oscillator is stretched to 36h and its interaction with a 24h oscillator results in alternative long and short days.

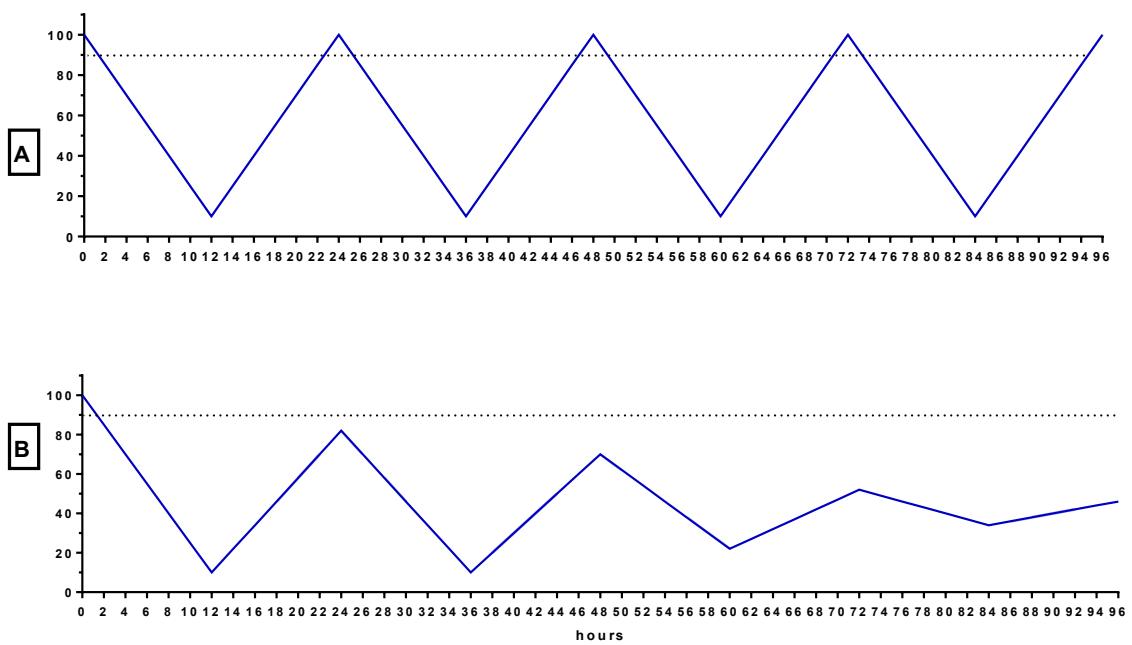


**Figure 1-8 Conceptual explanation for internal coincidence.** Consider a circadian-controlled gene (red) which is expressed rhythmically over 24h and a photo-responsive gene (blue) is expressed in response to light. The interaction of these genes is responsible for inducing a seasonal behaviour. In LD 8:16 (A), peak expression of the photoresponsive gene occurs 16h before the peak in the circadian gene. In a 36h Nanda-Hamner Cycle (8:28) (B), this period is 4h, conveying a long day. An LD 16:8 day (C) shows that there is an 8h period between the end of the photoresponsive peak and the circadian peak. Finally, a phase relationship between a photosensitive oscillator and non-sensitive oscillator shows stretching of the peaks resulting in alternate long/short days (D)

## The Hourglass Model

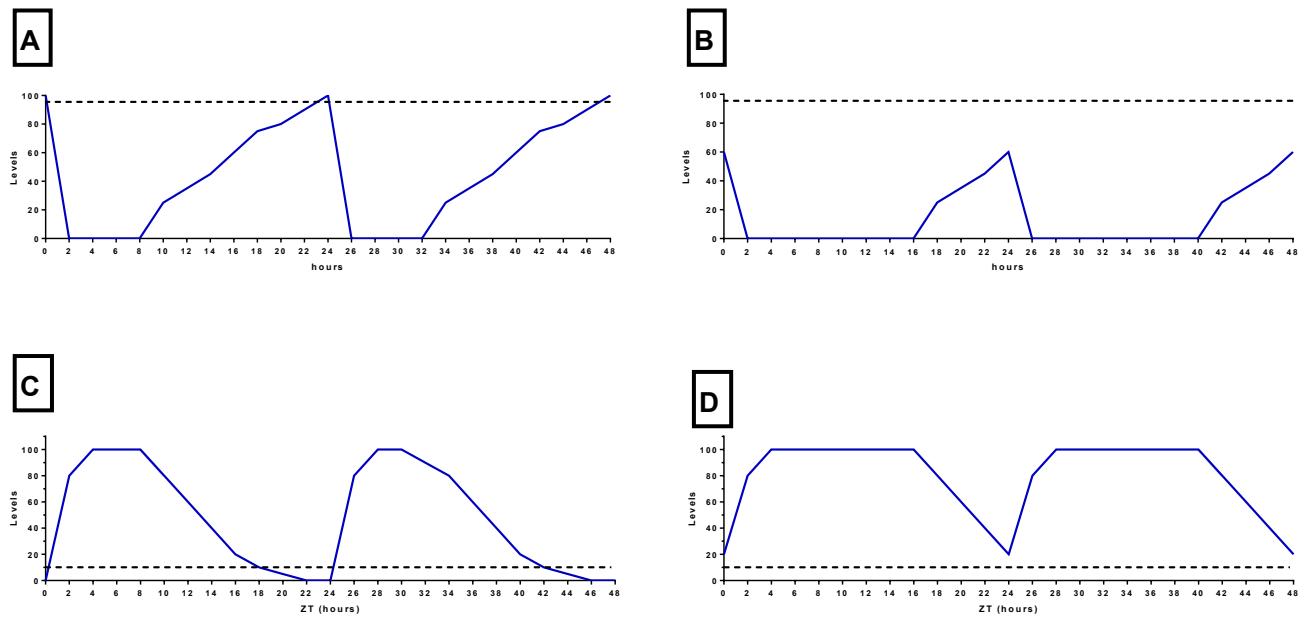
The hourglass model of diapause refers to phenotypes that do not fall under the oscillatory mechanisms evidenced with the internal and external coincidence models. The traditional example of this model is the work performed by Lees using the aphid *Megoura viciae* (Lees, 1973). The study was not on diapause, but on a different photoperiodic response in *M. viciae* which produce parthenogenic offspring (virginoparae) under long day conditions and reproductive offspring (oviparae) under short days. However, this response was not influenced by experiments using the Nanda-Hamner or Bünsow protocols indicating that it is very probable that no circadian clock involvement, but it also does not exclude the clock as a possible modulator (Meuti and Denlinger, 2013, Saunders, 2009).

Little work has been done to suggest how the hourglass model might ‘count’ the night or day length. One theory, suggested by Saunders (Saunders et al., 2002) is that of oscillatory damping (Figure 1-9) where the clock is wound down under diapause inducing conditions, and its ability to elicit behaviour and physiological changes (such as development) becomes more limited.



**Figure 1-9 Damping of circadian oscillators.** Under specific conditions, circadian oscillations are damped and the clock effectively switched off. When an oscillator fails to reach thresholds it signals that conditions are unsuitable for continued development.

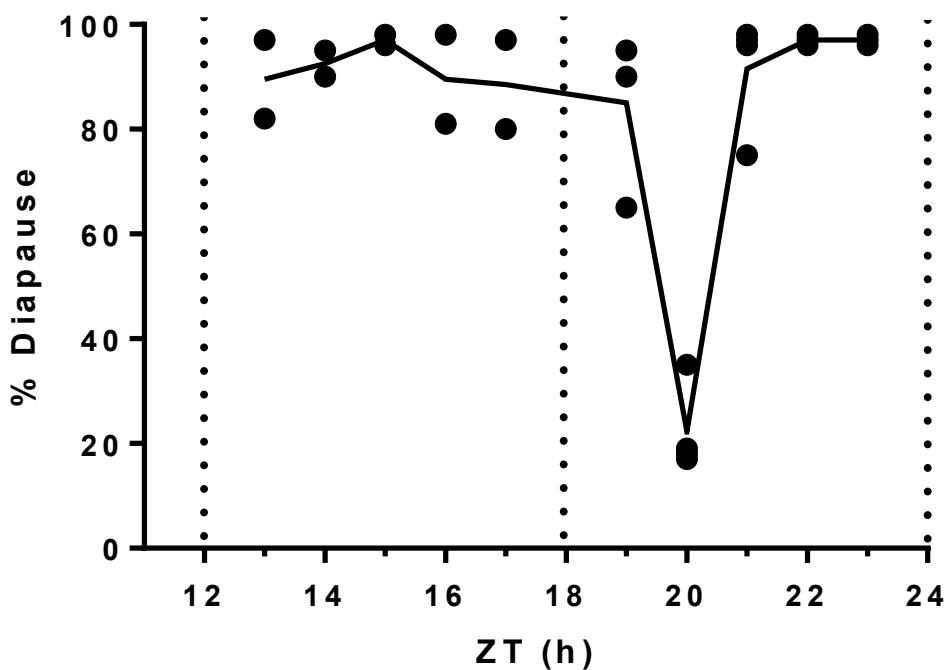
Another theory for the hourglass model relies on levels of a light sensitive molecule (Figure 1-10), for example a light degraded protein would increase in levels during the dark and high levels would signal a long night. In a short night the protein accumulates to low levels and does not achieve the threshold. The light pulse experiments cause degradation of the protein and it does not recover to reach the threshold, resulting in a short night like response. This model could work in a contrary manner, counting the day rather than the night with levels of a protein synthesised in light



**Figure 1-10 Conceptual explanation for the hourglass model.** A light sensitive protein reaches high levels during longer nights, crossing a threshold (A). In short nights (B) the protein does not have enough time to accumulate to the threshold. This could work in the opposite way with the days being counted by a molecule synthesised in light that falls below a threshold in short days (C) but not in long days (D)

The modelling of photoperiodism did not end at flowering plants and the validity of these models for explaining invertebrate photoperiodism has been assayed in a number of examples. At the time of publication in 2002, Saunders had identified 23 species of invertebrates in literature that had been tested for which model they most readily fit. Of these, 12 exhibited an hourglass diapause, eight supporting the internal coincidence model and three fitting the external coincidence pattern (Saunders et al., 2002) though this may be confounded by the assumption that having failed a test instantly means they fit the hourglass model. A classic example of an insect diapause that fits the external coincidence model is *Sarcophaga argyrostoma* in a study by Saunders (1979). *S. argyrostoma* exhibit a short day pupal diapause and were submitted to a Bünsow protocol with a 12h night (darkness ZT12-24). Animals were given light pulses at various points throughout the night

(Figure 1-11) and in experiments with a light pulse at 20h, a long day (low diapause) response was elicited, indicating high sensitivity 8h into a dark phase, but not high sensitivity before or after this point. The specificity of this period is very strong as at 19 and 21 hs, while there is some breaking of diapause, levels of diapause are notably lower. Using the model described earlier (Figure 1-6) it could be hypothesised that a light sensitive oscillating protein begins accumulating at lights off and reaches a threshold between 19 and 20h while light pulses before and after this time do not have the impact to prevent this threshold being reached.



**Figure 1-11 Calculating the photosensitive phase in *Sarcophaga argyrostoma*. Night occurs at ZT12 hs. A light pulse given at 20h initiates a long day (low diapause) response (reproduced from (Saunders, 1979)**

Experiments performed by Saunders (1990) utilised the Nanda-Hamner protocol in *D. melanogaster* and observed that *Drosophila* diapause is inducible under short photoperiods, but flies show a Nanda-Hamner response as the night length is artificially altered. These

experiments have more recently been replicated in a northern species, *Drosophila montana* which does not exhibit an internal coincidence phenotype, with the levels of diapause raising to near 100% in LD 12:10 and LD 12:12, but with levels consistently lowering as the night length is increased (Kauranen et al., 2013). It is important to note however that in this species, locomotor activity under DD conditions is often arrhythmic and under LL conditions it can be rhythmic (Kauranen et al., 2012) implying that the clock in this species may not be adapted in the same way as in *D. melanogaster* so *D. montana* may utilise the hourglass model for diapause induction, though tests using the Bünsow procedure have not been performed. Application of the Nanda-Hamner protocol has been indicated a positive response from a number of species, including *D. triauraria* (Yamada and Yamamoto, 2011), the coleopteran model *Colaphellus bowringi* (Wang et al., 2004) and, according to Saunders (1987) the hemipteran model *Pyrrhocoris apterus*. The *Pyrrhocoris* model posed an interesting result as its responses to the Nanda-Hamner experiment exhibited a robust rhythmic response to changes in phase, but unlike other models, it did not respond with peaks of diapause in phases of 24h, but in bouts of 16-17h.

As well as the important role that photoperiod plays in diapause induction, in some organisms, other factors can influence the induction of diapause. The daily changes in temperature (thermoperiod) also acts as an indicator of day length as the temperature will fall with the setting sun. *Pyrrhocoris* is one example, with insects exposed to short thermoperiod induces a stronger diapause than a short photoperiod at constant temperature (Kalushkov et al., 2001). The presence of allelochemicals can act as indicators. These chemicals may have numerous origins, depending on the conditions that the organism is trying to avoid. Spider mites for example respond to alarm pheromones

produced by conspecifics as an indicator of predation in the summer (Kroon et al., 2008). In response to this, the mites move away from their feeding sites and enter diapause to survive longer without food. Similarly, *Daphnia* appear to respond to kairomones produced by predatory fish to enter a predator-avoidance diapause (Pijanowska and Stolpe, 1996) as well as alarm pheromones from other *Daphnia* (Ślusarczyk, 1999). In other cases, the quality of food sources to herbivorous insects may also indicate an oncoming winter and food shortage in *Choristoneura rosaceana* which respond to altered chemicals in their food source (Hunter and McNeil, 1997).

### **1.3.3 Circadian control of photoperiodism**

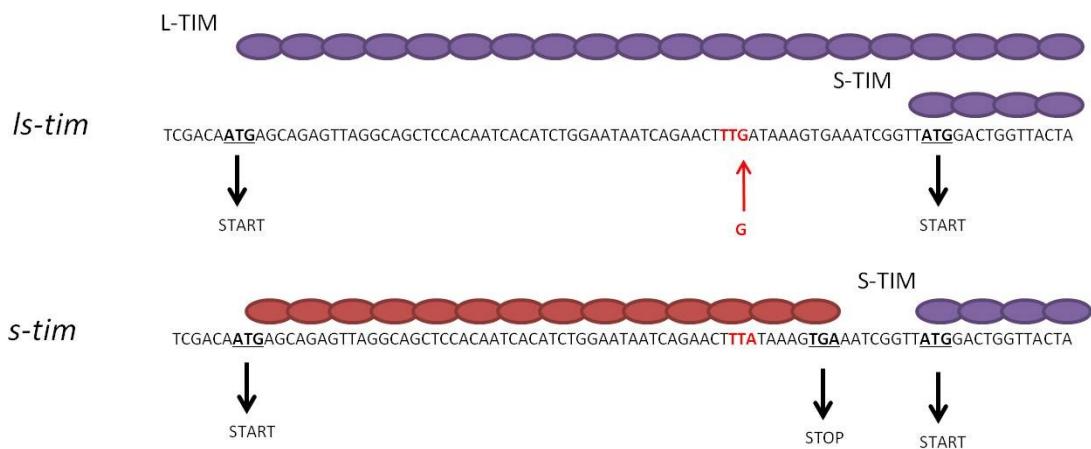
In order to induce photoperiodic diapause, an organism would require a mechanism that measures the photoperiod (or scotoperiod, the night) and translates the information into a signal to enter or exit diapause. It is unclear exactly how this mechanism works, but a number of models and analyses have been tested by which the circadian clock – or another oscillator – operates to signal the diapause response. The models in the previous section point to the role of an oscillator in maintaining these behaviours, with the possible exception of the hourglass model which may operate with the use of a non-circadian photosensitive element. In order to begin to predict which genes could be involved in the measurement, there are criteria that they must meet. A circadian component, which would oscillate and a photosensitive element would be required, or a module that is responsive to a zeitgeber which could be a thermosensitive molecule rather than a photosensitive one. It is tempting to make the assumption that the circadian clock is the driving force behind seasonal responses. The light sensitive TIM and the temperature sensitive PER makes for a well categorised system that influences gene expression in a circadian manner. Occam's

Razor implies that the simplest explanation is the most likely explanation, which is by no means a real scientific principle, but certainly a guide as to where to focus.

An early attempt to implicate the clock in diapause was undertaken by Saunders (1990) who used *per<sup>0</sup>*, *per<sup>S</sup>* and *per<sup>L</sup>* mutants and a *per* double deletion (which removes *per* from the chromosome without lethal effects) to show that a disrupted clock does not prevent photoperiodic diapause. Saunders has since suggested the possibility that his experiments did not take into account the effect of temperature, as *per<sup>S</sup>* and *per<sup>L</sup>* mutants' circadian rhythms move towards 24h as the temperature is lowered by 5°C (Konopka et al., 1989) and it is conceivable that as the temperature falls towards 12°C – as in a diapause experiment – the period effect of the mutants is diminished (Saunders, 2008, Saunders, 2004). Bradshaw (2010) listed this example among a number of other works implying that the circadian clock does not prevent photoperiodic diapauses. However, Bradshaw also points out that there is significant difficulty in proving the involvement of clock genes in circadian rhythms because of the pleiotropic influence of clock genes. This problem renders the testing of hypotheses using blunt ended instruments such as null mutants and RNAi knockdowns prone to the assumption that the influence of the clock on *everything* else is responsible for any results achieved, rather than the direct influence of the clock on diapause.

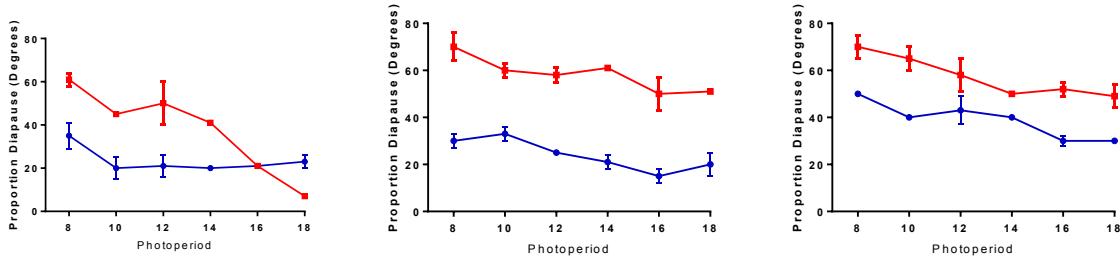
This leaves the researcher looking for sharper tools to study the role of the clock in diapause and few of these appear naturally. The discovery of a single nucleotide polymorphism (SNP) in *tim* under positive selection in Europe was particularly important because it renders flies with fully functioning clocks, but expression of different TIM isoforms, (Figure 1-12) (Tauber et al., 2007). This SNP results in the expression of two TIM isoforms, a long and a short form with the longer form much less readily degradable by light (Sandrelli et al., 2007). What is

important about the discovery of this natural variant however is that the mutation, tested in common backgrounds, is shown to influence diapause. This creates some evidence for a link between circadian and seasonal mechanisms.



**Figure 1-12 Diagrammatic representation of the single Guanine insertion in *timeless*. This SNP results in the novel recruitment of an upstream 23 residue ORF. The insertion eliminates a stop codon early in the transcript. This results in expression of a short TIM and a long TIM protein instead of just a short TIM as in the ancestral *s-tim*.**

Tauber *et al.* (2007) studied three natural populations from Europe, Bitteto, Salice (both Italy) and Houten (Netherlands). From each population, they extracted two separate populations, one carrying *ls-tim* and the other carrying the *s-tim* allele. They performed a series of diapause observations at 13°C with different photoperiods from LD8:16 to LD18:6. In each population (Figure 1-13), the extracted *s-tim* homozygotes exhibited a consistently lower level of diapause than *ls-tim* homozygotes across the experiment.

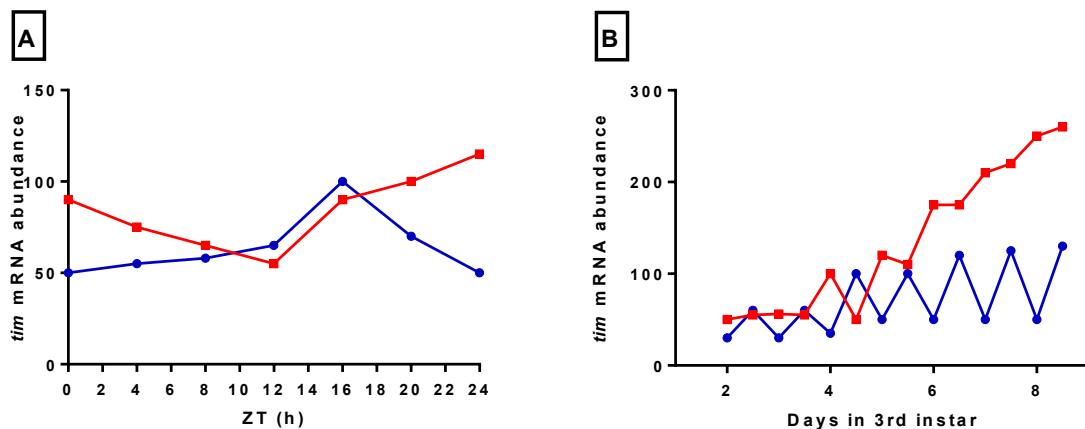


**Figure 1-13 Levels of Diapause in 3 populations from Bitetto (left) Salice (centre) and Houten (right) comparing the *ls-tim* (red) and *s-tim* (blue) females for comparison. The photoperiod is altered to match a scotoperiod to a 24h day. In each population, *ls-tim* flies exhibit a consistently stronger diapause across the experiment (reproduced from Tauber *et al.* 2007)**

The discovery of the effect of the novel and recently derived *tim* allele, *ls-tim*, is important for investigations into diapause in *Drosophila* and studies recently performed to observe clinal selection in Europe for this novel allele indicate that it is spreading throughout Europe from Southern Italy and may have originated as recently as 300 years ago (Zonato, personal communication).

Studies of *tim* and its role in the photoperiodic clock have been undertaken in species other than *D. melanogaster*. One set of studies was performed in another drosophilid, *Chymomyza costata*. A wild caught line from Japan failed to exhibit larval diapause under short day conditions and an autosomal recessive mutation was shown to be responsible (Riihimaa and Kimura, 1989). Further work with this line showed a number of mutations in the *tim* homologue, notably a deletion in the promoter region which deletes the E-box, a crucial element for the expression of *tim* (Stehlík *et al.*, 2008). Stehlík and colleagues also examined the levels of *tim* mRNA cycling in non-mutant flies and indicated that *tim* mRNA cycling is different in long and short day, showing a later peak in long days, 4 days into the fly's third instar (Figure 1-14). Furthermore, the transcription of *tim* in the larvae rises on a

daily basis starting at ~day 5 while short day flies continue to oscillate *tim* levels without the levels rising significantly



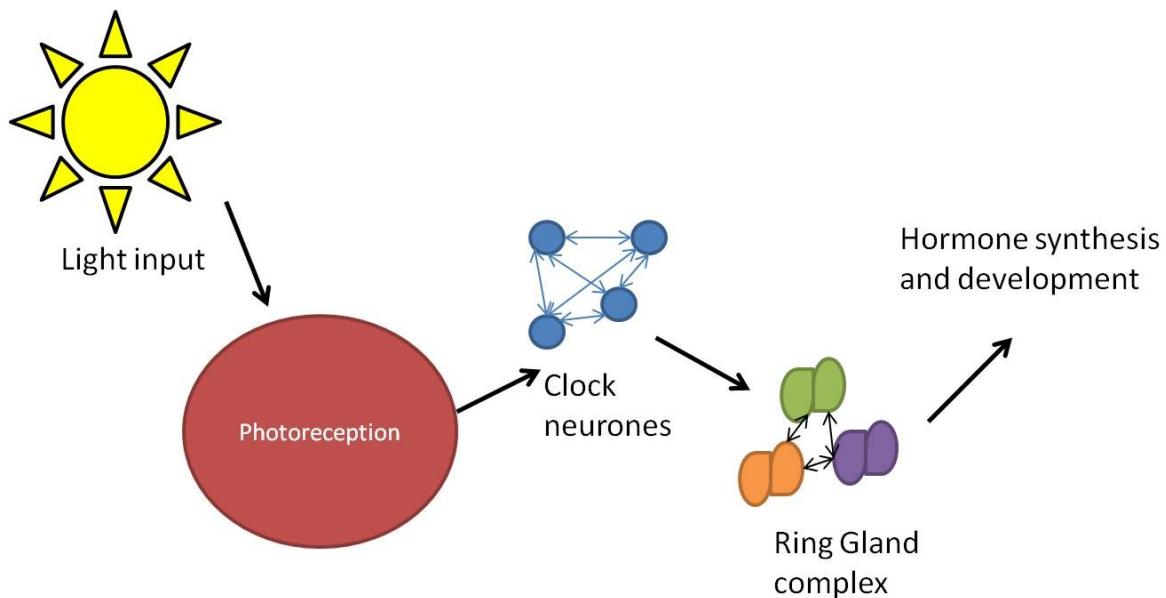
**Figure 1-14** *tim* mRNA cycling in *Chymomyza* comparing long day (red) and short day (blue). Daily cycling of *tim* after 4 days in the 3<sup>rd</sup> instar (A) shows that early onset of night in short days moves the peak of *tim* mRNA levels to 16h, but an oscillation of its transcription is still evident. Flies kept in long day regimes exhibit a later peak ~24h. Over a longer course (B) the long day flies *tim* levels continue to rise where the short day flies repeatedly oscillate transcription (reproduced from (Stehlík et al., 2008))

In spite of Saunders' negative findings with *D. melanogaster*, other models have been used to study the role of *per* in diapause. *Pyrrhocoris apterus* is one such model, which – similar to *Drosophila* – exhibits an adult reproductive diapause when larvae are submitted to short days and the diapause is broken in long days, unless a diapause stimulating low temperature is lowered (Socha, 1993). A wild line of insects was selected under diapause inducing photoperiod to create a wildtype (Wt) line that exhibited a photoperiodic diapause and a 'mutant' line that only entered diapause in response to low temperatures (Socha and Hodková, 1994). Wt bugs exhibit significantly higher levels of *per* and *Cik* mRNA in short days than in long days, but in non diapausing mutants, the levels of mRNA were similar in

both conditions and relatively similar to long day Wt (Syrová et al., 2003), indicating the putative involvement of clock gene expression in maintaining photoperiodic diapause. Subsequent analysis of *per* in these mutants by Doležel and colleagues (2005) indicated no sequence differences to Wt collected from the same locale, but a second mutant line, isolated from Lyon carried three SNPs in the second intron. Ultimately, it was unclear as to whether these *per* SNPs are important for diapause or how and if *per* has a role in photoperiodism (Doležel et al., 2007).

From a neurological viewpoint, and assuming the clock is involved in photoperiodism, it is interesting to understand how the signal of day length might be received into the circadian system, translated by ‘clock cells’ and then how it affects a physiological response. Obviously the clock is able to translate information on light signals using CRY, but the clock must have an influence outside of a handful of cells in the brain. PDF, discussed in section 1.2, is a neurotransmitter output of the clock that allows the clock cells to communicate with each other. Further investigation indicated that the clock neurones may innervate other cells in the brain and use PDF as their signal. Shiga and Numata (2009) investigated this in the blowfly *Protophormia terraenovae*, identifying clock cells by coexpression of PER and PDF proteins. The study indicated that ablation of some of these cells, the small ventral lateral neurones, would lead to a loss in of photoperiodic response in flies. The connection between these neurones and the *pars lateralis*, suggested an enticing putative link between the photoperiodic clock and the neurohormonal control of diapause. Similarly, clock cells have been shown to innervate the ring gland in the *Drosophila* brain which is a set of fused glands including the *corpora allata* (CA) (Siegmund and Korge, 2001). Juvenile Hormone (JH) as well as other hormones secreted from the ring gland are involved in development and

reproduction. This allows for the model outlined in Figure 1-15 where the clock is influenced by the light signal and the response generated in clock cells influences hormone synthesis and release.



**Figure 1-15 Basic model for a diapause pathway using the clock to relay light information.** Light into the system is received by CRY, which influences the circadian clock in clock cells. The clock output influences the synthesis and release of hormones which inhibit or promote development.

### 1.3.4 Hormonal control of diapause

JH is a putative element of the pathway that breaks (or induces) diapause in insects under control of the clock. The investigation into endocrine control of insect diapause has a long history and the insulin signalling pathway as well as the hormones synthesised from other constituents of the ring gland are well studied in their modulation of development. In *Drosophila*, a neurohaemal organ is found in the brain which consists of three fused glands including the CA, the *corpora cardiaca* (CC), the thoracic glands (TG) (Richard et al., 1989). JH was a hormone suggested to be synthesised in the CA of insects by Wigglesworth (1936) who studied the role of the CA in development of *Rhodnius prolixus*. He termed this “inhibitory hormone”, as it actively prevented metamorphosis in early nymphal stages. The

effect of this hormone was elicited by transferring the CA of 3<sup>rd</sup> or 4<sup>th</sup> nymphal stages into a 5<sup>th</sup> stage nymph (which would ordinarily metamorphose into an adult), which resulted in a 6<sup>th</sup> or even 7<sup>th</sup> nymphal stage, a condition termed morphostasis. These experiments were replicated in Cecropia moths where the more active CA of adult moths were transplanted into pupae which then failed to moult or developed into an intermediate between pupae and adult (Williams, 1959). The existence of this hormone was confirmed and the structure identified as methyl *trans,trans,cis-10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate* (JH)(Röller and Dahm, 1970).

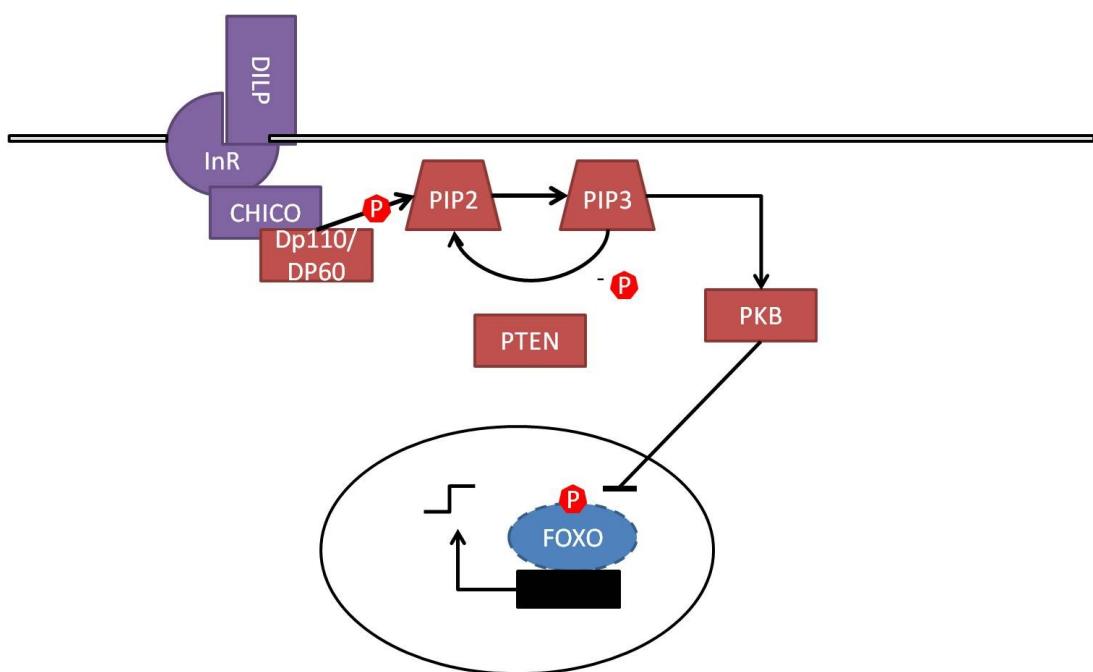
The findings of transplantation experiments indicated a possible role for high levels of JH in maintaining diapause. However, experiments using topically applied synthetic JH on diapausing *Hypera postica*, revealed that it terminated diapause (Bowers and Blickenstaff, 1966). Similar observations were made using topically applied JH in *Riptortus pedestris* (Numata and Hidaka, 1984) and *D. melanogaster* (Saunders et al., 1990) with both studies indicating that in these insects, blocking JH synthesis prevents the ovaries from taking up vitellogenin, resulting in diapause. The evidence began to mount in favour of the idea of adult diapause acting to block JH synthesis in a range of insects including butterflies (Benz, 1972) and mosquitoes (Spielman, 1974). Removal of the CA in larvae of *Ostrinia nubialis* however, resulted in a rapid development from larva to pupa to adult under diapause inducing conditions and application of JH to these insects resulted in pupal diapause (Yin and Chippendale, 1979), also supported in other insects with a pupal diapause (Yagi and Fukaya, 1974). This is an indicator of two different mechanisms inducing two different types of diapause, with pupal diapause being elicited by high JH titres and adult diapause caused by low JH titres.

Such examples indicate the difficulties of comparing different species of insect for diapause as there are inconsistencies in how different insects modulate the condition. As a rule, in larval and pupal diapausing species, diapause causes a block between the brain and the prothoracic gland (PTG) and a block to ecdysteroidogenesis while adult diapauses are associated with the absence of JH (Denlinger, 2002). Diapause in *Drosophila* is largely associated with functional insulin signalling with mutants for insulin and insulin receptor genes displaying a diapause phenotype under normal conditions (Brogiole et al., 2001, Tatar et al., 2001b, Tu et al., 2005). *D. melanogaster* insulin like peptides (DILP) are encoded for by seven different genes, *Dilp1-7* which all show a degree of homology to human insulin (Claeys et al., 2002). These peptides are synthesised in the *pars intercerebralis* (PI) (certainly DILP2, 3 and 5) and the ovaries (DILP5) (Broughton et al., 2005). The PI directly innervates the CA (Siegmund and Korge, 2001) indicating a likely role for insulin in regulating JH synthesis.

Tatar and colleagues (2001b), showed that mutants for the insulin receptor (*InR*) exhibited diapause-like symptoms as adults with no vitellogenesis in the ovaries as well as an extended life span and unusually small size. Mutations in other insulin signalling pathway genes result in similar phenotypes including the receptor substrate CHICO (Clancy et al., 2001, Tu et al., 2005). Overexpression of transcription factor FOXO (blocked by insulin signalling), a situation achieved when insulin signalling is blocked, also acts to increase lifespan, with evidence suggesting that it has a role in oxidative stress protection (Giannakou et al., 2004). Experiments using the *InR* mutants showed that a topical application of JH would result in reduced lifespan (closer to normal) and would allow normal

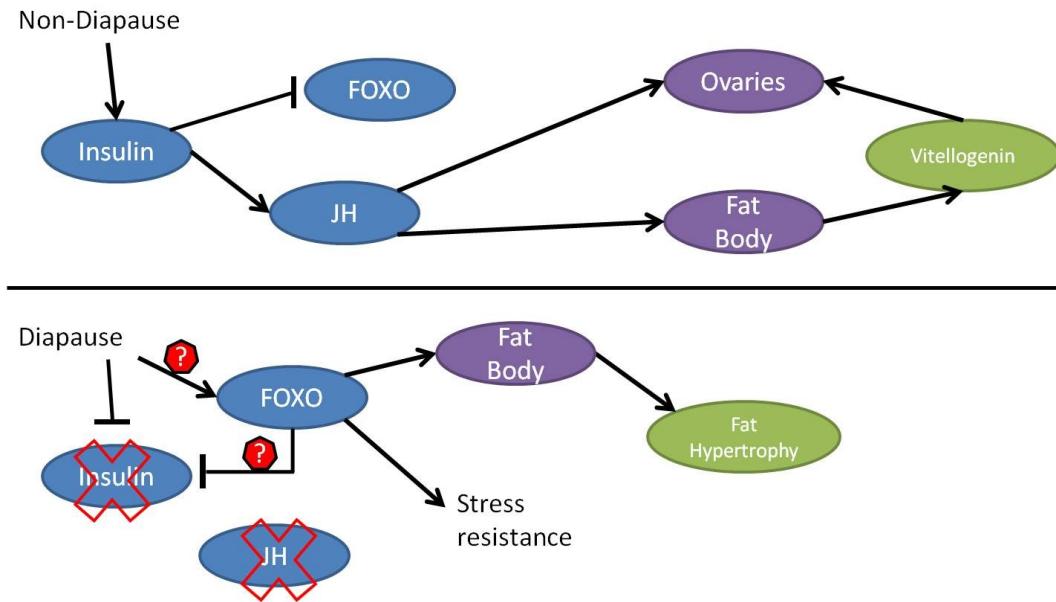
reproductive behaviours (Tatar et al., 2001b). The evidence indicates that insulin signalling may modulate the JH titres in *Drosophila* and therefore be responsible for diapause.

Insulin signalling acts as a cascade (Figure 1-16). DILPs bind the InR which attracts CHICO as a substrate. CHICO works in tandem with DP110 and DP60, which act as catalyst and regulator of phosphatidylinositol-3-kinase (PI3K) which phosphorylates phosphatidylinositol (PIP)2 to create PIP3, a secondary messenger. This reaction is reversed by PTEN which switches off the pathway in the absence of DILPs. Higher levels of PIP3 stimulate activity of kinases, including protein kinase B (PKB) which phosphorylates the forkhead transcription factor FOXO, which moves into the nucleus and activates transcription, influencing growth, development and metabolism (Garofalo, 2002, Giannakou and Partridge, 2007, Williams et al., 2006).



**Figure 1-16 Overview of the insulin signalling pathway in *Drosophila*.** DILPs bind the InR while CHICO acts as a substrate to activate Dp110 and Dp60. Dp110 acts as a catalyst and Dp60 acts as the regulatory subunit for PI3K which phosphorylates PIP2 to form PIP3, a reaction antagonised by PTEN. The secondary messenger PIP3 activates a series of kinases including PDK1 and PKB which phosphorylates FOXO, preventing transcription.

Limited work has been performed studying the role of insulin signalling in *Drosophila* diapause. Williams *et al.* (Williams et al., 2006) used GAL4 overexpression of *Dp110* to see if this had an effect on the propensity to diapause and observed a significant reduction in diapause in flies kept under diapause inducing conditions. In addition to this experiment, they took two wild lines with a high and low diapause level. These lines were crossed to deficiencies for *Dp110* and then Wt *Dp110* was reinserted using a P-element which resulted in the two lines exhibiting similar levels of diapause. This experiment effectively traced the high diapause response phenotype to the *Dp110* locus. Subsequent to this, the *InR* and *Chico* components of the insulin signalling pathway were studied by Paaby and colleagues (2010). They discovered a number of SNPs in CHICO, but failed to find any relationship to diapause. In *InR* however, they discovered a polymorphic region which was characterised by six indel polymorphisms. Of the alleles observed, two were of particular interest, 248 and 254 (named based on the length of the PCR fragment). 248 was more prevalent in more northern areas of North America while 254 was more prominent in the south. The cold tolerance of 248 was enhanced, implying these polymorphisms help flies adapt to the relevant environmental conditions. The experiment was replicated in Australia with a corresponding outcome, i.e. increased prevalence of 254 in the northern warmer climate and 248 in the southern cool regions. Relatively speaking, little evidence has been produced for a role for FOXO in *Drosophila* diapause. A study using RNAi knockdown in the mosquito *Culex pipiens* however has been performed, indicating that knockdown of FOXO results in shorter lifespan and reduced fat storage, key to diapause, indicating a strong role for FOXO as a pro-diapause signal.

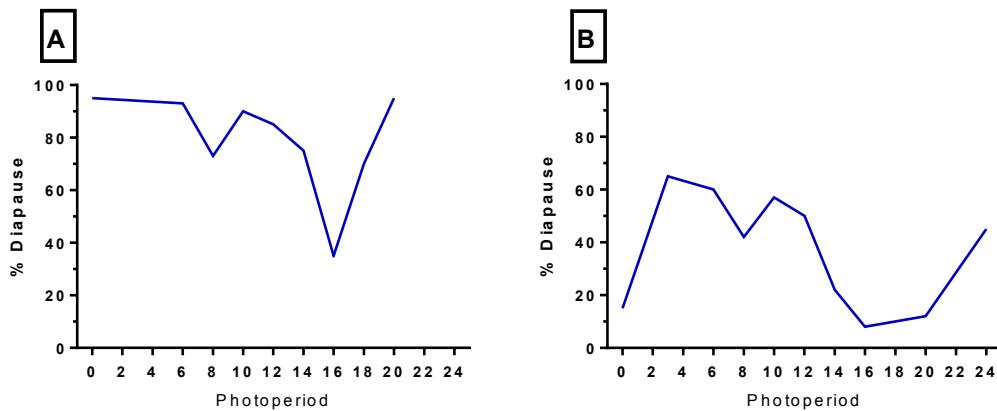


**Figure 1-17 Insulin and JH signalling role in diapause.** In non-diapause conditions, insulin is synthesised and JH acts on the fat body to synthesise vitellogenin and the ovaries to enable them to take up vitellogenin. FOXO synthesis is inhibited through insulin signalling. In diapause conditions, insulin synthesis is blocked and FOXO is upregulated, stimulating the fat body to store fat stress resistance and longevity are both increased.

### 1.3.5 Diapause in *Drosophila*

While *D. melanogaster* originated in sub-Saharan Africa, they have successfully colonised Europe within the last 10-15,000 years (Lachaise et al., 1988) as well as conquering the Americas and Australasia within the last few hundred years with human migration from Europe and Africa (Keller, 2007). This emigration has brought *Drosophila* into contact with more variable temperate environments. As mentioned earlier, Saunders and colleagues (1989) produced the earliest evidence for diapause in *D. melanogaster* and observed that under short photoperiods, between 2-12h per day, flies exhibited intermediate levels of diapause, averaging 40-60%. Notably, diapause levels were drastically reduced under DD conditions, however under LL conditions, over 40% of flies exhibited diapause (Figure 1-18B), further indicating the importance of light and photoreception. Lowering the temperature to 10°C however, removed the effect of photoperiod and resulted in a

stronger, more robust diapause (Figure 1-18A). A photoperiod of 16h still induced lower relative levels, but DD conditions did not. Saunders *et al.* also observed a spontaneous exit from diapause after 6-7 weeks in their experiments.



**Figure 1-18 Photoperiod response curves for *Drosophila* diapause.** At 10°C (A) high levels are observed at all photoperiods except LD 16:8. At 12°C (B) photoperiod is more important with 14-22h photoperiods resulting in low diapause (Reproduced from Saunders *et al.* 1989)

The recent *Drosophila* exodus to cooler clines may explain why the diapause phenotype in *Drosophila* is relatively weak, relying on low temperature for its induction (Emerson *et al.*, 2009b, Saunders *et al.*, 1989, Tatar *et al.*, 2001a) and there is indeed a question as to the relevance of *Drosophila* diapause model in photoperiodism.

My studies will aim to investigate the role of clock genes in *D. melanogaster* diapause, focusing on the novel *ls-tim* mutant and studying the effects of *per* splicing. As there are indications that the *D. melanogaster* diapause is weak, experiments have been designed to see if the length of the diapause can be extended further using more natural conditions. Furthermore, this project will look to better understand the diapause in *Drosophila*, investigating the ancestral nature of the diapause before flies migrated into Europe and the similarities between diapause in *D. melanogaster* and other species in the *D. melanogaster*

subgroup. The initial (and ultimately unsuccessful) part of this project however will aim to follow up on a study performed by Ed Green in this laboratory which identified a number of putative novel clock genes.

## **2 General Materials and Methods**

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### **2.1 Fly husbandry**

Maize food was prepared using the Manchester Recipe (7litres water, 555g maize, 350g brewer's yeast, 89.5g Agar, 555g glucose, 94.5ml 20%nipagen and 21ml propionic acid). As standard, flies to be used for experiments were raised in vials (Size 25mmØ x 95mm) at 25°C in LD 12:12. Stocks were kept at 18°C with a 12h photoperiod. To expand large populations of flies rapidly, pots were used (Size 60mmØ x 130mm).

### **2.2 Fly locomotor activity**

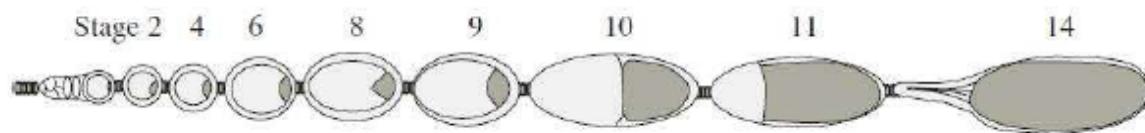
Locomotor activity was measured using the Trikinetics *Drosophila* Activity Monitoring Systems (DAM) (Trikinetics inc., MA, USA) following the protocol described by Rosato and Kyriacou (2006). This method requires flies to be kept in glass tubes with food at one end and placed into a monitor. This monitor produces an infra red beam and a computer records every incident where the beam is crossed. Monitors were kept in incubators at 25°C and LD cycles of 12:12 for two full days to entrain and then DD to measure the period for the subsequent seven days. Data was analysed using the Befly! Excel macro, developed by Dr Ed Green from our laboratory, to generate individual profiles for each fly's activity and produce a mean period or identify flies that are arrhythmic (Allebrandt et al., 2013).

### **2.3 Diapause**

On the day of the beginning of the experiment, fly pots were emptied first thing in the morning. Within 2h, collections of recently emerged flies were placed into food vials and placed immediately into an incubator under diapause inducing temperature and photoperiod conditions. Several such collections were performed over the day. Each tube

contained between 25 and 40 flies (male and female). The tubes were immediately placed into the incubator 35cm from the light source.

Diapause was scored using a method outlined by Schmidt (2008) using the developmental stage definitions described by King (1970). The flies were dissected in phosphate buffered saline solution (PBS (NaCl, KCl, Na<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>HPO<sub>4</sub>)) and their ovaries scored as *diapause* or *non diapause*. Figure 2-1 shows the stages of ovariole development and as a rule, ovaries where yolk protein had begun to be visibly deposited (stage 8 or later) were recorded as *non-diapause* and where there was no sign of this (stage 7 or earlier) the record was *diapause*. Non-diapause was defined by the presence of an individual developing ovariole with yolk present rather than the whole ovary and each tube generated a percentage population score for diapause to give an individual replicate. Dissections were all performed on the same Leica S6E microscope with total magnification of ~17.5x to ensure consistency. Where it was unclear if there were yolk deposits, magnification was increased to inspect more closely.



**Figure 2-1 Development of ovarioles in *Drosophila* in 14 discrete stages with the developing oocyte in grey. Stages lower than 8 are considered previtellogenic and scored as “diapause” while 8 and up were scored as “non-diapause” (Ogienko et al., 2007)**

## **2.4 Commonly Used Molecular Techniques**

### **2.4.1 DNA and RNA Extraction**

#### **2.4.1.1 *DNA extraction***

To extract DNA, unless stated otherwise, a “fly squash” method described by Gloor *et al.* (1993) was used. This method involves pulverising a whole fly in 50 $\mu$ L of buffer – containing Squishing Buffer (SB)(10mM Tris-Cl, 1mM EDTA and 25mM NaCl at pH 8.2) and proteinase K (diluted into SB at 200 $\mu$ g mL<sup>-1</sup>). The fly is then incubated at 37°C for 25 minutes and then the proteinase K is inactivated with a 95°C temperature step for 3 minutes.

#### **2.4.1.2 *Extracting DNA for non-lethal genotyping***

In other experiments, non-lethal genotyping is necessary and to perform this, a method described by Carvalho (2009) was utilised. In this method, wings were removed from an individual fly and placed into the bottom of a PCR tube. 10 $\mu$ l of proteinase K (diluted to 400 $\mu$ g mL<sup>-1</sup> in SB) was pipetted over the wings and to ensure they were submerged, they were spun down for one minute in a centrifuge and – where necessary – pushed down using a pipette tip. This was then incubated for 1h at 37°C and proteinase K was inactivated at 95°C for 2 minutes.

#### **2.4.1.3 *RNA Extraction***

Because RNA typically exists in low quantities in an individual fly, and its extraction is prone to loss and degradation, one fly will rarely yield sufficient RNA for an experiment. Typically, at least 8 bodies of flies are required for reasonable amount of RNA. For experiments where heads were required, at least 20 heads are removed by freezing flies on dry ice or liquid nitrogen and vortexing them before sieving the heads out separately from the body, wings

and legs. RNA extraction was performed using the Norgen total RNA Purification Kit (Norgen Biotek Corp.). Once the RNA is extracted, some of it was treated with DNaseI (Invitrogen) to further purify the RNA. For many applications, cDNA must be synthesised and in these cases, the Invitrogen Superscript protocol was used. This utilises SuperscriptIII reverse transcriptase, RNaseOUT ribonuclease inhibitor and Oligo(dt)<sub>20</sub> primer (Invitrogen). Once the cDNA is made, it can be treated exactly as normal DNA within a sample

## **2.4.2 Polymerase Chain Reaction**

KapaTaq (Kapa Biosystems) was used as 1 unit per 20 $\mu$ l reaction (0.2 $\mu$ l), paired with a 10x buffer which was added as 2 $\mu$ l per 20 $\mu$ l reaction. Primers (Sigma-Aldrich) are designed to bind 3' and 5' to the specific target site and these are added at 10nM per 20 $\mu$ l reaction. In situations where additional primers are used in order to multiplex the PCR and amplify two fragments, all extra primers are added at 10nM at the expense of water. To act as a substrate Deoxyribonucleotide Triphosphate (dNTP) is added (Kapa Biosystems). This is a mixture containing dATP, dTTP, dCTP and dGTP and 4nM of the mixture (1nM of each dNTP) is added per 20 $\mu$ l reaction. DNA is also added to the reaction and this is largely dependent on the concentration of the DNA. Typically, 2 $\mu$ l of DNA is used, unless it is highly concentrated and could lead to over amplification. To make the total volume to 20 $\mu$ l, purified water is added. In cases where RNA amplification is the goal, RNA is extracted and converted to cDNA as described in 2.4.1.3, and the cDNA is treated in the same way as genomic DNA. This is referred to as reverse-transcriptase PCR (RT-PCR).

For the reaction, there are several steps. A heated lid was utilised at 110°C to prevent any components from condensing at the top of the tube. An initial 95°C denaturation step was performed for 2 minutes in the first step of the reaction. The next step involves 35 cycles of

3 steps, a 1 minute denaturation at 95°C, 30 seconds of annealing at a temperature 5°C below the optimal melting temperature ( $T_m$ ) and an extension at 72°C for 1 minute per kilobase (kb) of DNA product. A final extension takes place at the end of the cycles for 6 minutes.

DNA can then be separated by agarose gel electrophoresis. This typically involves heating agarose in TBE buffer (89mM Tris, 89mM boric acid, 2mM EDTA – pH 8.0) and adding ethidium bromide which binds the DNA and glows under UV light to visualise amplified DNA. Gels used throughout are typically 1.2% (1.2g per 100ml TBE) and ethidium bromide was added at 7 $\mu$ l per 100ml. Using a DNA ladder can help to confirm the length of a fragment, but can also be used as a marker for quantification by RT-PCR

### **2.4.3 *ls-tim/s-tim* Genotyping**

The *ls-tim/s-tim* allele is frequently with diapause levels (Tauber et al., 2007). To genotype this polymorphism the method was used that is described by Tauber *et al.* (2007) and takes advantage of a single nucleotide insertion in the 5' region of the *tim* gene. This allows two sequence specific primers ('GA' for *ls-tim* and 'AT' for *s-tim*) to be used in separate PCRs. A reverse primer that was common to both sequences ('tim3') was used and multiplexed with 2 control primers ('C3' and 'C5') to ensure that *tim* is being amplified in the PCR.

GA primer *TGGAATAATCAGAACTTTGA*,

AT primer *TGGAATAATCAGAACTTTAT*,

tim3 primer *AGATTCCACAAGATCGTGTT*,

C5 primer *CATTCATTCCAAGCAGTATC*

C3 primer *TATTCATGAACTTGTGAATC*.

Two mastermixes were prepared as below, one mastermix for the AT primer and another for the GA primer. A PCR reaction was performed as per the description in section 2.4.2 but half volumes to a total volume of 10 $\mu$ l per reaction. The thermocycler programme described in 2.4.2 was used with an annealing temperature of 55°C.

Samples were run out on a 1.2% Agarose gel with ethidium bromide (140 $\mu$ L per litre of gel). Samples were run on the gel with the  $\Phi$ X HaeIII ladder (New England Biolabs) for comparison. A band ~693 bases in length indicates a positive for *GA* or *AT*, depending on the mastermix used for that sample. A band of this length in both samples indicates that the fly is heterozygous. A second band ~487 bases indicates a positive for the control. Each fly must have a positive for the control to be sure of confirming the genotype.

#### **2.4.4 Sequencing**

This was outsourced but the samples were prepared here. For most single sequence reads, level 3 sequencing was performed using the PNACL facility at Leicester University was used. This required a reaction to be performed before sending the sample. The quantity of DNA required for the reaction is between 50 and 100ng. The concentration is assessed by nanodrop prior to performing the level 3 reaction. Big Dye v3.1 sequencing dye (Invitrogen) was added at 1 $\mu$ l per 20 $\mu$ l reaction, along with buffer at 3.5 $\mu$ l. 15nM of a primer that binds in the sequence is added and between 50 and 100ng of DNA, with water to make up the rest of the reaction. The reaction was run on a thermocycler for 1 minute at 96°, followed by 25 cycles of 30seconds at 95°C, 5 seconds at 50°C and 60°C for 4 minutes. Samples were then cleaned using Performa DTR gel filtration cartridges (EdgeBio) and sequenced by PNACL.

# 3 Diallel cross screening for candidate clock genes

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## 3.1 Introduction

In this laboratory, Dr Ed Green performed a circadian screen of genes using RNAi, whose microarray profiles from a half diallel cross had suggested that they may correlate with subtle changes in rhythmic behaviour (Green, 2010). The diallel cross is a method of discovering the underlying polygenic architecture of a phenotype (Hayman, 1954). In these experiments, Green took flies carrying subtle phenotypic mutations (each described in Table 3-1) and backcrossed each into a *Canton-S* background for six or more generations. Each of these lines was then crossed to each other to form transheterozygotes, i.e. *Adf/Csp*, *Adf/Mth*, *Csp/Mth* etcetera. These transheterozygote flies were then assayed for various behavioural differences, including circadian locomotor activity to find which mutants were extremely deficient in their behaviours or fell at an extreme end of a behavioural profile, for example, those that have long and short rhythms. This can indicate which of the mutants express certain abnormal behaviours and see if this is rescued by other mutants. Revisiting the circadian example, would a short period mutant and a long period mutant produce a normal period transheterozygote and would two long period mutants result in an extra long period? Microarrays performed on the transheterozygotes to see which genes are up or down regulated offered a correlational approach to identifying putative genes that could be contributing to these behavioural differences. A similar study was performed to uncover the genetic basis of aggression in *Drosophila*, identifying a number of genes which pleiotropically act to influence aggressive behaviour (Zwarts et al., 2011).

**Table 3-1 A list of gene mutants used in the diallel cross experiments (reproduced from Green 2010)**

Allele	Gene Name	Gene Function
<i>Adf<sup>mal</sup></i>	<i>Adh transcription factor 1 (nalyot)</i>	Transcription factor; enhances <i>Adh</i> transcription. Mutants have defects in learning, memory and larval locomotor activity.
<i>Csp<sup>P1</sup></i>	<i>Cysteine string protein</i>	Exocytosis; stabilizing and controlling release of neurotransmitters at neuromuscular junction. Mutants have defective courtship behaviours.
<i>mth<sup>1</sup></i>	<i>methuselah</i>	G-protein coupled receptor activity; plays role in longevity through response to environmental stresses; mutants have neurophysiology defects.
<i>trf<sup>d</sup></i>	<i>TBP-related factor</i>	Transcription regulation. Mutant males sterile and show behavioural defects and hyperactivity.
<i>Itp-r83d<sup>i5B4</sup></i>	<i>Inositol tri-P receptor</i>	Signal transduction and ion transport; mutants have neuroanatomy and flight behaviour defects.
<i>Pen<sup>k14401a</sup></i>	<i>Pendulin</i>	Protein transporter; mutants may be sterile and show defects in geotaxis behaviours.
<i>nmo<sup>P1</sup></i>	<i>Nemo</i>	Serine/threonine kinase. Plays role many biological processes including eye development, geotaxis, wing development and regulation on WNT signalling.
<i>cnk<sup>k16314</sup></i>	<i>connector enhancer of ksr</i>	Protein binding; RAS protein signal transduction. Involved in eye and wing development

Numerous genes were found to have putative circadian roles, but only a number were selected for further analysis including neurotransmitters and kinases (Ed Green, personal communication). Previously, Dr Green has tested knockdown of these genes using the UAS-GAL4 system with RNAi, specifically focusing on knocking down the genes in clock cells using *timGAL4* or in all neuronal cells using *elavGAL4*. Some of the knock-down flies revealed statistically significant changes in circadian phenotypes, but the biological significance of these results was unclear. By using *dicer2* in the *timGAL4* line, it may be possible to improve

the strength of the knockdown and, if the genes are involved in the clock – validate the early results of this screen.

The primary advantage of using RNAi over other methods such as mutagenesis is that using the GAL4 system, the genes can be knocked down in specific cells and tissues (Dietzl et al., 2007). For example, researchers interested in the effect of a gene on the clock can use *timGAL4* which will express RNAi only in clock cells. This is important because genes have pleiotropic functions and may be expressed in numerous tissues and so performing a knockdown in all tissues could drastically affect its function and result in changes in behaviour not associated with the clock, but also dysmorphia or even lethality.

Of the genes discovered by Dr Green's screening method and subsequent analysis, those listed in Table 3-2 were significant or near significant in their period changes and were followed up in my study.

**Table 3-2 Table showing a list of results achieved by Dr. Green using RNAi knockdown in locomotor activity experiments and the period achieved using *timGAL4* or *elavGAL4* drivers**

Gene	Period with <i>timgal4</i> (hs)	Period (h) using <i>elavGAL4</i>	Synonyms (Flybase)	Reported Function
<b>CG17818</b>	24.1	-	<i>rdgBβ – retinal degeneration gene β</i>	Phospholipase C-dependent phototransduction (Fullwood et al., 1999)
<b>CG7156</b>	24.2	23.6	Homologue of RPS6KC1	Mediates initiation of protein synthesis (Zhang et al., 1999)
<b>CG11440</b>	24.5	23.3	<i>laza/lazaro</i>	Phototransduction – regulates <i>rdgBβ</i> (Kwon and Montell, 2006)
<b>CG9784</b>	24	23.9	<i>Inositol polyphosphate 1-phosphatase - IPP</i>	Phosphatase – may have a role in synaptic transmission (Majerus et al., 1999)
<b>CG4656</b>	23.3	23.8	<i>Rassf</i>	Tumour suppression (Polesello et al., 2006)
<b>CG8548</b>	23.9	24	<i>Karyopherin α1/Importin α1</i>	Nuclear transport/meiosis (Chook and Blobel, 2001)
<b>CG8426</b>	27.3	-	<i>Lethal(2) NC136/NOT3</i>	Heart function regulator Transcription
<b>CG1171 (GD)</b>	25.1	-	<i>Akh – Adipokinetic hormone</i>	Lipid mobilisation (Stone et al., 1976)
<b>CG8772</b>	Untested		<i>nemy – no-extended memory</i>	Learning and memory (Iliadi et al., 2008)

Of these genes one result stands out in particular. The result achieved with *CG1171* (also known as *Adipokinetic hormone (Akh)*) contradicts the literature. It is an antagonistic hormone to the insulin signalling pathway (Lindemans et al., 2009, Lorenz, 2003, Staubli et al., 2002) and experiments have shown that in crickets it is expressed in a rhythmic manner (Subrata et al., 1993), but other experiments have shown knockdown does not disrupt circadian rhythms in *Drosophila* (Lee and Park, 2004). Lee and Park used *AkhGAL4* lines to drive *rpr* to ablate all cells expressing *Akh* and traced its expression to the CC, showing no

evidence for its expression in clock cells. The results for locomotor activity in their experiments indicated that there was no influence over the circadian rhythms.

## 3.2 Aims

This study aims to continue the work of Dr Green and test the RNAi lines that he had examined using a UAS-*Dicer2* driver to establish if the original effects of knockdowns can be enhanced. In addition to this, the study will focus on the *Akh* knockdown that gave a significant phenotype with *Dicer2* enhancement in spite of contradictory evidence from Lee and Park's study (Lee and Park, 2004). This will be backed up with the use of a second RNAi line and the use of an *AkhGAL4* line used in Lee and Park's study to drive *reaper* and *head involution deficiency* to ablate *Akh* expressing cells and the use of additional RNAi and overexpressing lines as well as RNAi for the *Akh* receptor.

## 3.3 Materials and Methods

### 3.3.1 Using *timGAL4>Dicer2* to enhance RNAi knockdown

Crosses were performed to drive the expression of the relevant RNAi constructs. Males of the RNAi lines were crossed to female GAL4 flies. The RNAi flies are listed in Table 3-2, showing the period that Dr Green had obtained using *timGAL4* drivers. This experiment will utilise a *timGAL4* driver with *Dicer2* recombined onto the second chromosome (Provided by Ed Green). The GAL4 driver is balanced over *Curly of Oster* (*CyO* a homozygous lethal balancer that gives curly wings) on the second chromosome. After the cross, all straight winged offspring will carry the driver and RNAi. In addition to the lines already tested by Green, a second line of *Akh* RNAi will be used from the VDRC using a  $\phi$ c31 construct which utilises a bacteriophage DNA integrase to integrate information into the genome (Allen and

Weeks, 2006, Venken et al., 2006). This line shall be referred to as the *Akh* KK line and the original *Akh* line will be referred to as the GD line (VDRC terminology). All RNAi lines used here were acquired from the VDRC.

### **3.3.2 Akh Expressing Cell Ablation**

An *AkhGAL4* line was acquired from Bloomington, originally used in Lee and Park's experiment with cell ablation (Lee and Park, 2004). Two were available from Bloomington, one carried the GAL4 p-element on the second chromosome balanced with *CyO* and the second line has an insert on the third which was the line used for the majority of the work. An additional line prepared by Lee and Park with the *AkhGAL4* insert on the X chromosome was not available from Bloomington. The acquired GAL4 lines were crossed with *UAS-rpr;UAS-hid* to ensure that cells expressing *Akh* were ablated. The resulting female offspring were run in a locomotor activity experiment.

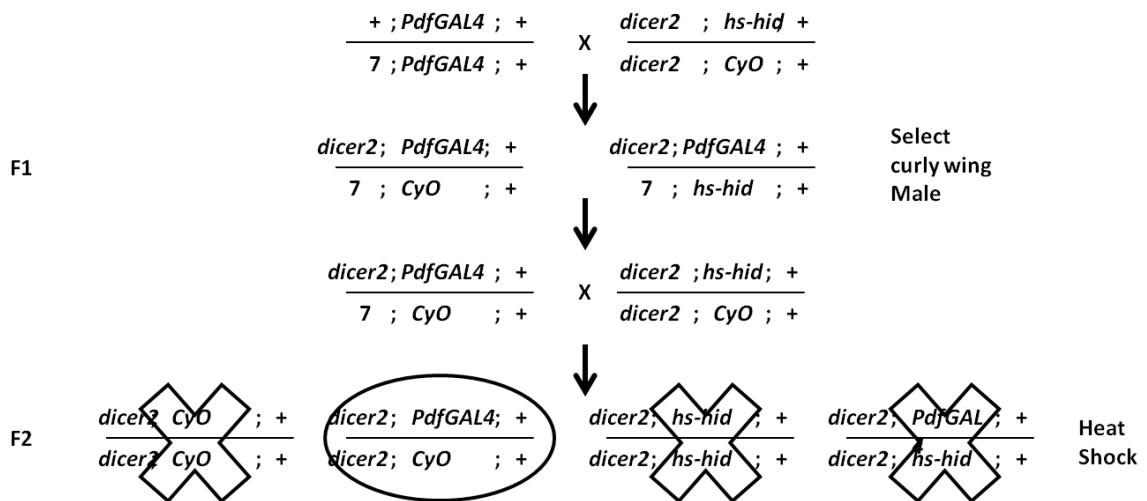
In order to confirm that the knockdown was successful, RT-PCR was performed. *AkhGAL4* flies were crossed with *UAS-rpr;UAS-hid* flies. Females from the cross, the GAL4 and *rpr/hid* line were collected at two days post emergence at ZT6. 8 whole bodies were used for the RNA extraction. A 10 $\mu$ l PCR reaction was performed and primers for *Akh* were used *AAGAATCCTTGCTAGTGCTGTG* (*Akh\_f*) and *ATCTCGAGCAGCATTTCGTT* (*Akh\_r*). A multiplex reaction was performed in order to use an internal control to ensure that there is RNA in the samples. *rp49* is a common control for RT-PCR and primers have previously been used in publications so it is possible to use those previously designed (Matsuno et al., 2009) *GACAATCTCCTTGCCTTCT* (*rp49\_f*) and *CACCGGATTCAAGAAGTTCC* (*rp49\_r*). An annealing temperature of 58°C was used for the PCR and noRT negative controls were used as well as a water negative control.

### **3.3.3 Bloomington *Akh* stocks**

In addition to the experiments using the VDRC stocks and using *rpr-hid* stocks to ablate *Akh* expressing cells, an additional RNAi stock for *Akh* was acquired from Bloomington as well as a UAS overexpression stock. The RNAi stock comes from the Harvard Transgenic RNAi Project (TRIP) stocks. Because it had been shown in Lee and Park's (2004) experiments that *Akh* is only expressed in the CC and not the clock neurones targeted by *timGAL4* the decision was made to use the *AkhGAL4* driver to perform these knockdown and overexpression experiments as it should be more effective.

### **3.3.4 Creating *PdfGAL4>dicer2* stocks**

*PdfGAL4* flies were provided by João Gesto (Leicester) as a potential alternative to *timGAL4* and this line was used to produce a *PdfGAL4>dicer2* line for further comparison. A line from VDRC (60013) carrying *dicer2* on the X chromosome can be used to create a GAL4 lines that express GAL4 and DICER2. The line has *dicer2* on the X chromosome and a heat shock inducible *hid* (*hs-hid*) on the second balanced over *CyO*, i.e. *dicer; hshid/CyO;+*. By using successive heat shock (37°C) and selection, resulting flies would carry *PdfGAL4* and *dicer2* as in Figure 3-1



**Figure 3-1 Cross for producing *PdGAL4>dicer2* lines.** *PdGAL4* males were crossed with *dicer2* females. From the F1 generation, curly winged males are selected and crossed back to the *dicer* line. At F2, homozygous *CyO* is not viable and heat shock will kill any flies carrying *hs-hid* leaving only *PdGAL4* balanced over *CyO*. Successive generations may be selected to eliminate *CyO*

## 3.4 Results

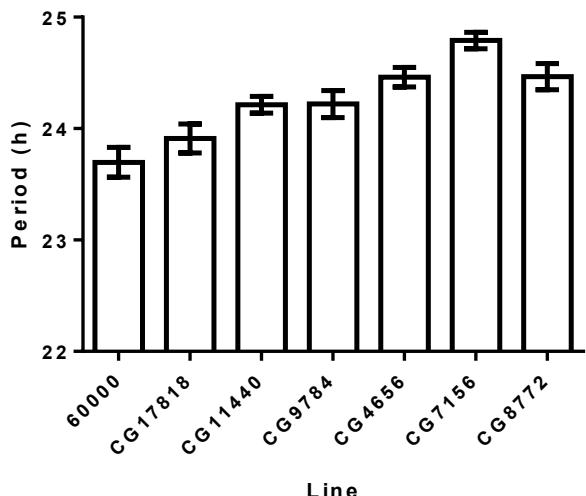
### 3.4.1 Using *timGAL4>dicer2* drivers to enhance knockdown of candidate genes

The downregulated flies were compared to a control which consisted of a line that forms the background for the GD RNAi lines (60000) that had been crossed to the driver. Results are compared in Table 3-3 and Students t-test provided a P value (Table 3-3)

**Table 3-3 – Results for *timGAL4/dicer2* knockdown of putative clock genes. Period length, the number of flies used in analysis and the P value of a Students T-test compared to the control results.**

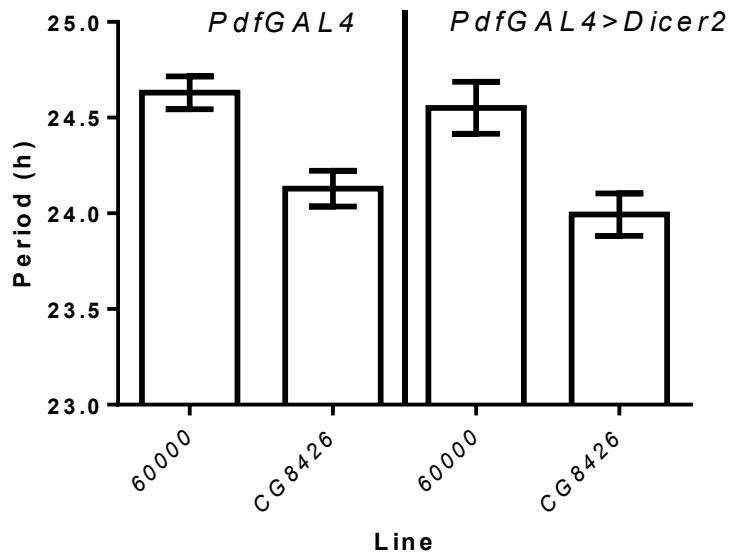
Genotype	Period (h)	Green result (h)	n	P = control	%
<b>Arrhythmic</b>					
<b>CG17818</b>	23.9 ±0.13	24.1	31	0.26	12.9
<b>CG7156</b>	24.79 ±0.07	24.2	32	<0.00001	21.9
<b>CG11440</b>	24.21 ±0.08	24.5	32	<b>0.0088</b>	9.4
<b>CG9784</b>	24.22 ±0.12	24	24	<b>0.0062</b>	12.5
<b>CG4656</b>	24.46 ±0.09	23.3	32	<b>0.00001</b>	21.9
<b>CG8548</b>	30.68 ±0.39	23.9	30	<0.00001	93.3
<b>CG8426</b>	<b>Knockdown Lethal</b>				
<b>60000</b>	23.69 ±0.13		22	-	4.5

The results are largely significant, with the exception of *CG17818*. The margin of the period extension however is small in all of these lines with non exceeding 25h. Comparison to the results achieved by Green using *timGAL4* alone reveal a larger difference between the control and the knockdown as *dicer2* should enhance the efficiency of the knockdown and therefore, the effect on period. In the case of the lines described above, Green showed a period shortening effect relative to his controls in all but *CG11440*. However, all of the results achieved using the *dicer2* driver show a period lengthening effect relative to my control. Figure 3-2 shows a comparison of the periods achieved in these experiments compared to a control of *60000*.



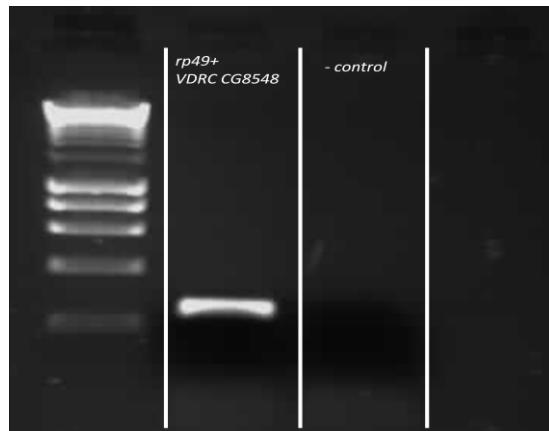
**Figure 3-2 Period achieved after RNAi knockdown. *CG8548* is excluded because of its very long period and *CG8426* was lethal and it was not possible to test it. All genes tested, excluding *CG17818* exhibit a statistically significant, but marginally longer period after knockdown. Mean  $\pm$  SEM**

*CG8426* could not be tested because knockdown with *timGAL4>dicer2* resulted in no viable offspring. This experiment was repeated using a *PdfGAL4* and a *PdfGAL4>dicer2* line. *PdfGAL4>dicer2* was then used to perform further experiments (Figure 3-3). One-way ANOVA was performed to compare the lines with Bonferroni multiple comparisons. ANOVA indicated that there was significant variation in the experiment ( $F=8.06$ ,  $P<0.0001$ ) and Bonferroni confirmed that the *PdfGAL4* knockdown was lower than its control ( $P<0.001$ ) and the *PdfGAL4>dicer2* knockdown was lower than its own control ( $P<0.001$ ). There was no difference between the controls or the two knockdowns ( $P>0.05$ )



**Figure 3-3 Results of using *PdfGAL4* and *PdfGAL4>dicer2* to drive expression of *CG8426* RNAi. In both circumstances, the expression of RNAi shortens the period, but *dicer2* does not enhance the effect. Mean  $\pm$ SEM**

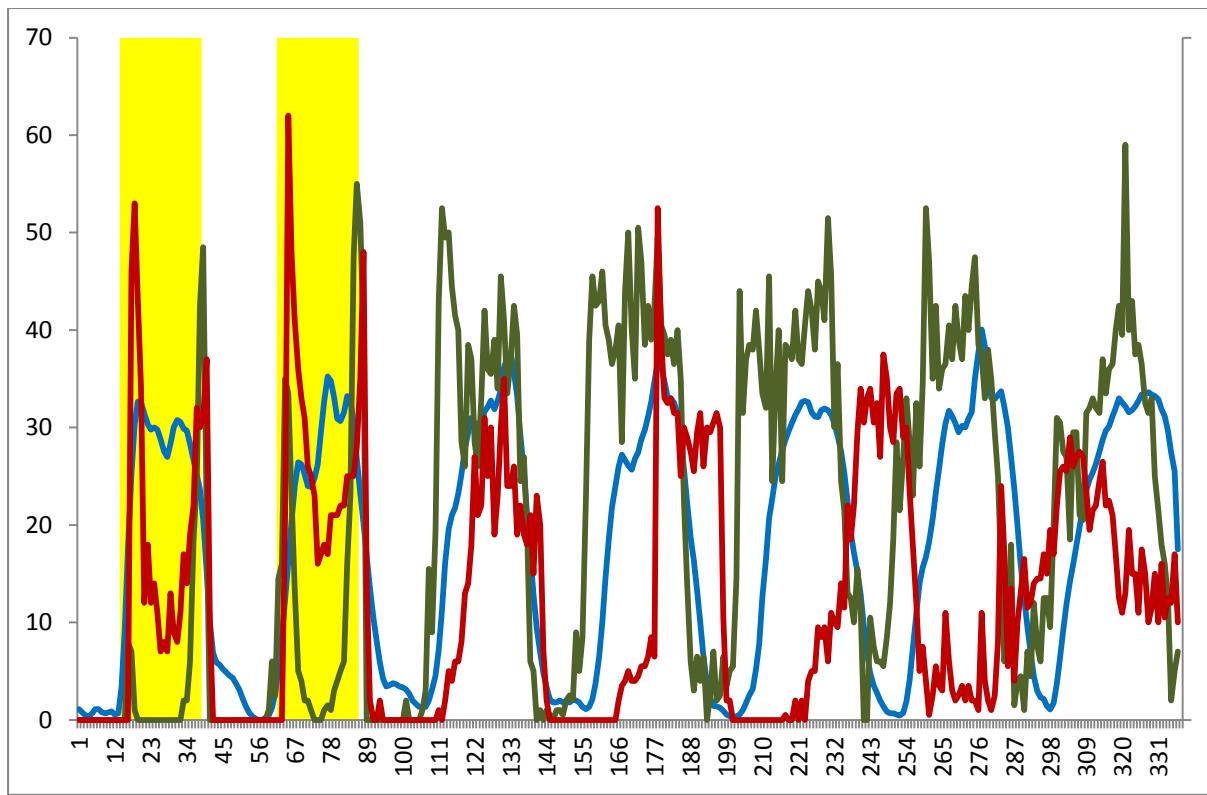
*CG8548* gave a very long period ~30.7h, but more interesting was the level of arrhythmicity with only two flies displaying a circadian rhythm and the other 28 being arrhythmic. A PCR was performed on the *CG8548* RNAi line to confirm that the flies carried the RNAi transgene. The PCR utilises a primer that targets the *CG8548* transgene in the p-element (ATTCGACGGCGAACAA) and a primer to target *hsp70* (GCAACTACTGAAATCTGCCAAG), another sequence found in the p-element as well as a pair of primers to amplify *rp49* control sequence (*q\_rp49\_f* GACAATCTCCTTGCCTTCT and *q\_rp49\_r* CACCGGATTCAAGAAGTTCC) to act as a control. This would amplify that region and confirm that a p-element carrying *CG8548* is present in the flies. The result indicated that no *CG8548* RNAi construct was present in the line (Figure 3-4). The strong period lengthening result of this line is therefore enigmatic



**Figure 3-4 PCR to confirm presence of CG8548 RNAi insert. Multiplexing *rp49* and *CG8548* RNAi primers gave a positive control band for *rp49*, but no evidence of the *CG8548* insert. This implies that the stock is contaminated and no longer carries the RNAi of interest.**

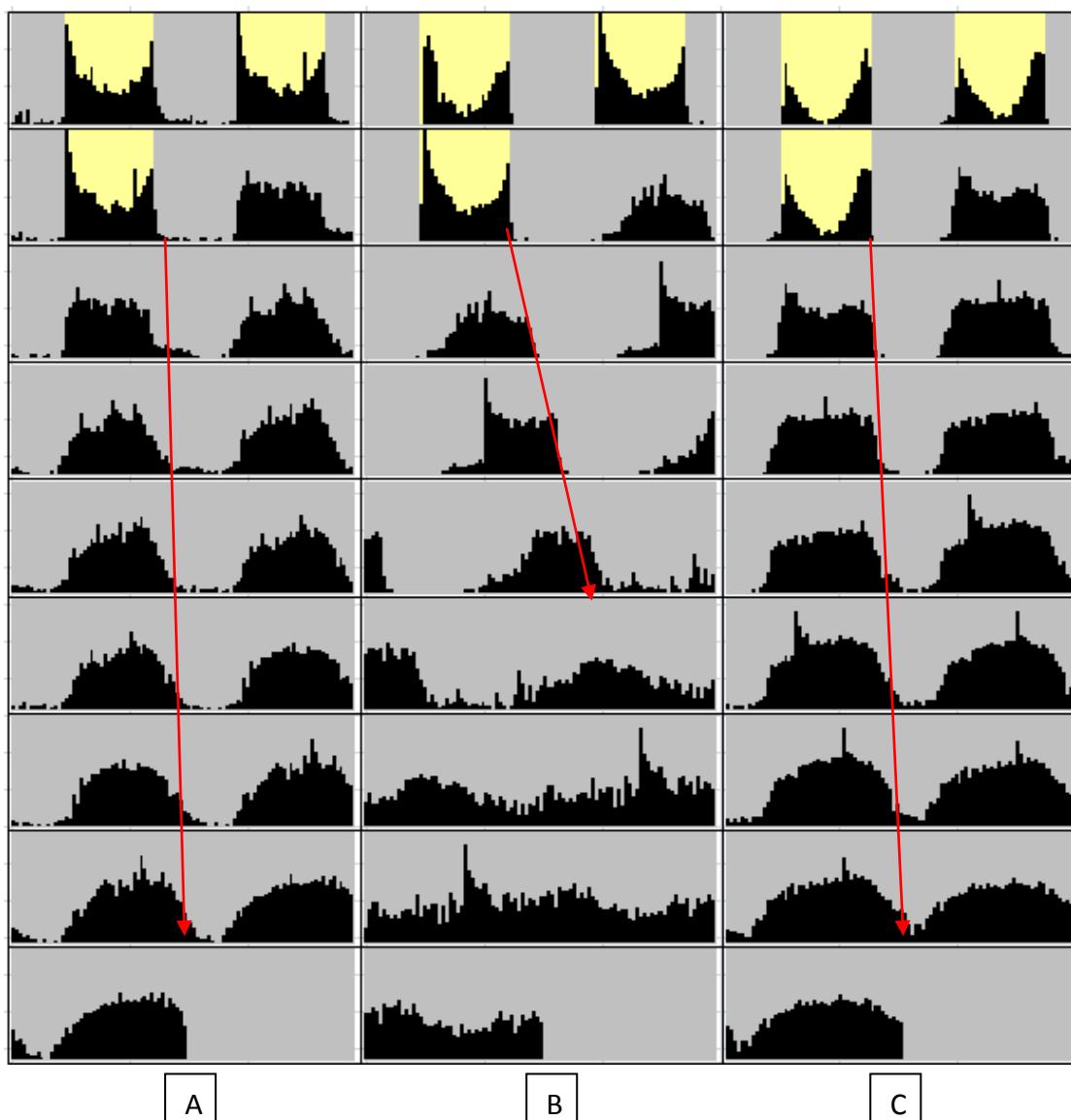
### **3.4.2 The Effect of *Akh* on the Circadian Clock**

Results from the original *Akh* RNAi knockdown experiments reveal a long period (median=30.6h ±0.6 n=29) after entering DD. Knockdown using the alternative KK line however gives a shorter period (median=24.4h ±0.3 n=72) similar to the *timGAL4>dicer2* control (median=24.7h ±0.1 n=29), a result that seems clear from Figure 3-5 where control and KK line traces are similar, whereas knockdown with the original GD line appears out of phase after three days in DD.



**Figure 3-5 - Histogram of activity in *Akh* knockdowns. The control *timGAL4>dicer2* (green), *timGAL4>dicer2>RNAi KK* (blue) and *timGAL4>dicer2>RNAi GD* (red). Yellow shading indicates lights on during entrainment. While the KK line deviates slightly from the control, the GD line differs significantly and is completely out of phase after 3 days in DD.**

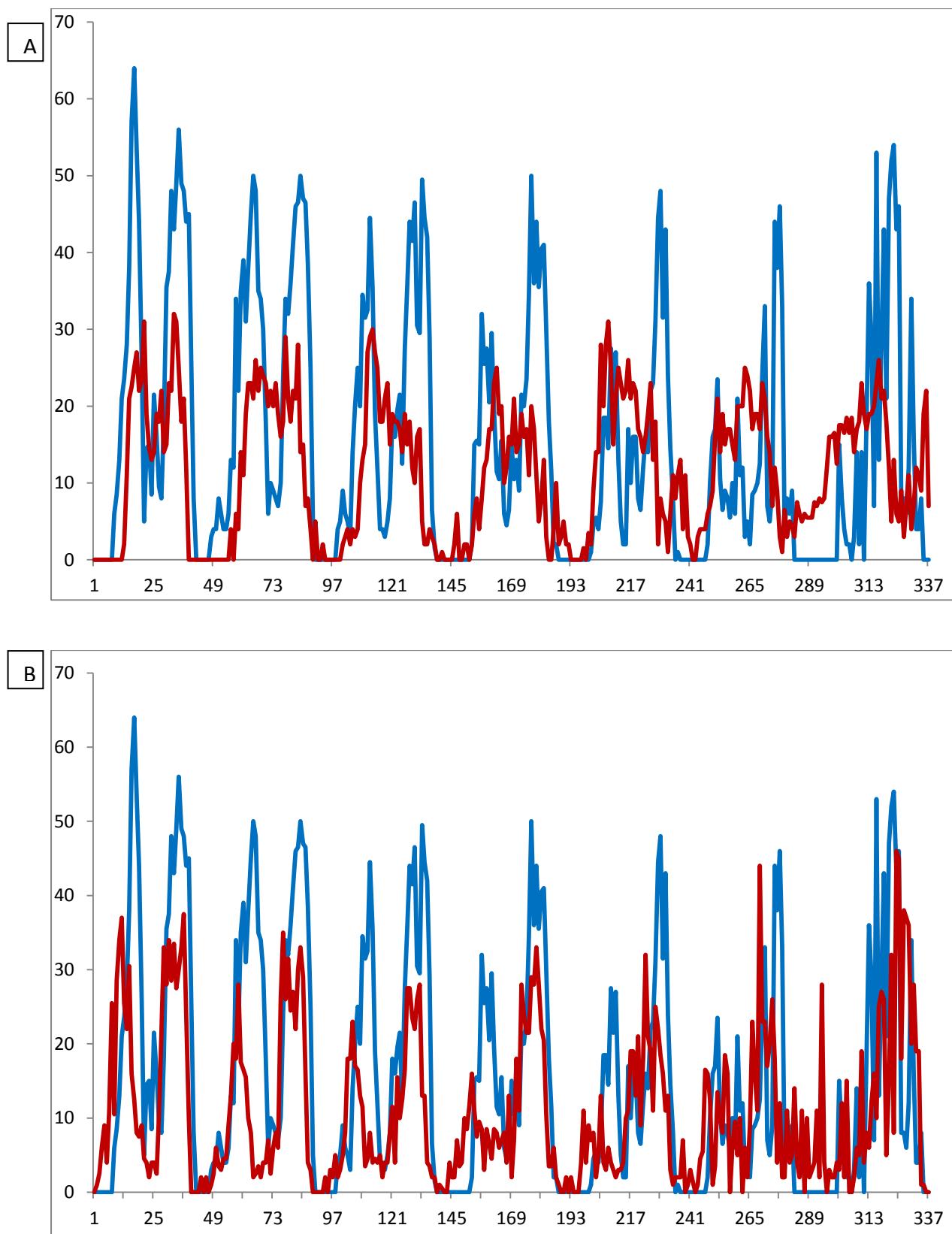
The effect knockdown is evident from the shifting evening peak in the actograms (Figure 3-6) which show a significant deviation in the GD line and by the fourth day there is no discernible rhythm to the flies' behaviours. ANOVA was performed to compare the results of knockdown using the GD and KK line with the *timGAL4>dicer2* control with Dunnet's multiple comparisons test to compare both knockdowns to the control. This confirmed that the KK line and the control are similar ( $P=0.69$ ) but the GD period is significantly longer ( $P<0.0001$ ).



**Figure 3-6 – Acto-histograms of circadian activity monitoring in the *Akh* activity experiment. Yellow shading shows lights on – grey is lights off. Control flies (A) are compared to the GD RNAi knockdown (B) and KK RNAi knockdown (C). The red arrows show the deviation of the onset of evening peak during the course of the DD experiment**

The results using RNAi with the *timGAL4>dicer2* driver meant it was unclear as to whether the GD line was behaving abnormally or the KK line did not knock down *Akh*. Three experiments were performed using the *AkhGAL4* driver from Lee and Park (Lee and Park, 2004). The first experiment utilised the *AkhGAL4* driver to ablate *Akh* expressing cells in the CC. This was achieved by using a line carrying the UAS-*rpr* and UAS-*hid* overexpression cassettes, whereas Lee and Park had only driven *rpr* in their experiments. The results for these experiments are shown in Figure 3-7. The results do not indicate any strong

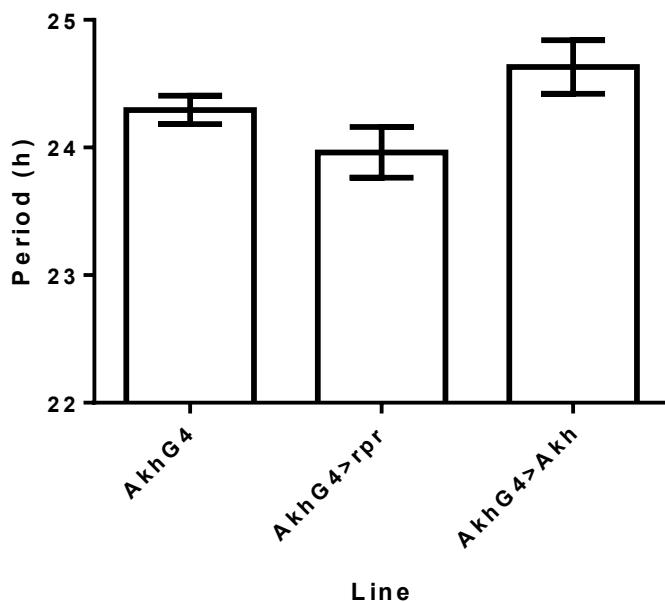
differences between control and experimental lines with the *AkhGAL4* control giving a median period of 24.3h  $\pm$ 0.11, (n=22), the *Akh* cell ablation experiment giving a median period of 24h  $\pm$ 0.20 (n=16) and the overexpression experiment giving a median period of 24.4h  $\pm$ 0.21 (n=17). One way ANOVA and Dunnet's post-test were performed to compare over expression and cell ablation with the *AkhGAL4* control independently and showed that neither overexpression ( $P=0.27$ ) nor cell ablation ( $P=0.29$ ) were significantly different to the control. The period differences are not large between the driver control and either experiment (Figure 3-8)



**Figure 3-7 – Histograms showing the use of *AkhGAL4* drivers to assess the role of *Akh* in circadian activity. In cell ablation experiments (A), the *AkhGAL4* control (blue) elicits a similar behaviour to the *Akh* cell ablation experiment (red) indicating that losing the *Akh***

cells has no influence on the period. Similarly, using the *AkhGAL4* to overexpress *Akh* (B) indicates that overexpression of *Akh* (red) has a similar period to the *Akh>GAL4* control (blue)

ANOVA of the data indicates significant variance between the groups ( $F=3.53$ ,  $P=0.036$ ), but Dunnet's multiple comparisons test, comparing each line to the control, indicates that cell ablation and overexpression have no effect on period length.



**Figure 3-8 Effect of *Akh* cell ablation and overexpression on period. Cell ablation gave a marginally shorter period than the control while overexpression gave a marginally longer period. Mean  $\pm$  SEM**

The results lend further doubt to the idea that *Akh* could be involved in influencing circadian rhythms. After ablating all cells involved in *Akh* synthesis, testing a second RNAi line and overexpressing *Akh* the only conclusion that can be drawn is that the *Akh* line tested by Green is having an effect other than *via Akh* knockdown

### **3.4.2.1 Testing for Off-Target effects of *Akh* RNAi**

The results for the study of *Akh*'s involvement in the clock imply that a problem may lie within the RNAi line originally studied. Using dscheck, (described by (Naito et al., 2005)) a

number of off-target genes that may be targeted by an RNAi can be identified. The results (Figure 3-9) indicate a possible off-target effect on *per*. Performing a sequence alignment with *per* and the RNAi Sequence (Figure 3-10) indicates a sequence within the GD line – absent in the KK line – that may also have an interaction with *per* that is 17 bases with one mismatch.

[dsRNA Design page](#) / [dsRNA Verification page](#)

### off-target candidate sequences [\[help\]](#)

```
mis=0 mis=1 mis=2 name of off-target candidate sequence
204   6     2     gi|17647146|ref|NM_079194.1| Drosophila melanogaster CG1171-PA (Akh) mRNA, complete cds
0     2     5     gi|24654805|ref|NM_138200.2| Drosophila melanogaster CG12030-PA (CG12030) mRNA, complete cds
0     2     4     gi|24639386|ref|NM_080317.2| Drosophila melanogaster CG2647-PA (per) mRNA, complete cds
0     2     3     gi|24639526|ref|NM_166977.1| Drosophila melanogaster CG13021-PB, isoform B (CG13021) mRNA, complete cds
0     2     2     gi|24656042|ref|NM_139462.1| Drosophila melanogaster CG13800-PA (CG13800) mRNA, complete cds
```

**Figure 3-9 Screen Capture of the result for RNAi off target matches for the *Akh* RNAi. The primary strong result is for *Akh*, but in addition to this, *CG12030* and *per* are listed as possible additional targets.**

AAGCAATTATAGAAAGAATCCTGCTAGTGCTGTGTTAATTACTTACCAGCTGTCTCCCTT  
CGAGCTCAATGGGTATAAAAGCAGGGCTGAATCGAAGTGGACCAGCATAGAAACTCAGAATGAA  
TCCCAAGAGCGAAGTCCTCATTGCAGCCGTGCTTCA[**TGCTGCTGGCCTGCGTCCAGTGTC**  
AAGTGAGTGAATTCATATCCCGCTGCAGGATATCCCTTCTGATGTCTCATTTATTTCT  
CTATATAGTTGACCTCTCGCCGGATTGGGGCAAGCGTTGGTGGGGCAGGAGCTGGCCTGGAA  
**ACCTTTTCGAGACACAGCAGGGCAACTGCAAGACCTCCAACGAAATGCTGCTCGAGATCTT**  
**CCGCTTCGTGCAATCTCAGGCACAGCTTTCTGGACTGCAAGCACCACGAGTAGATAGCTG**  
TAGGCCAACAGATCCTGGTCCGGATCTGGACGATGTCTAGCACGCACACACATACACTCT  
ATATATATAATTACCCGTATATACAGCACGTTATCAAGGTAGTCGTAGTCGGCATTAGATT  
TAATGGAACACTCCTTCTCAACAATAAGCGCATCCGAAATT

AAGCAATTATAGAAAGAATCCTGCTAGTGCTGTGTTAATTACTTACCAGCTGTCTCCCTT  
CGAGCTCAATGGGTATAAAAGCAGGGCTGAATCGAAGTGGACCAGCATAGAAACTCAGAATGAA  
**TCCCAAGAGCGAAGTCCTCATTGCAGCCGTGCTTCA[**TGCTGCTGGCCTGCGTCCAGTGTC****  
AAGTGAGTGAATTCATATCCCGCTGCAGGATATCCCTTCTGATGTCTCATTTATTTCT  
CTATATAGTTGACCTCTCGCCGGATTGGGGCAAGCGTTGGTGGGGCAGGAGCTGGCCTGGAA  
**ACCTTTTCGAGACACAGCAGGGCAACTGCAAGACCTCCAACGAAATGCTGCTCGAGATCTT**  
**CCGCTTCGTGCAATCTCAGGCACAGCTTTCTGGACTGCAAGCACCACGAGTAGATAGCTG**  
TAGGCCAACAGATCCTGGTCCGGATCTGGACGATGTCTAGCACGCACACACATACACTCT  
ATATATATAATTACCCGTATATACAGCACGTTATCAAGGTAGTCGTAGTCGGCATTAGATT  
TAATGGAACACTCCTTCTCAACAATAAGCGCATCCGAAATT

**Figure 3-10 The sequence for Akh aligned to RNAi sequences. Aligning the sequences used for making the RNAi lines in green for the KK line (top) and the GD line (bottom). Blue highlighting represents a 17bp per-like sequence that may be influenced by RNAi knockdown. This sequence is not present in the KK line, but it is in the GD line.**

To confirm that the results are due to an inadvertent knockdown of *per*, a qPCR was performed. *elavGAL4>dicer2* flies were crossed with *Akh* RNAi (GD) flies to induce a knockdown in all neuronal cells. RNAi was extracted from the heads of the resulting offspring, *elavGAL4>dicer2* flies and *Akh* RNAi (GD) flies. Primers were designed for *per* with the consideration that they should flank the target region, but avoid binding to the target (Holmes et al., 2010) the 5' primer being *q\_per\_F* ACAACAAGAAATACACGGACAG and the 3' primer *q\_per\_R* GATCTTGCTCTCAGATGTGC. *rp49* primers described in section 3.4.1 was used as a housekeeping gene for relative expression. A Brilliant II 1-step SYBR-green mastermix kit (Agilent) was used for qPCR according to manufacturers instructions. The results of the qPCR were run through the REST programme (Pfaffl et al., 2002) which uses the pfaffl method (Pfaffl, 2001) to calculate the level of downregulation and the level of

significance (Table 3-4) which indicated that *per* mRNA transcripts are significantly lower in the knockdown than in either of the controls.

**Table 3-4 Pfaffl analysis showing RNAi knockdown of *per* in *elavGAL4>dicer2>AkhRNAi*. Levels of *per* mRNA transcripts are lower in the knockdown relative to the GAL4 control (top) and the RNAi control (bottom)**

Relative Expression Results – <i>elavGAL4&gt;dicer2</i> vs. <i>elavGAL4&gt;dicer2&gt;Akh RNAi GD</i>							
Parameter	Value						
Iterations	2000						
Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
<i>rp49</i>	REF	0.9751	1.000				
<i>per</i>	TRG	0.9894	0.063	0.022 - 0.184	0.011 - 0.265	0.000	DOWN
<b>Interpretation</b>							
<i>per</i> is DOWN-regulated in sample group (in comparison to control group) by a mean factor of 0.063 (S.E. range is 0.022 - 0.184).							
<b><i>per</i> sample group is different to control group. P(H1)=0.000</b>							
Relative Expression Results - <i>Akh RNAi GD</i> vs. <i>elavGAL4&gt;dicer2&gt;Akh RNAi GD</i>							
Parameter	Value						
Iterations	2000						
Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
<i>rp49</i>	REF	0.9751	1.000				
<i>per</i>	TRG	0.9894	0.330	0.171 - 0.631	0.123 - 0.938	0.000	DOWN
<b>Interpretation</b>							
<i>per</i> is DOWN-regulated in sample group (in comparison to control group) by a mean factor of 0.330 (S.E. range is 0.171 - 0.631).							
<b><i>per</i> sample group is different to control group. P(H1)=0.000</b>							

## 3.5 Discussion

### 3.5.1 The Influence of *Akh* Knockdown on Circadian Rhythms

Ultimately, the results for this experiment indicated a low likelihood that *Akh* is involved in modulating circadian behaviour. This result was not unanticipated due to previous work by Lee and Park (2004). The primary issue comes from the fact that *Akh* is not expressed in clock cells, but in a very specific set of cells in the CC. The results achieved previously by Lee and Park (2004) showed that loss of *Akh* resulted in no effect on circadian rhythms and the method of ablating *Akh* expressing cells is a more effective knockdown than using RNAi. Experiments performed here supported the data achieved by Lee and Park, by using *AkhGAL4* to drive in one instance *UAS-rpr;UAS-hid* to ablate the cells expressing *Akh*, but also the addition of another *Akh* RNAi line, and overexpression of *Akh* - all of which showed no effect on circadian rhythms.

This cautionary tale reveals that a second independent RNAi line, if available, is required to confirm initial results and to check for possible off-target effects using programs such as dsCheck (Naito et al., 2005). Although the VDRC make every attempt to inform customers of possible off-target effects, their system only gives warning when the RNAi has 19bp continuous sequence homology with another gene. In this case, there is a 20bp match with a single mismatch and in some cases, this is enough to result in an off-target match (Saxena et al., 2003).

### 3.5.2 Testing Other RNAi Lines for Putative Clock Gene Studies.

The range of other genes that were tested in the study also indicated limited likelihood of circadian behavioural modification when knocked down. While all of the results indicated statistical significance, the significance - biologically speaking - of a rhythm shift to 24.8h is

not impressive. A more ideal control than used here when performing knockdowns would be to use both parental lines to minimise the effect of genetic background or insertional effects of p-elements. The lethality associated with *CG8426* knockdown suggests a problem with Green's results which indicated that knockdown using *timGAL4* extended the period to over 27h.

Similarly, experiments with *CG8548* indicated that the line had been contaminated and could not be used for comparison. The long period achieved using this line was intriguing, but attempting to find out what had been influenced would have used up a great deal of time and resources. It is unclear as to whether this contamination had occurred before Dr Green used it in his experiments or whether it was contaminated before it was tested here.

One thing worth noting with regards to these results is that enhanced knockdown generally gave a longer period than the control, much of the evidence achieved by Green had shown that these genes indicated a shorter period. Again the comparison between the two sets of results is disturbing and hints at very small effects, if any, of RNAi on these genes.

## 4 Winter simulation experiments

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### 4.1 Introduction

Saunders *et al.* (1989) demonstrated the role of photoperiod in the diapause of *D. melanogaster*, but also acknowledged that temperature was the strongest factor for its induction. However, a number of other insect models were shown to have a photoperiodic-induced diapause and were well established within the field before Saunders' research. Among these are the pitcher plant mosquito *Wyeomyia smithii* (Bradshaw, 1980), the jewel wasp, *Nasonia vitripennis* (Saunders, 1965) and silkworms - including *Bombyx mori*, which was shown to be photoperiodic as early as 1933 (Williams, 1942, Lees, 1955). In fact silkworms formed the bulk of the earliest diapause research (Henneguy, 1904, Shelford, 1929, Lees, 1955, Williams, 1959, Williams and Adkisson, 1964). *Drosophila* is, by some, considered a poor model for diapause research (Emerson *et al.*, 2009a, Tatar *et al.*, 2001a). Indeed, even Saunders' original work showing *Drosophila* exhibited a photoperiodic diapause implied that the diapause is weak and that photoperiodicity was lost at temperatures below 13°C (Saunders *et al.*, 1989). The major advantage of using *D. melanogaster* comes with the genome sequence (Adams *et al.*, 2000) and the molecular and genetic tools available to researchers.

A number of genes have been investigated for their roles in *Drosophila* diapause. Williams and Sokolowski (2008) suggested that there are three particularly important genes in *Drosophila* diapause, based on research into naturally occurring mutations, their effects on diapause and their latitudinal distribution. The first of these genes is *tim*, (Sandrelli *et al.*, 2007, Tauber *et al.*, 2007), and a naturally occurring N terminal polymorphism influencing diapause was discussed in the Introduction. A mutation identified by Williams in the PI3K

gene, *dp110*, was shown to give rise to different diapausing phenotypes and the distribution of this mutation was more common in the north and less so in the south of North America, implying balancing selection (Williams et al., 2006). The third comes from an analysis of *couch potato (cpo)* mutants. Natural variation in this gene was shown to have a cline in North American *Drosophila* populations and variants have different effects on diapause (Schmidt et al., 2008). Elevated *cpo* transcript levels have been identified as having a role in diapause in the mosquito *Culex pipiens* (Zhang and Denlinger, 2011).

Saunders' original experiments showed that the *Canton-S* line exit diapause spontaneously within 6-7 weeks at 12°C LD 10:14 (Saunders et al., 1989). Emerson et al. (2009a) similarly concluded that the diapause remission was evident in North American populations of *D. melanogaster*, comparing 25 day and 28 day diapause results with 12 day results achieved in an earlier paper (Tauber et al., 2007). In this laboratory, a study of both North American and European lines under constant conditions for 12 or 28 days has observed spontaneous remission at 28 days (Valeria Zonato, personal communication). Inevitably, winter in temperate clines lasts longer than four weeks. In Leicester, UK (52°38'N), the time between the mean high temperature lowering to 12°C and returning to 12°C is approximately 5 months from October 28<sup>th</sup> to March 31<sup>st</sup> (<http://weather.UK.msn.com>). The time between lowering to 12°C and beginning to rise from the depths of winter is approximately 2.5 months (January 9<sup>th</sup>). Clearly, a diapause that only lasts one month implies that the insects are no longer delaying maturation according to their environment and brings into question the validity of the experimental paradigm used to test diapause in *D. melanogaster*.

*Drosophila simulans* is another closely related species within the *Sophophora* subgroup of *Drosophilidae* first recognised as a separate species in 1919 (Sturtevant). Much like *D.*

*melanogaster*, *D. simulans* originated in sub-Saharan Africa and gradually colonised Europe through human trade and migration (David et al., 2007). However, unlike *D. melanogaster*, *D. simulans* do not appear to have adapted to the variable climate and particularly harsh winters of Northern Europe. It is reported that they do not have a diapause response and in colder areas it has been implied that the populations die out and the regions are repopulated in spring by migration (Schmidt et al., 2005b, Boulétreau-Merle et al., 2003). Nevertheless, data published so far on *D. simulans* has been performed with limited 28 day single temperature experiments with little or no information on the lines used. Zonato (2012) indicated that *D. simulans* from Rende (Italy) exhibit a diapause in short day and long day conditions at 12 °C and show a typical remission effect in longer 28 day experiments. In order to better compare diapause in *D. simulans* to *D. melanogaster*, winter simulation experiments might prove useful.

It could be that the diapause remission reported above indicates that *Drosophila* do not diapause, but under cold conditions, their development is delayed thereby extending the life cycle. However, this is unlikely as Saunders (Saunders, 1990, Saunders et al., 1989), as well as others have shown that photoperiod can influence diapause and complete darkness impedes diapause at 12°C, thus partially nullifying the role of temperature.

## 4.2 Aims

The current paradigm for diapause analysis in *Drosophila* considers only a continuous state of temperature and photoperiod, not a more realistic winter where the temperature gradually falls along with the photoperiod. The objective here is twofold. First, the maintenance of diapause will be assessed in a more realistic winter simulation and compared to the typical single temperature/photoperiod experiments at 12°C and the lower

constant temperature of 8°C. In addition, there will be a comparison of flies carrying the *ls-tim* and *s-tim* alleles to see if *ls-tim* flies better maintain diapause under this paradigm as reported under constant condition paradigms (Tauber *et al.* 2007). This paradigm will additionally be assessed in the diapause of *D. simulans* to revisit the findings of Boulétreau-Merle and colleagues (2003) suggesting that there is no diapause in this species.

## 4.3 Materials and Methods

### 4.3.1 Fly Lines

*Houten* flies, for the *ls-tim* and *s-tim*, comparison were acquired from Dr João Gesto (University of Leicester). They were extracted from a Northern European population from Houten, Netherlands (52°N, 5.2°E). By performing a series of backcrosses for 8-10 generations Dr Gesto produced congenic populations, one homozygous for *ls-tim* and the other for *s-tim* (Gesto, 2010). In addition to the *Houten* populations, a population was prepared for *D. simulans*. Three isofemale lines from Rende (southern Italy) were used (Donated by Dr Mirko Pegoraro, University of Leicester). Five mated females from each line were placed into a bottle to give rise to the *SREN* population. As the *SREN* population originates from Southern Europe, a direct comparison with a Northern European population would not be appropriate. For this reason, a similar protocol was used to produce a *D. melanogaster* population from the same region. Ten isofemale *D. melanogaster* lines (Also donated by Dr Pegoraro) were available from a similar area and the population (*MREN*) was prepared in the same way as the *SREN*, using 5 mated females from each isofemale line. The *MREN* population is polymorphic for *ls-tim/s-tim* so because *D. simulans* are monomorphic for the ancestral *s-tim* allele (Rosato *et al.*, 1997, Tauber *et al.*, 2007), I selected for *s-tim* using the non-lethal *tim* genotyping method (section 2.4.3).

#### **4.3.2 Winter simulation diapause experiments**

Using Sanyo MIR-254 incubators, the user can set temporal and temperature programs in a link function to perform them in sequence without interruption. The programmes therefore were set as in Table 4-1, starting at 12°C LD 10:14, falling by 1°C and the photoperiod shortening by 30 minutes per week, similar to winter in Northern Europe. Once at 8°C, LD 8:16, the incubator maintained the flies for 4 weeks, before the next set of programs began to raise the temperature and photoperiod at the same rate as the lowering. A large number of flies (approximately 30-40 vials of each genotype each carrying ~20-30 mixed male and female flies) were collected and placed randomly in the incubator on the same day. Vials were selected at timepoints (2, 4, 8, 10 and 12 weeks) randomly and females were assessed for diapause as described in chapter 2.3. Controls were maintained at constant temperature/photoperiod, comparing 12°C/LD 10:14 and 8°C/LD 8:16.

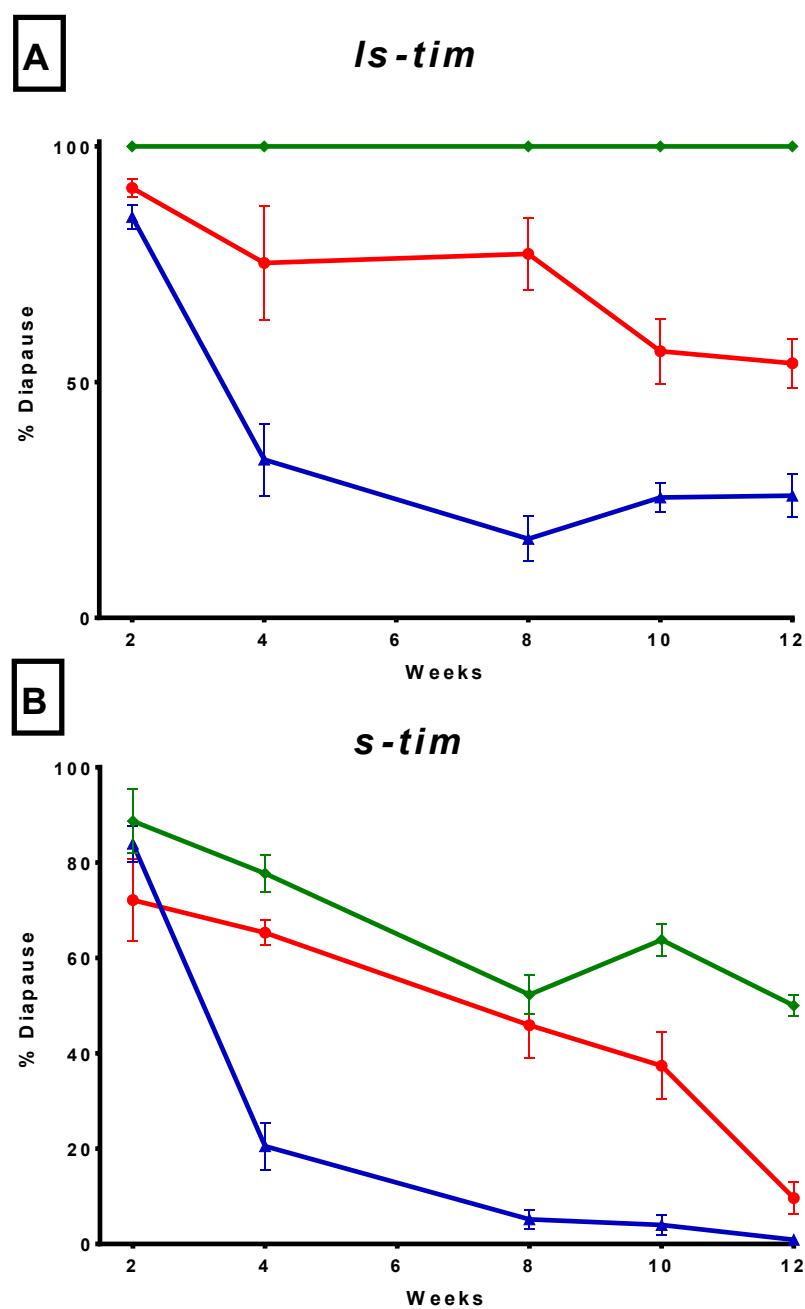
Programme name	Temperature (°C)	Photoperiod (h)	Programme Length (weeks)
#1LACWINTER	12	10	1
#2LACWINTER	11	9.5	1
#3LACWINTER	10	9	1
#4LACWINTER	9	8.5	1
#5LACWINTER	8	8	4
#6LACWINTER	9	8.5	1
#7LACWINTER	10	9	1
#8LACWINTER	11	9.5	1
#9LACWINTER	12	10	1

**Table 4-1 – The sequence of programmes used to set up an artificial winter. Rows in grey denote weeks at the end of which samples were taken**

## 4.4 Results

### 4.4.1 Northern European *D. melanogaster* Populations

The results illustrated in Figure 4-1 indicate clear differences between the conditions, but also between the lines in each condition. The most striking difference is clear at 8°C where the *ls-tim* line reaches 100% diapause at all time points. By 12 weeks however, the mortality rate was high and the final point at 8°C is represented by a solitary female fly. In comparison, the *s-tim* flies at 8°C gradually enter remission, though they do remain above 60% diapause by the end of the experiment.



**Figure 4-1** Winter simulated diapause experiments in *D. melanogaster*. *Is-tim* (A) and *s-tim* (B) genotypes tested in the three experimental conditions, winter simulation (red), constant 12°C LD 10:14 control (blue) and constant 8°C LD 8:16 control (green). Winter simulation experiments involve a 30 minute decline in photoperiod and 1°C per week until 4 weeks, where it is maintained at 8°C LD, before rising at the same rate for the final 4 weeks controls mimicking the highs and lows of the simulation. Mean ± SEM - *Is-tim/s-tim* winter (n=906/647 or 7/5 replicates per timepoint), 12°C (n=588/654 or 6/6 replicates per timepoint) and 8°C (n=215/293 or 5/5 replicates per timepoint)

Three-way ANOVA compared the influences of allele, time and conditions, excluding the 8°C control which was incomplete and lacked variability (i.e. variance=0). The results for the ANOVA and interactions are in Table 4-2.

**Table 4-2 Three-way ANOVA results.**

Effect	SS	Degrees of freedom	MS	F	P
time (weeks)	8.075	4	2.019	55.1	0.000*
Conditions	3.953	1	3.953	108.0	0.000*
Allele	2.284	1	2.284	62.4	0.000*
time (weeks)*conditions	1.444	4	0.361	9.9	0.000*
time (weeks)*allele	0.578	4	0.144	3.9	0.005*
conditions*allele	0.026	1	0.026	0.7	0.405
time (weeks)*conditions*allele	0.132	4	0.033	0.9	0.465

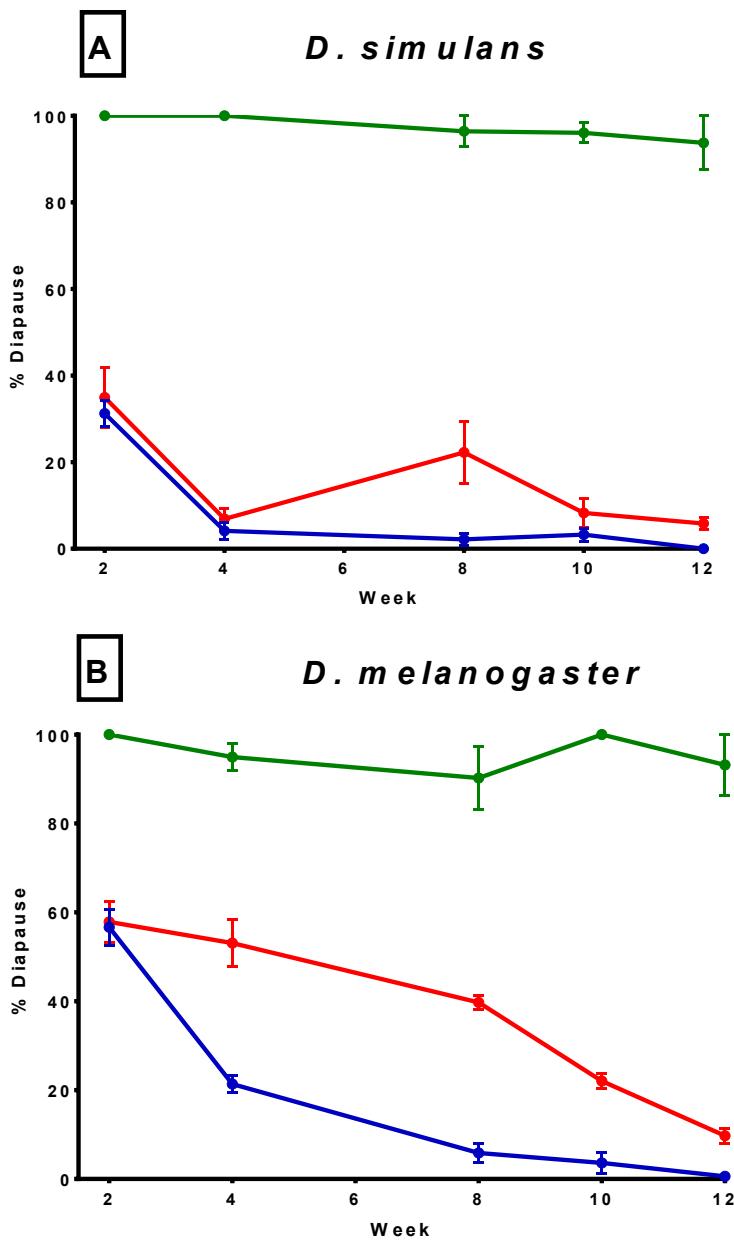
Each of the factors, time, conditions (simulation and 12°C constant conditions) and allele, influence the experiment, but the three-way interaction between factors was not significant ( $P=0.465$ ). The interaction of conditions and allele also do not show significance ( $P=0.405$ ) implying that under both sets of conditions, both alleles are behaving in a similar manner. Interaction of time and allele however indicates that the alleles behave differently over the period of time in the experiment ( $P=0.005$ ) which is evident from Figure 4-1 as *ls-tim* flies maintain stronger diapause throughout the experiment in both constant 12°C and winter simulation conditions. The interaction of time and conditions indicates that there is a significant difference between the way that the two conditions influence diapause over the time of the experiment ( $P<0.001$ ), with both lines exhibiting a stronger remission effect under constant experimental conditions by week 8.

Newman-Keuls post-hoc tests (Appendix A) indicated that *ls-tim* winter simulation results differed from the control results at week 4 ( $P=0.0004$ ). The same observation was made in *s-*

*tim* flies ( $P=0.001$ ), showing the effects of remission in constant conditions. The earliest evidence of remission under winter conditions in *s-tim* flies is at 8 weeks, at which point, diapause levels are lower than they were at two weeks ( $P=0.03$ ). Some ‘remission’ is observed in the *ls-tim* lines as the level of diapause drops at 10 weeks ( $P=0.007$ ). This may be considered active development as this is 2 weeks into the upward swing towards spring. The effect of genotype under control conditions does not show any difference until week 10, where, as expected, levels of diapause have lowered significantly more in *s-tim* flies compared to *ls-tim* ( $P=0.01$ ). Under simulation conditions however, the differences between the two lines are evident as early as eight weeks ( $P=0.01$ ).

#### **4.4.2 Comparing species in winter simulation experiments**

The results for the Rende populations, comparing *D. melanogaster* and *D. simulans*, show that *SREN* flies exhibit a weaker diapause than *MREN* flies under constant 12°C and winter simulation conditions after two weeks, with less than 40% of *SREN* flies exhibiting diapause compared to near 60% of *MREN*. After four weeks, the level of diapause in *SREN* drops below 10% where over 20% *MREN* flies in the 12°C control are still in diapause. The levels of diapause do rise again at 8 weeks in the *SREN* winter simulation, but generally remain low.



**Figure 4-2 Results for the winter simulation and control diapause experiments using the Rende populations. Comparing *D. simulans* (A) and *D. melanogaster* (B).** Simulation (red), 12°C control LD 10:14 control (blue) and constant 8°C LD 8:16 control (green). Winter simulation experiments involve a 30 minute decline in photoperiod and 1°C per week until 4 weeks, where it is maintained at 8°C LD, before rising at the same rate for the final 4 weeks controls mimicking the highs and lows of the simulation. Mean  $\pm$ SEM - MREN/SREN winter (n=522/605 or 6/6 replicates per timepoint), 12°C (n=454/487 or 6/6 replicates per timepoint) and 8°C (n=206/208 or 4/4 replicates per timepoint)

The 8°C control for both species show well maintained diapause remaining above 90% after 12 weeks for both species. Three-way ANOVA compared the influences of species, time and conditions, excluding the 8°C control to make the results more comparable with those in section 4.4.1 and variance is still clearly very low. The results for the ANOVA and interactions are in Table 4-3. 8°C controls were tested by linear regression which indicated that the slopes were equal between *MREN* and *SREN* ( $F=0.18$ ,  $P=0.67$ ).

**Table 4-3 Three-way ANOVA analysis for *SREN* and *MREN* populations**

Effect	SS	Degrees of freedom	MS	F	P
time (weeks)	4.871	4	1.218	63.74	0.000*
conditions	1.540	1	1.540	80.63	0.000*
Line	1.399	1	1.399	73.24	0.000*
time (weeks)*conditions	0.480	4	0.120	6.28	0.000*
time (weeks)*Line	0.600	4	0.150	7.85	0.000*
conditions*Line	0.153	1	0.153	8.00	0.006*
time (weeks)*conditions*Line	0.097	4	0.024	1.27	0.285

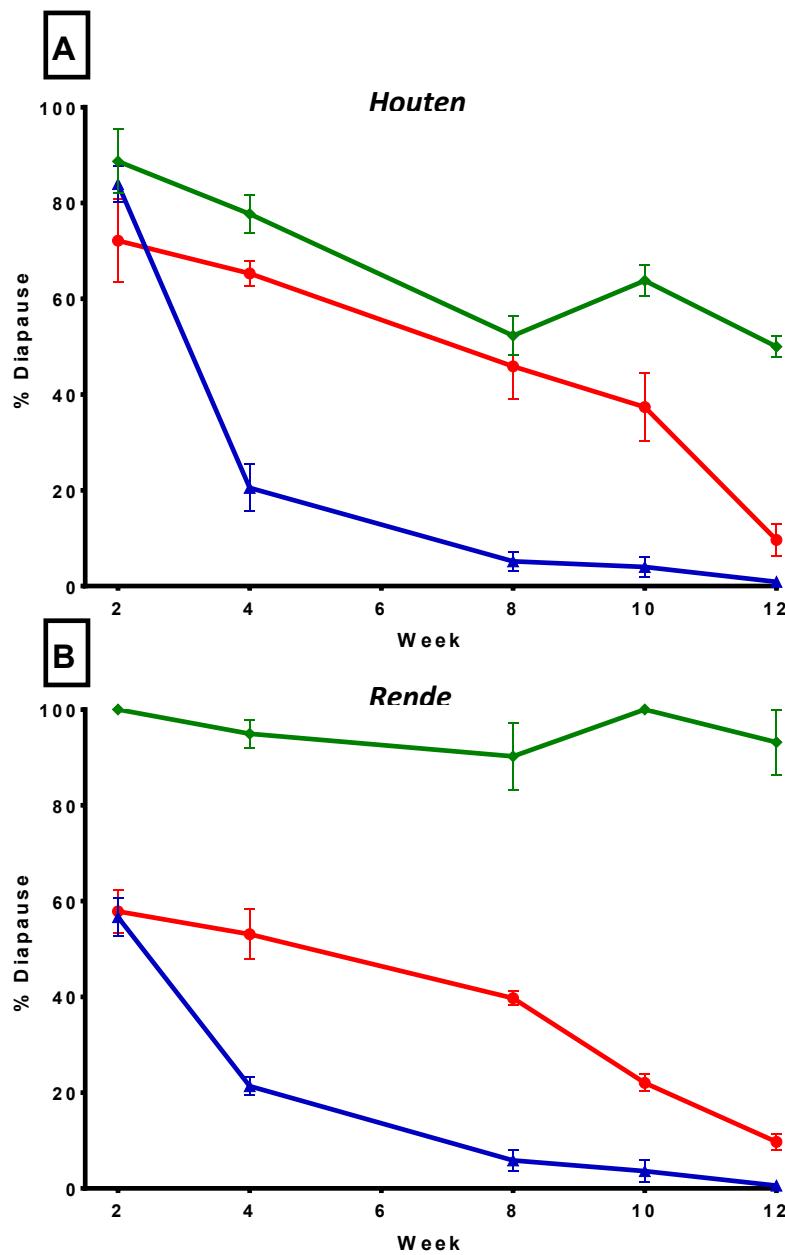
Individually, each of the factors, time, conditions and species, influence the experiment ( $P<0.001$  for all), but the overall three-way interaction between factors was not significant. The interaction of conditions and species is significant ( $P=0.006$ ) implying that under the different conditions, the two species react differently, as is evident from Figure 4-2. The significant interaction of time and species ( $P<0.001$ ) is revealed by *SREN* flies exhibiting a much weaker diapause phenotype than *MREN*. The interaction of time and conditions ( $P<0.001$ ) indicates that there is a significant difference between the way that the two conditions influence diapause over the course of the experiment which is clear from the results achieved for *MREN*, but less so for *SREN* flies.

Newman-Keuls post-hoc tests (Appendix B) revealed that *MREN* winter simulation results differed from the control results at week 4 ( $P=0.0002$ ), evidence for a remission effect in control conditions that is delayed in winter simulations. However, differentiation between simulation and control was only evident at 8 weeks in *SREN* flies ( $P=0.001$ ) and not at any other point in the experiments. The earliest evidence of exiting diapause under winter conditions in *MREN* flies is at 10 weeks, at which point, diapause levels are lower than two weeks ( $P=0.0003$ ). Remission is clear at week 4 in *SREN* flies however ( $P=0.0002$ ). The differences between the species under control conditions is evident from week two ( $P=0.01$ ) with *SREN* flies exhibiting a much lower level of diapause.

Using linear regression, it is possible to see how much of an effect the simulation experiment had on the trajectory of *D. simulans* diapause. Comparing the simulation and 12°C control lines it was indicated that the slope is different between these two experiments ( $F=11.8$ ,  $P=0.001$ )

#### **4.4.3 Comparing MREN and Houten *s-tim***

While the *MREN* line was primarily being used as an *s-tim* line with similar geographical location to the *D. simulans* *SREN* flies, it also serves as a comparison with the northern European populations from *Houten* to see if there is a difference in how they respond to changing and constant conditions. The results (Figure 4-3) indicate that both lines react to conditions in a similar fashion, with the exception of the 8°C control where a much stronger remission event is observed in *Houten* flies.



**Figure 4-3 Results comparing *Houten s-tim* (A) and *MREN s-tim* (B) in simulation experiments. Under simulation (red), constant 12°C LD 10:14 control (blue) and constant 8°C LD 8:16 control (green). Winter simulation experiments involve a 30 minute decline in photoperiod and 1°C per week until 4 weeks, where it is maintained at 8°C LD, before rising at the same rate for the final 4 weeks controls mimicking the highs and lows of the simulation. Mean  $\pm$ SEM - *Houten/Rende* winter ( $n=647/522$  or 5/6 replicates per timepoint), 12°C ( $n=654/454$  or 6/6 replicates per timepoint) and 8°C ( $n=293/206$  or 5/4 replicates per timepoint)**

Three way ANOVAs were performed comparing the effects of time, line and conditions (Table 4-4). Constant 8°C controls were excluded from analysis for better comparison to analyses performed in previous sections.

**Table 4-4 Three-way ANOVA analysis for *Houten* and *MREN s-tim* populations**

Effect	SS	Degrees of freedom	MS	F	P
time (weeks)	9.289	4	2.322	120.4	0.000*
conditions	2.887	1	2.887	149.7	0.000*
Line	0.199	1	0.199	10.3	0.002*
time (weeks)*conditions	1.151	4	0.288	14.9	0.000*
time (weeks)*Line	0.267	4	0.067	3.5	0.011*
conditions*Line	0.011	1	0.011	0.6	0.445
time (weeks)*conditions*Line	0.075	4	0.019	1.0	0.427

Individually, each of the factors, time, conditions and line, significantly influence the experiment revealing *Houten* to have a generally higher level of diapause over the experiment ( $P=0.002$ ). However, neither the three-way interaction between factors, nor conditions x line were as significant ( $P=0.427$ ,  $P=0.445$ ) indicating that both lines are influenced in a similar manner. The effect of line x time ( $P=0.011$ ) further confirms a different trajectory for *Houten* versus *MREN* over the course of the experiment.

Newman-Keuls post-hoc tests (Appendix C) revealed that at week 2 *MREN* diapause is lower relative to *Houten* under simulation ( $P=0.04$ ) and in the 12°C control ( $P=0.002$ ) conditions, supporting the implication above that *MREN* flies generally exhibit a lower diapause than *Houten*. The first indicator of diapause termination under simulation are at 10 weeks in *MREN* ( $P=0.0004$ ), but earlier in *Houten* after 8 weeks ( $P=0.002$ ). In constant 12°C controls however, remission is observed at four weeks in both lines ( $P=0.0004$  *MREN* and  $P=0.0001$  *Houten*).

Linear regression on the two 8°C controls revealed a significant difference ( $F=15.4$ ,  $P=0.0003$ ) in that the *Houten* control has a significant slope ( $F=36.8$ ,  $P<0.0001$ ), whereas the *MREN* control does not deviate significantly from a slope of zero ( $F=0.4$ ,  $P=0.5$ ).

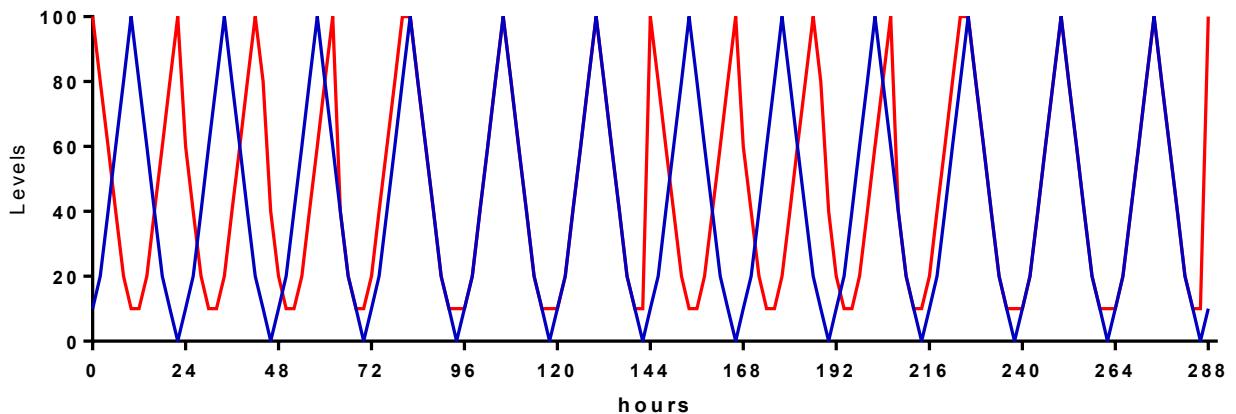
## 4.5 Discussion

### 4.5.1 Winter simulation experiments indicate a robust diapause in *D. melanogaster*

A number of diapause studies utilised four weeks of constant conditions (Schmidt et al., 2005b, Boulétreau-Merle et al., 2003, Emerson et al., 2009a, Schmidt et al., 2005a). From my work comparing diapause at different times after induction, four weeks under constant 12°C is likely to result in a remission effect in all lines tested. In this respect, a two week experiment would be better than a four week experiment when assessing diapause under these conditions, supporting Saunders' (1989) findings that 12 days was the appropriate period for assessing diapause. However, all three lines of *D. melanogaster* in these experiments, exhibited a longer and more robust diapause in winter simulations.

The mechanism behind this is unclear, but the theory that *Drosophila* do not exhibit a diapause, but rather a slow development does not hold up to observations that have been made previously, namely the photoperiodic work performed by Saunders (1989) and Tauber et al. (2007) and the evidence from the Nanda-Hamner experiments (Saunders, 1990). One possible explanation for the discrepancy between constant conditions and the winter simulation is that flies require thermal feedback to regularly reset the diapause signal. Considering the internal coincidence model described earlier (section 1.3.2), as this is the model *Drosophila* have been suggested to use (Saunders, 1990), it could be imagined that an oscillator phase is altered by the change in conditions (such as the thermal resetting of

per cycles observed by Sidote *et al.* (1998)), but gradually re-entrains to the correct phase if there is no further change in the environmental conditions, thus, the influence of the diapause inducing conditions is lost (Figure 4-4).



**Figure 4-4 Conceptual graph of rhythm resetting under changing conditions. One of the two oscillators is initially tuned out of phase by changes in temperature, but gradually re-entrains to the conditions, before another change in temperature introduces another phase shift.**

While purely speculative, this feedback model reinforces the diapause response and would also explain why remission was observed in insects under constant conditions. Using a strong signal, such as a very low temperature it may be that the clock stops or the oscillators are uncoupled. Under cold conditions in the wild, Menegazzi *et al.* (2013) found little evidence of PER or TIM expression in fly brains so perhaps the clock is kept in a phaseless state and this stimulates diapause. Remission does not appear to be common in insect models as many insects exhibit a highly robust diapause. Perhaps the ~10,000 years since *Drosophila*'s relatively recent dispersal into Europe (Lachaise *et al.*, 1988) has not been long enough for *Drosophila* to evolve a robust photoperiodic diapause.

While less practical to perform, the winter/spring simulation experiments have revealed a more realistic separation of the diapause exhibited by *ls-tim* and *s-tim*, *D. melanogaster* and *D. simulans* and *s-tim* flies from northern and southern Europe, compared to a snapshot of diapause taken at two weeks. An ideal scenario would be to test the simulation conditions while only changing the photoperiod or, only changing the temperature to see if either has an independent effect on diapause maintenance. Defining diapause using the negative marker of undeveloped ovaries does present a problem and further work to develop a method of diagnosing diapause would mark a further improvement in methodology.

#### **4.5.2 Winter simulation experiments suggest selective advantage for the novel *ls-tim* allele in *Drosophila***

The results indicate that there is a significant difference between the *ls-tim* and *s-tim* *D. melanogaster* lines derived from Houten. This supports earlier work suggesting that the novel *ls-tim* allele bestows a more robust diapause phenotype and may be advantageous to flies in temperate climates (Tauber et al., 2007). One difference between *ls-tim* and *s-tim* flies is the extended length of diapause observed in *ls-tim* flies after conditions begin to improve, relative to the *s-tim* fly response which is to rapidly terminate diapause. This might seem like a disadvantage as waiting until further into spring gives flies a narrower period for mating. However, late in winter when the *s-tim* flies may begin to terminate diapause they may still find themselves in a period of poor quality mating sites with mating and egg laying options very limited. Selection will favour a balance between resisting the winter and having a longer growing season in the spring. If the advantage of an earlier diapause in winter for *ls-tim* outweighs the disadvantage of a shorter growing season *ls-tim* will be favoured and spread throughout Europe, as has been observed (Tauber et al., 2007).

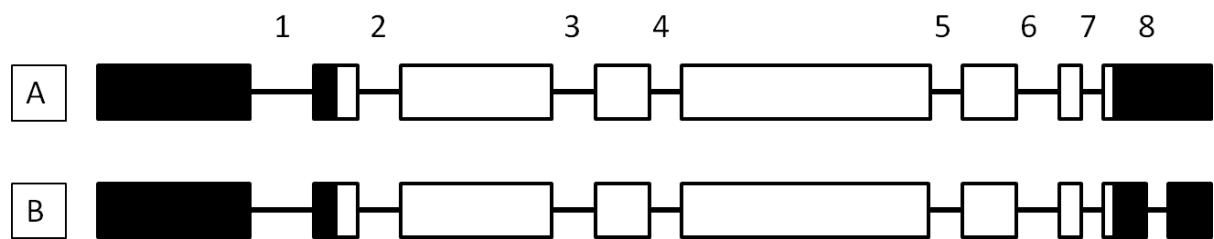
#### **4.5.3 Comparing populations of *D. melanogaster* and *D. simulans* from a southern European location**

The *D. simulans* population tested in these experiments do not maintain diapause at 12°C and the level of diapause is not much higher under winter simulation. This implies that *D. simulans* have not adapted to the temperate nature of more northern regions. The results are consistent with the view that *D. simulans* do not diapause, but rather they move into northern Europe from southern Europe and are purged by the cold winter, after which repopulate annually from the south (Boulétreau-Merle et al., 2003). This could also explain the comparative lack of genetic variability of *D. simulans* across Europe (Andolfatto, 2001, Capy et al., 1993, Hyttia et al., 1985, Lachaise et al., 1988, Singh et al., 1987).

# 5 Effects of *per* splicing on diapause

## 5.1 Introduction

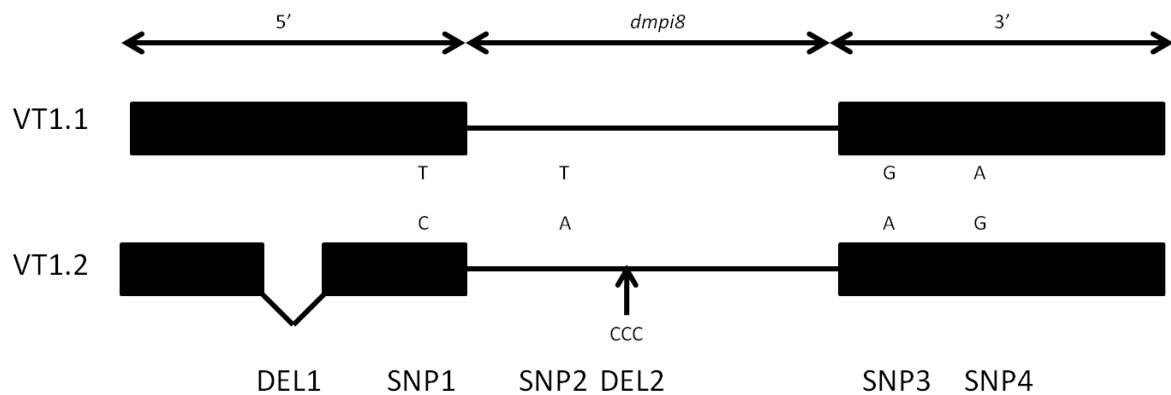
Splicing of the *per* 3'UTR was discussed in the Introduction as a method of seasonal adaptation in order to maximise the foraging time for insects during cold days and avoiding desiccation with a siesta at the height of the day when it is likely to be warmest (Collins et al., 2004, Majercak et al., 2004, Majercak et al., 1999). The variation involves an additional intron excised from *perB* termed *dmpi8* (Figure 5-1). Cheng and colleagues (1998) created transgenic flies in a *per*<sup>01</sup> background that carry a transgene of a splice-locked *perA* or *perB* variant or the wildtype *perG* that can express both the A and B isoforms. This splicing event is thermally regulated in *D. melanogaster* though in *D. yakuba* this is mediated by photoperiod, not temperature (Low et al., 2008). Higher *perB:perA* ratio is observed under cooler conditions (Majercak et al., 2004) implying that the splicing event may play a role in ‘colder’ phenotypes such as diapause which is to be investigated in this chapter.



**Figure 5-1 Exon diagram of the *per* spliceforms *perA* and *perB*. An additional intron – *dmpi8* – is excised in the *perB* 3'UTR.**

Low and colleagues (2012) discovered, through sequencing a number of wild *D. melanogaster* samples in North America, that two different haplotypes (Figure 5-2) for the *per* 3'UTR existed, differing by six polymorphisms, two deletions and four SNPs. While no clinal distribution was found for these polymorphisms, the investigation uncovered a role

for a third and fourth SNP, 3' to the *dmp18* intron making splicing more efficient. If splicing is more efficient, and occurs more often, these flies might potentially be better adapted to cooler conditions. Here, I extend the analysis of Low *et al.* to Europe in order to survey the variation that might exist in the *per* 3' region



**Figure 5-2 Diagram of the VT1.1 (wildtype) and VT1.2 (novel) haplotypes described by Low *et al.* (2012).**

### 5.1.1 Putative roles of microRNA in *per* splicing

One hypothesis for how the removal of this intron could influence seasonal behaviour is through the involvement of microRNAs. miRNAs were first described by Lee (Lee *et al.*, 1993) as a short non-coding RNA that influenced translation of another gene by complementary binding to the 3' UTR. Many miRNAs have been described, each with the function of regulating expression of - in some cases - thousands of genes through partial complementarity. It is possible to identify some of the miRNAs that can target *per* by using the available databases. Relative to the study of human and mouse miRNAs, fewer of these databases have records for *Drosophila* miRNAs. Dweep *et al.* (2013) reviewed a number of databases and software for their sensitivity and accuracy for predicting miRNAs and their target sites. The two primary issues that arise when studying miRNAs in *Drosophila* is the relative lack of bioinformatic tools, in addition to the fact that that miRNA/mRNA

interaction is still so poorly understood it makes prediction complicated and requires experimental confirmation (Betel et al., 2008, Dweep et al., 2013, Ebert et al., 2007, Jin et al., 2010, Kertesz et al., 2007, Krek et al., 2005, Vatolin et al., 2006). As well as testing for miRNAs that target an intron, in some cases, an intron can form a hairpin that would code for a miRNA and so the possibility that *dmp18* generates a miRNA was also tested

## 5.2 Aims

The work described in this chapter represents an attempt to study the potential effects of the *per* splice isoforms on diapause, another seasonal adaptation. To this end, the use of *per*<sup>01</sup> flies constitutively expressing specifically splice form A, splice form B or a transgene that can generate both are used to test whether these spliceforms have an effect on diapause.

In addition to this, flies carrying the VT1.1 and VT1.2 haplotypes were tested for their diapause induction and maintenance abilities by using flies carrying a transgene of each haplotype in the same *per*<sup>01</sup> background. The SNP variation around the splice site will also be investigated in European populations which are ancestral to their North American cousins to find evidence of. Finally, a bioinformatic study of the potential role for miRNAs in the *per* splicing pathway will be reported

## 5.3 Materials and Methods

### 5.3.1 Fly lines

The *per* splice transformants were acquired from Isaac Edery (Rutgers University, NJ). The artificial construct is inserted into *per*<sup>01</sup> flies. For each construct (A, B and control G) two

lines were used, A18, A29, B11, B12, G27 and G32 and *per*<sup>01</sup>. Flies were genotyped for *Is-tim/s-tim* and all were homozygous for the *s-tim* allele.

Additional flies were received from Isaac Edery (Rutgers University, NJ.). These flies were again, engineered in a *per*<sup>01</sup> background to carry either the VT1.1 or VT1.2 haplotype (Low et al., 2012).

### **5.3.2 Variations in the *per* 3' UTR in European populations**

For the population experiments on SNP variation, DNA samples were kindly donated by Dr. Valeria Zonato and Dr. Mirko Pegoraro (Leicester). All of the samples have been assayed to ensure they are *D. melanogaster* beforehand. All of the DNA samples used were from male flies, in some cases a single wild caught male was used and in others, a single male was selected from the first generation from captured isofemale lines. Males were used because they only carry one copy of the sex linked *per* gene which results in fewer complications when sequencing than using females that may carry two different *per* alleles.

As a proof of concept and to test the primers, a PCR was performed. For this, flies from the *Houten* lines were tested. A male each - from the *Is-tim* and *s-tim* lines - was used and a fly-squash DNA extraction performed (2.5.1) and a PCR performed using the forward primer *CCGCCAACACAAGGTATG* and reverse primer *GTTGGCGTTGGCTTTCG* in a 20µl reaction. The thermocycler would follow a standard protocol for PCR (outlined in 2.4.2)

Samples were diluted in 6x loading dye and run on a 1.2% agarose gel. Bands were cut from the gel and DNA purified from the gel using the Zymoclean Gel DNA Recovery Kit (Zymosearch, USA). Samples were sequenced according to the sequencing method described earlier (2.4.4).

After the initial test, further experiments were performed using males from 10 Zambian isofemale lines.

Ultimately, large numbers of samples were required to infer *per* 3' UTR variation within Europe. It was also important to select flies from a range of areas to establish if there was any evidence for a cline. For the full experiment, the same methodology was used for the PCR. However, the large numbers of samples makes using gel extraction difficult. For this, the ExoI/SAP method was used. This method, described by Werle *et al.* (1994) utilises exonuclease I (ExoI) and an alkaline phosphatase to digest PCR reagents without digesting the PCR products. ExoI (Thermo Scientific) was used in conjunction with FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific) in a ratio of 0.5:1 $\mu$ l per reaction. This was added to 5 $\mu$ l of sample and incubated at 37°C for 15 minutes followed by an 85°C denaturation for 15 minutes. After this, sample concentration was quantified using Nanodrop and all samples normalised to a similar concentration ~50ng $\mu$ l<sup>-1</sup> and 3.3 $\mu$ l of this was added to the sequencing reaction plate (end concentration ~11ng $\mu$ l<sup>-1</sup>) with 1 $\mu$ l of the forward primer (at 15mM) and 10.7 $\mu$ l of water and completed plates were sent to Eurofins for sequencing.

### **5.3.3 Diapause Experiments**

Diapause experiments were undertaken using the standard diapause experiments outlined in the general materials and methods section. The experiment using *per* splice locked flies was performed at LD 10:14 and 12°C. Experiments using the VT1.x haplotypes compared the lines at LD16:8 and LD8:16 to assess the influence of photoperiod on diapause.

### **5.3.4 MicroRNA targeting of the *per* 3' UTR a computational study**

This project involved bioinformatic analysis of likely target sites and using programs to find likely miRNAs. We start off at a disadvantage with the *Drosophila* model as many programs that are available for such analysis are geared around the use of mammalian models and so human and mouse miRNA databases are considerably more advanced. The primary problem with miRNA analysis is that no one is entirely sure how they work in a model system and predictions must be backed up with experimental evidence (Betel et al., 2008, Dweep et al., 2013, He and Hannon, 2004, Jin et al., 2010, Lewis et al., 2003, Ørom and Lund, 2010, Yang et al., 2008). The algorithms consider a variety of factors when calculating likelihood of interactions including a BLAST method to score complementarity, but also considers free energy values of mRNA folding, i.e. how accessible the putative target site is.

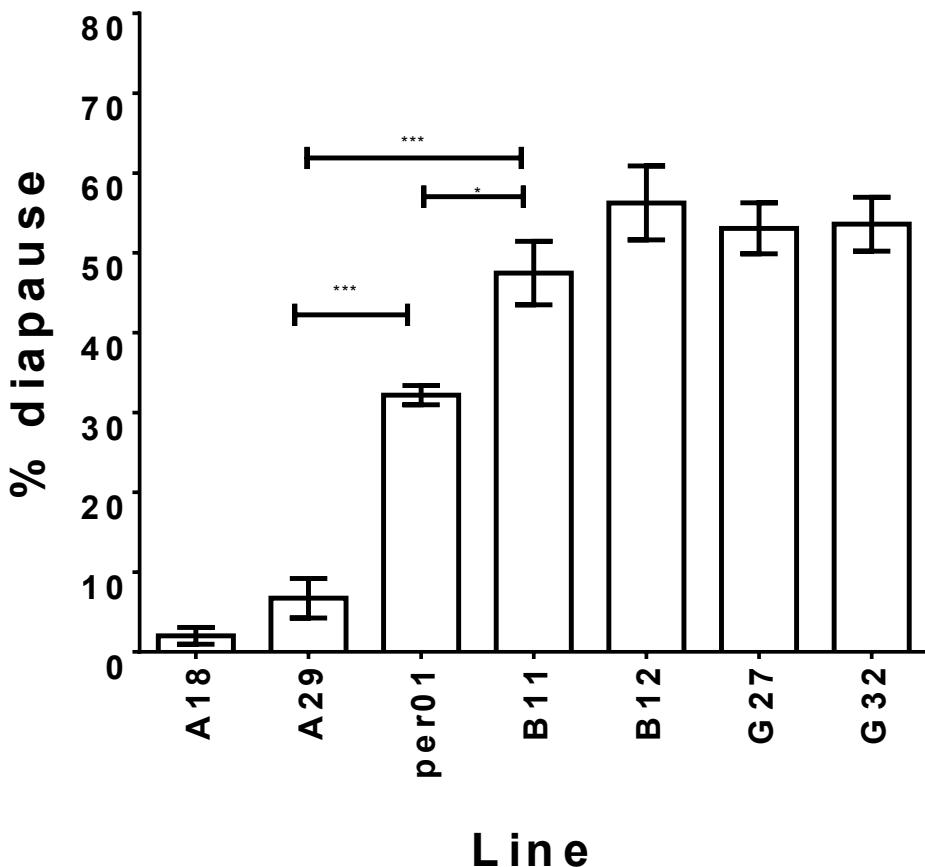
One database that gives information on *Drosophila* miRNAs is microRNA.org (Betel et al., 2008) which has a database of *Drosophila* mRNAs and miRNAs and predicts the putative binding sites. In addition to the factors outlined above, microRNA.org also considers how well conserved an interaction is when awarding a score and PicTar (Krek et al., 2005) works in a similar manner. Both operate as a database of potential interactions, but this is not always an ideal method. PITA (Kertesz et al., 2007) operates in a different manner, allowing users to input their sequence (or sequences) of interest and compare them to a selected miRNA (or miRNAs), in a similar manner to RNAHybrid (Rehmsmeier et al., 2004).

For the purposes of this project, each of the methods outlined above were tested and the most likely results were considered for future investigation.

## 5.4 Results

### 5.4.1 Diapause in flies only expressing *perA* or *perB*

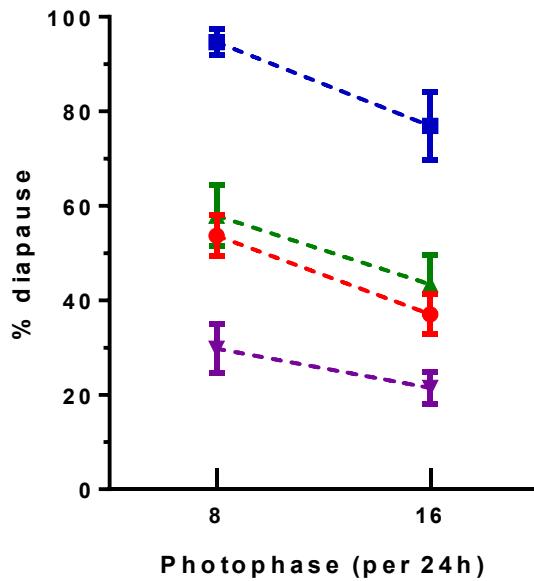
remarkably, the results indicate that flies locked into expression of *perA* only, suffer a dramatic block to diapause induction in 12°C LD10:14 ( $F=54.7$ , DoF=6  $P<0.0001$ , see Figure 5-3). The mean level of diapause in these lines is less than 5%. *perB* flies exhibit a similar level of diapause to the control *perG* flies, averaging at approximately 55% between the two lines, *perG* the same. *per<sup>01</sup>* flies exhibit an intermediate diapause between *perA* and *perG* lines, approximately 33%. This value is markedly lower than Saunders *et al.* (1989) achieved (~60% LD 10:14 12°C), but is in line with a result achieved more recently in this laboratory indicating a level of approximately 30% (LD 8:16 12°C) (Gesto, 2010). Tukey's multiple comparisons test (Appendix F) showed that *per01* exhibited significantly lower diapause levels than *perB* and *perG* lines while being significantly higher than the *perA* lines (P values ranging from <0.02 to 0.0001).



**Figure 5-3 Comparison of diapause levels in *per* splice locking lines (LD 10:14 12°C). Mean  $\pm$  SEM, n=11 replicates for each line.**

#### 5.4.2 Diapause in *per* 3'UTR natural variants

When lines were *tim* genotyped, the VT1.1a line was shown to be *ls-tim* homozygous while the other lines were all *s-tim*. Sequencing confirmed that each line carried the relevant *per* allele. Two-way ANOVA of diapause revealed significant photoperiod ( $F(1,63) = 14.05$ ,  $P=0.0004$ ) and line ( $F(3, 63)=4$ ,  $P<0.0001$ ) effects, but no interactions ( $F(3, 63) = 0.3419$ ,  $P=0.8$ ). Tukey's multiple comparisons indicated that VT1.1a had a high level of diapause in both long and short day compared to VT1.1b ( $P<0.0001$ ), similarly, VT1.2a exhibited a higher level of diapause than VT1.2b ( $P<0.001$ ) (Figure 5-4).



**Figure 5-4 Photoperiod response in *per* UTR variants. Comparing long (LD16:8) and short (LD8:16) photoperiods at 12°C in VT1.1a (red), VT1.1b (blue), VT1.2a (green) and VT1.2b (purple). Mean  $\pm$ SEM Replicates by line 8:16/16:8 VT1.1a (n=9/9) VT1.1b (8/9) VT1.2a (5/11) VT1.2b (9/11)**

### 5.4.3 Variation of the *per* 3'UTR in Europe

#### 5.4.3.1 Initial tests with Houten and Zambian flies

The initial testing with *per* 3' UTR primers on the Houten flies showed signs for variation in the 3' UTR in European flies. Aligning the results of the *ls-tim* and *s-tim* flies gave the same result for both. Aligning these to the haplotypes described by Low et al. (2012) however indicated additional variation within European flies (Appendix D). Flies carried some aspects of VT1.1 and some of the VT1.2 haplotypes as well as some unique SNPs not described previously

Ancestral populations from isofemale lines from Zambia provide an interesting comparison. The sequencing results from 10 Zambian isofemale lines (Appendix D) showed some of the variations exhibited in the VT1.2 and VT1.2 haplotypes, but not all. Flies were all VT1.1 type for DEL1, but all VT1.2 type for DEL2 and SNP4. Other VT1.2 variations were present in

varying degrees - SNP1 (6/10), SNP2 (1/10) and SNP3 (8/10). All four novel variations found in *Houten* flies (Appendix D), were all present in Zambian populations to varying degrees and an additional 11 SNPs unique to Zambian flies.

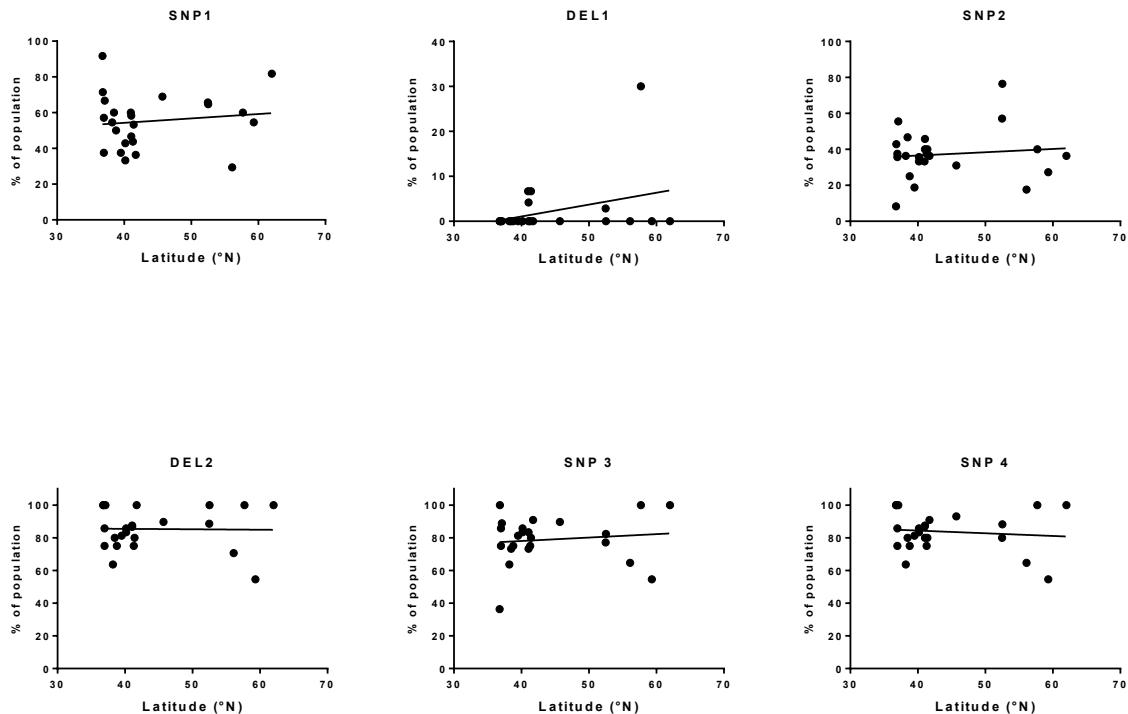
#### **5.4.3.2 Variation in a European-wide screen**

Results from a wider screen in Europe yielded a large amount of data. In total, 355 samples gave data covering 426bp of sequence. Data beyond 426bp was removed as there was a lot of noise in a number of samples and poor sequence. The samples covered areas of Scandinavia, England, Northern Italy and Spain. Using DnaSP (Librado and Rozas, 2009) Tajima's D-test of the sequences indicated that there was no significant selection evident ( $D=-1.1 P>0.1$ ) (Tajima, 1989). Tajima's test is often considered too conservative for analysis (Simonsen et al., 1995) so Fu and Li's statistic (Fu and Li, 1993) was used with the same result ( $D=-0.11, F=-0.67 P>0.1$ ). Haplotypes were calculated using DnaSP (Librado and Rozas, 2009) and Arlequin (Excoffier and Lischer, 2010) and aligned with comparison to VT1.1 and VT1.2 (Appendix E). A total of 24 distinct phenotypes were described from the sequencing data. These haplotypes were ordered by prevalence in the regions tested and the SNPs and deletions that were common were assessed for clinal variation in their distribution as well as the distribution of the haplotypes.

Looking at the SNPs and deletions described by Low (2012) first, there was evidence for the presence of each of them in Europe (Figure 5-5). The DEL2, SNP3 and SNP4 variations – all of which occur in *dmp18* or 3' of it – were very common in Europe (86%, 80% and 84% respectively). DEL1 however is rare (2%) and SNP1 and SNP2 are reasonably common (57% and 39%). None of them showed significant latitudinal clinal distribution by linear regression

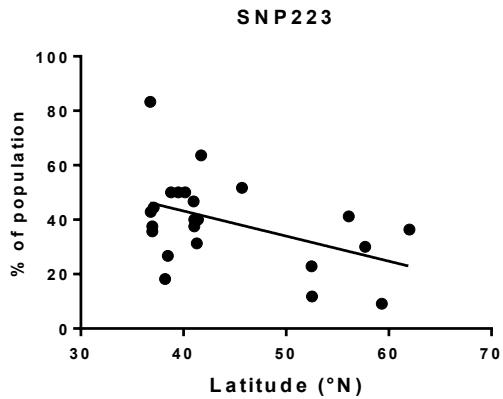
(SNP1 P=0.55, DEL1 P=0.11, SNP2 P=0.62, DEL2 P=0.94, SNP3 P=0.5893 and SNP4 P=0.62).

Indications are that SNP3 and SNP4 are both typically inherited together with 79% of the flies carrying both SNPs and only 6% carrying one of SNP3 and SNP4, but not both, supported by DNAsp linkage disequilibrium tests ( $P<0.0001$ ) (Librado and Rozas, 2009).



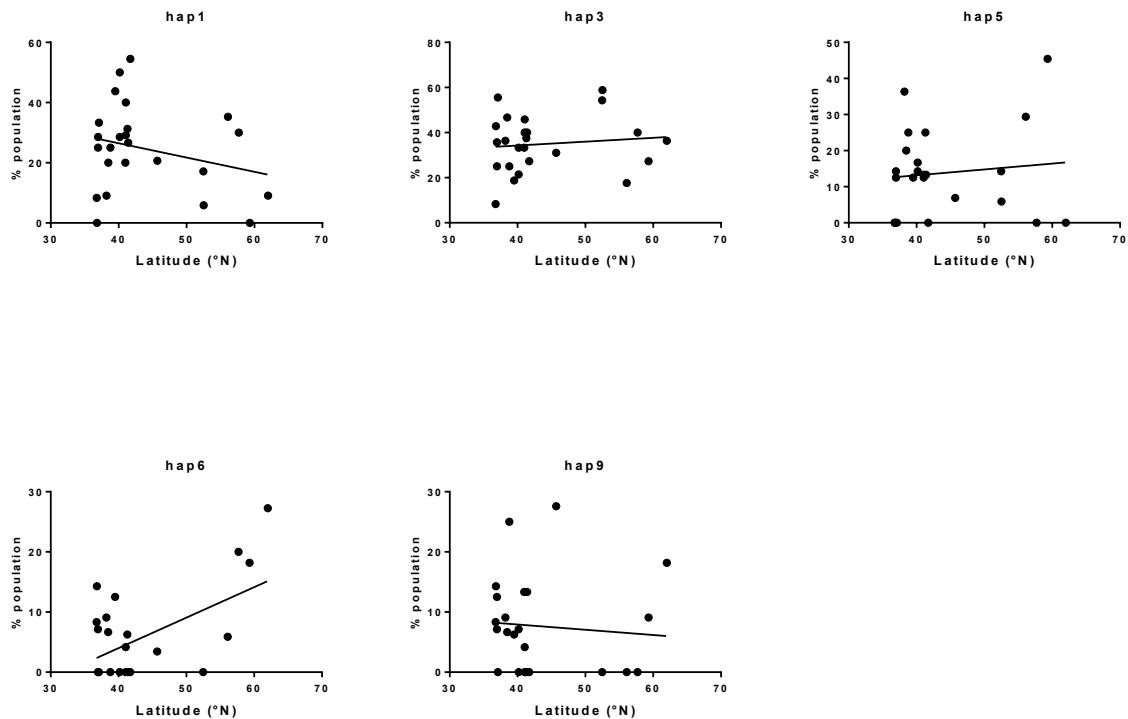
**Figure 5-5 Spatial distribution of the SNPs and deletions described by Low *et al.* All of the variants are found in European populations but there is no obvious clinality in their distribution.**

As mentioned previously, a small screen of flies from Zambia indicated that a SNP was common (90% prevalent n=10) that was not seen in the VT1.1 or VT1.2 haplotypes. The SNP (referred to henceforth as SNP223) was prevalent within populations across Europe (38%), less common than in the Zambian test (90%). The distribution of this SNP showed a clinal distribution across Europe using linear regression (Figure 5-6) indicating that this thymidine/adenosine substitution is more common in the south than it is in the north of Europe)



**Figure 5-6 Linear regression of the presence of SNP223 in Europe.  $R^2=0.2107$   $P=0.024$ . Indicates a higher presence in the South of Europe than in the North**

While 24 haplotypes were discovered, five of these made up 87% of the samples, many of the haplotypes being found in only a few samples. Hap1, Hap3, Hap5, Hap6 and Hap9 (Appendix E) were the most common within the samples tested (24%, 36%, 13%, 6% and 8% respectively). Linear regression (Figure 5-7) indicated that of these only Hap6 showed a clinal distribution ( $R^2=0.2854$ ,  $P=0.0072$ ). It is worth noting that it is only present in 21 samples (~6% of the population), but is present in Højbjerg (6%), Stockholm (18%), Korpilahti (27%), Gothenburg (20%) in Northern European populations, but present as very small percentages in 8/19 Spanish populations and Treviso. Korpilahti, Stockholm and Gothenburg represent the three most northern populations sampled.



**Figure 5-7 Linear regression of the most common haplotypes in Europe. Haplotype 6 shows a latitudinal distribution with increased prevalence in the North ( $R^2=0.2854$   $P=0.0072$ ) though it represents only 6% of all samples.**

Distribution of the most commonly occurring haplotypes by country (Figure 5-8) indicates the frequency that some of the alleles have in different regions. Hap6 indicates the most striking variation in distribution, common only to Scandinavia, though there is little else clear from these results. A breakdown of the samples by origin is shown in Table 5-1.

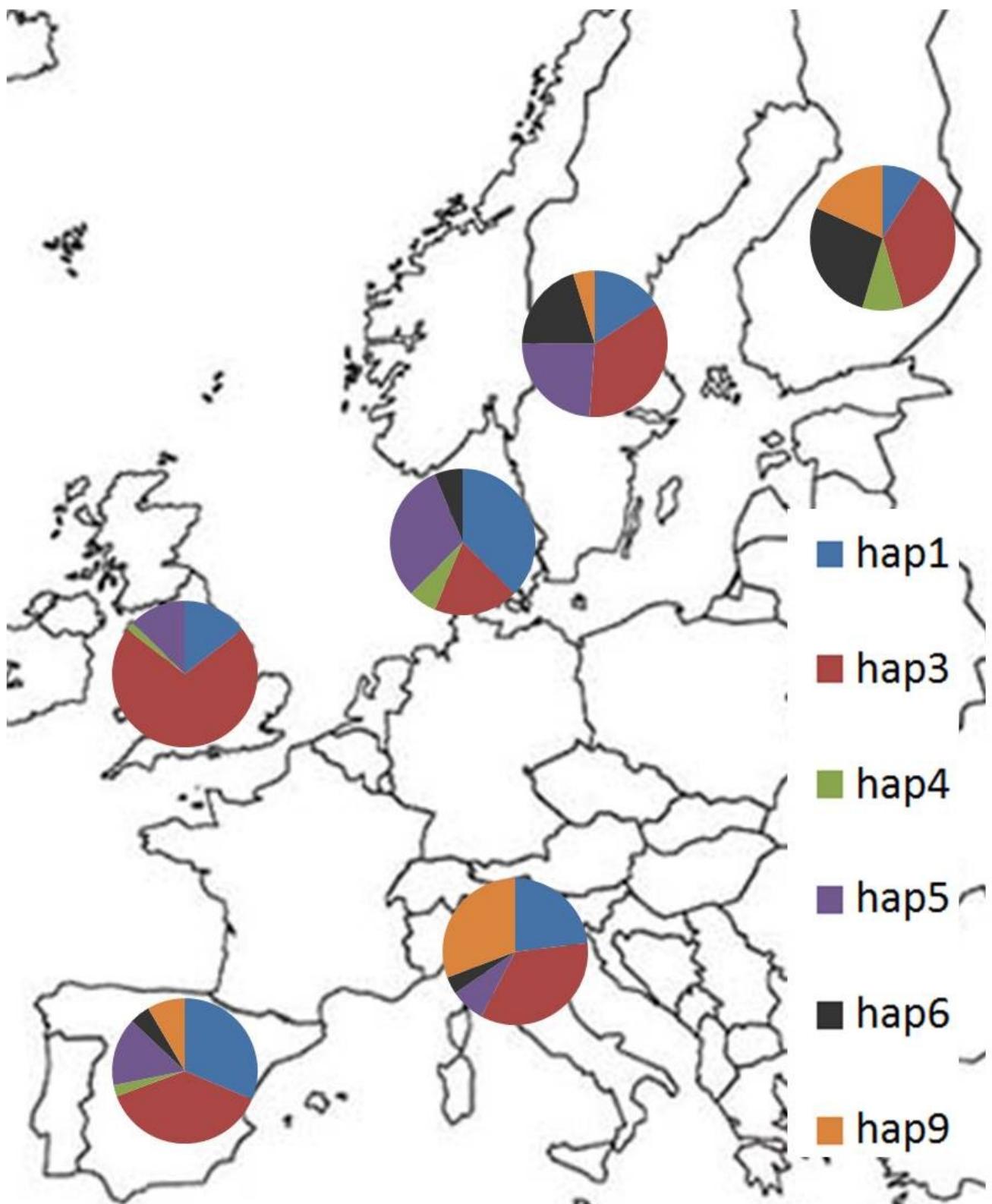


Figure 5-8 Map showing the distribution of the seven most common haplotypes across Europe by country.

**Table 5-1 The frequency of the most frequently occurring haplotypes by latitude within the countries sampled.**

Country	Latitude	n	Haplotype Frequency					
			hap1	hap3	hap4	hap5	hap6	hap9
Spain	36.77	12	8.3	8.3	0.0	0.0	8.3	8.3
	36.82	7	0.0	42.9	14.3	0.0	14.3	14.3
	36.97	8	25.0	25.0	0.0	12.5	0.0	12.5
	36.97	14	28.6	35.7	0.0	14.3	7.1	7.1
	37.1	9	33.3	55.6	0.0	0.0	0.0	0.0
	38.21	11	9.1	36.4	0.0	36.4	9.1	9.1
	38.48	15	20.0	46.7	0.0	20.0	6.7	6.7
	38.78	4	25.0	25.0	0.0	25.0	0.0	25.0
	39.49	16	43.8	18.8	0.0	12.5	12.5	6.3
	40.16	14	28.6	21.4	14.3	14.3	0.0	7.1
	40.16	6	50.0	33.3	0.0	16.7	0.0	0.0
	40.99	15	20.0	33.3	0.0	13.3	0.0	13.3
	41.05	15	40.0	40.0	0.0	13.3	0.0	0.0
	41.05	24	29.2	45.8	0.0	12.5	4.2	4.2
	41.31	16	31.3	37.5	0.0	25.0	6.3	0.0
	41.42	15	26.7	40.0	0.0	13.3	0.0	13.3
	41.73	11	54.5	27.3	9.1	0.0	0.0	0.0
Italy	45.71	26	20.7	31.0	0.0	6.9	3.4	27.6
UK	52.48	35	17.1	54.3	2.9	14.3	0.0	0.0
	52.53	17	5.9	58.8	0.0	5.9	0.0	0.0
Denmark	56.11	17	35.3	17.6	5.9	29.4	5.9	0.0
Sweden	57.7	10	30.0	40.0	0.0	0.0	20.0	0.0
	59.33	11	0.0	27.3	0.0	45.5	18.2	9.1
Finland	62.02	22	9.1	36.4	9.1	0.0	27.3	18.2

#### **5.4.4 Micro RNA targeting in the 3' UTR of of *per* splice variants.**

Results from the screens for miRNA targets in *per* are outlined in Table 5-2. The only miRNAs that are confirmed by more than one method is *mir-276a* and *mir-276b*. The score given by microRNA.org is higher than the cut off of -1, but evidence for targeting from PITA suggests they may have a reasonable chance of binding. *mir-932* is the only significantly likely miRNA described by microRNA.org, but its target falls outside of the *dmp18* intron, meaning its target is unlikely to be influenced by splicing. The PicTar database is an example of the difficulty of predicting miRNAs in *Drosophila* as *per* was not found in their database at all.

PITA indicates that *mir-278* is a strong candidate for a miRNA that targets the *per* intron which may be influenced by splicing, but there is no indication using other methods that this may be the case

**Table 5-2 Predictions of miRNAs targeting the *per* 3'UTR.**

Software	miRNAs Predicted	Score (perA)	Score (perB)	Comments	Cut-off score	
microRNA.org	276a	-0.4273	N/A	-1 usually used as cut off, but both 276a and 276b target the intron	<-1	
	276b	-0.4273	N/A			
	932	-1.1335	N/A	3' of <i>dmp18</i>		
PITA	278	-15.49	N/A	Targets the <i>per</i> intron	<-10	
	iab-4-5p	-12.29	-12.75	lower score in the spliced form		
	375	-11.07	-11.07			
	954	-10.73	-10.48			
	34	-10.65	-10.54			
	276a	-10.54	-0.66	more reassuring score than with microRNA.org		
	276b	-10.54	-0.66			
PicTar	<i>per</i> not found in the database					
RNAhybrid	1015	0.06	0.04	More likely binding with <i>perB</i>	<0.05	

The tests using MiPred to find potential for *dmp18* to act as a pre-miRNA indicate that it is unlikely that the intron forms a hairpin to synthesise miRNAs (Jiang et al., 2007).

## 5.5 Discussion

The results using *per<sup>0</sup>* flies confirm earlier findings (Gesto, 2010, Saunders, 1990, Saunders et al., 1989) that expression of functional PER is not essential for diapause induction. Although lower levels of diapause were indicated here and by Gesto (2010) there is still evidence that some flies can induce diapause. These results do however suggest that splice variant B is critical for diapause. As we already know that splice variation in *per* influences

the siesta under warmer and cooler temperatures (Cheng et al., 1998, Collins et al., 2004, Low et al., 2008, Majercak et al., 2004), the results offer a possible role linking the circadian clock to diapause through a shared thermal sense. How splice variants that do not alter the protein coding may influence the phenotype is unclear. Splicing in untranslated regions is rare, especially in insects, with an estimated 5-6% of all metazoan genes anticipated to exhibit binding in the 3'UTR (Hong et al., 2006) and trying to predict how or why it might happen is difficult. In a number of cases, it has been shown that 3'UTR splicing can make mRNA less stable, for example or the *TCRζ* gene which becomes destabilised after splicing in the 3' UTR which leads to a break down in its complex formation with CD3 (Tsuzaka et al., 2003). However, in other cases, the splicing event can help stabilise a gene as described by Sandberg *et al.* (2008) observing that T-cells splice a number of genes in the 3'UTR when proliferating, and forcing cells to express full length mRNAs (blocking the splicing) results in poor proliferation as the mRNA is degraded.

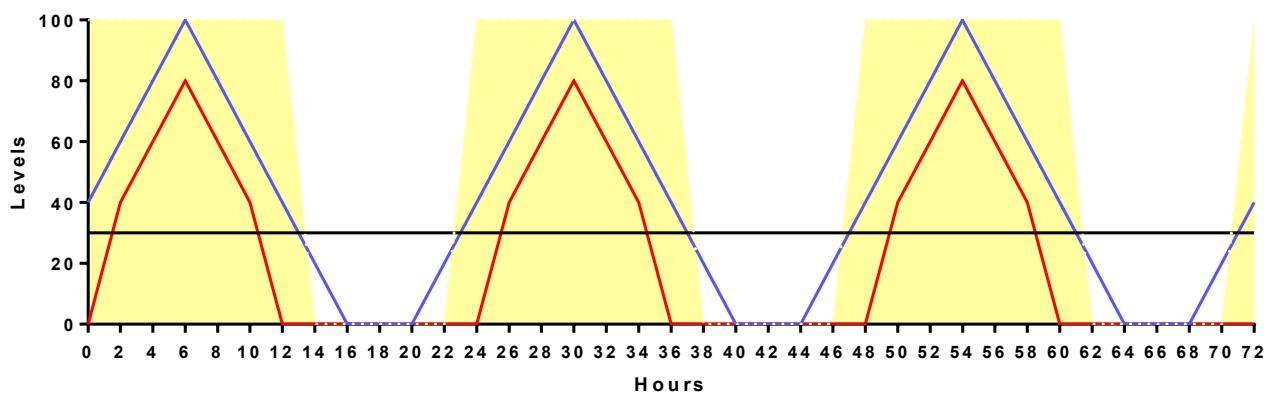
Explaining the phenomenon is difficult, but one possible explanation comes from a similar event observed in the human gene *PER2*. In this case, a SNP in the 5' UTR of the gene confers no structural protein change, but alters the shape of the mRNA, resulting in the "lark" phenotype, a tendency towards morningness (Carpen et al., 2005). Reviewing this data, the authors suggested the altered shape allowed more efficient translation into protein and faster accumulation of PER2 protein in the nucleus, as implied in the *per* splicing of the fly siesta behaviours. However, previous work has shown that *per*<sup>01</sup> flies, which produce no functional protein, do maintain some level of diapause so functioning *per* is obviously not required for diapause. This implies that *per*<sup>B</sup> may not be necessary for diapause, but *per*<sup>A</sup> is required to prevent it.

A further explanation may lie in the nonsense mediated degeneration (NMD) system. NMD is a defence mechanism that exists in cells to prevent expression of proteins that may be harmful to the cell (Hentze and Kulozik, 1999). In the case of introns that are found in the 3'UTR, it is thought that this mechanism is able to recognise the splice sites for the intron and the NMD system *assumes* that the stop codon is in the wrong position and targets the mRNA for destruction. This offers a neat explanation for my results. Theoretically, as the temperature is reduced, splicing is increased and the NMD recognises this as a signal to block expression, switching off the clock and entering diapause, a theory that would fit the circadian damping effect described in section 1.3.2. This theory has two problems. First, results with *per*<sup>01</sup> flies show that a loss in functional PER does not prevent diapause. In addition, these experiments utilise flies that artificially splice *per*. Because of this, there is no splice complex for the NMD system to recognise. Therefore it can be concluded that the product of the splicing event and not the act of splicing has an effect on diapause. This is different to the results achieved by Majercak (1999) which showed that the act of splicing is necessary to induce the siesta behaviours. This could be influenced by miRNAs. Results from bioinformatics screens indicate that *perB* and *perA* are potentially prone to degradation from miRNAs and some of these may influence the two transcripts differently. Of particular interest would be those that target the intron as a suppression of *per* when only *perA* is expressed. There was no evidence however that the *dmpi8* intron may itself form a miRNA to influence other genes through modulated splicing.

Ideally, more experiments would provide a better view of what is happening with these flies. Testing a range of photoperiods as Saunders had done previously would give a better indication of any influence that the variants have on photoperiod. Any differences in critical

day length may indicate a role for photoperiodism effects of splicing and how the splice variation influences – or is influenced – by the clock.

To try and understand why mutants like *per*<sup>01</sup> can still diapause while implying that the clock still has some involvement, it is important to consider how these disruptions effect downstream gene expression. Visiting the old adage, “even a stopped clock tells the right time twice a day” it is important to remember that losing a functional clock does not cease activity or the downstream gene expression in an organism, it merely loses its ability to maintain rhythmic activity. The concept of a broken clock telling the time right twice per day could actually come in useful when considering clock mutants that diapause. The action of the clock is not just to elevate, but to suppress and if, over the course of a day, an important protein for photoperiodic response is expressed, albeit at lower levels, it will still result in a mild response (Figure 5-9).



**Figure 5-9 The broken clock hypothesis.** A protein is expressed in a rhythmic manner and if its profile fits the blue line, with some expression early in the night phase, diapause is induced. If it fits the red profile with expression only in the light phase, diapause is not induced. An arrhythmic profile (black) would see some expression during dark phases, signalling diapause inducing conditions.

### **5.5.1 Variation of the *per* 3' UTR**

There is considerable variation within the 3'UTR of *per* in Europe and without further experiments, one can only speculate on the importance of these SNPs. Low (2012) indicated that the role of two SNPs 3' of the *dmp18* intron was to increase the likelihood of splicing. A screen across Europe indicated that both of these SNPs are very common. Similarly, DEL2 which occurs in the intron is also common. SNP223 is the only SNP shown in this study to have a significant latitudinal cline. It was discovered initially in Zambian flies and was not found in Low's USA haplotypes, but here it is shown to be increasingly rare in Northern Europe and more common in Spain. This might indicate that there is a disadvantage to carrying this SNP in the north, but further evidence for a role and the presence of the SNP in northern clines would be required. Of the haplotypes defined in this study, only one produced a significant latitudinal cline, being more common in the north than the south. Primarily, this is down to the lack of SNP223 in the haplotype. Numerous novel SNPs were identified in this survey, but there was little evidence for selection. Further investigation of SNP223 may be interesting, particularly to see if it has any influence on splicing efficiency or diapause as its position 3' of *dmp18* means it is in a similar location to SNP3 and SNP4.

It was indicated here that the two haplotypes described by Low (Low et al., 2012) may have an influence on the ability of flies to enter diapause under photoperiodic induction. The results indicated a weak, but significant difference between the effect of photoperiod between lines carrying VT1.1 and VT1.2. Further investigation into this would be interesting and reproducing the photoperiod response curves produced by Saunders (1989) might reveal changes in the critical day length as well as other differences in photoperiodic influence of diapause. Ensuring that the P-elements are inserted into the same background

is important and this would give more consistent results as the levels of diapause between lines fluctuated demonstrably within these four lines. Comparing the effect of the haplotype in different *tim* genotypes might also be of some interest to see if there may be some influence of *ls-tim* and *s-tim* over the phenotypes.

## **6 Ancestral origins of diapause in *D. melanogaster* and related species**

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### **6.1 Introduction**

*Drosophila melanogaster* was first identified from samples obtained in Europe, but its origins are in Sub-Saharan Africa (Lachaise et al., 1988, Lachaise et al., 2004). An important question remains over how *Drosophila* have adapted from tropical regions with little seasonal variation in temperature and photoperiod to the variable northern latitudes where the temperature and conditions swing wildly within the space of a year. On the one hand is the suggestion that *Drosophila* have adapted in Europe to recognise oncoming winter conditions and prepare for them by entering diapause (Tauber et al., 2007, Saunders, 2008, Saunders, 2009, Saunders et al., 2002, Schmidt et al., 2005a). On the other is the idea that *Drosophila* already had the machinery to survive harsh weather conditions in Africa and that this machinery was later utilised to adapt flies to Europe and the rest of the world (Pegoraro et al., submitted).

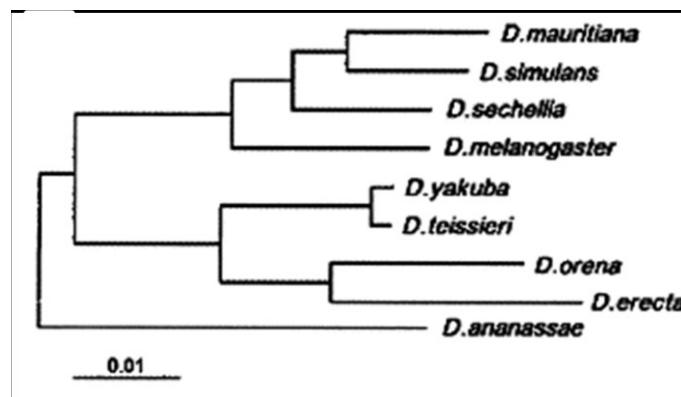
Pegoraro and colleagues tested a number of *D.melanogaster* populations originating from America, Africa and Europe, as well as the sibling species *D. simulans* from Italy and Africa. They analysed the different responses to long and short day photoperiods (LD 16:8 and 8:16) over the course of a long 28 day and short 12 day experiment. What they observed was a propensity to diapause across all the populations tested, even those from Africa. Some responses were stronger than others, but the results indicate an ancestral basis for diapause. They hypothesised that diapause was initially a stress response evolving to wet and dry season conditions in Africa. It is not unusual to see invertebrates under stress halt

reproduction or even reabsorb the ovaries when mounting an immune response (Hopwood et al., 2001), when starved (Isabel et al., 2005, Ward and Dixon, 1982) or dehydrated (Crawford and Warburg, 1982). So the diapause response may be a reaction to an initial stress of cold weather and the remission effect observed in chapter 4 occurs if conditions do not worsen – as they do when the animals are held at diapausing threshold constant of 12–13°C.

The concept of diapause is a contentious subject in *Drosophila*. The idea that *Drosophila* exhibit a diapause is supported by the work presented earlier in this thesis (Chapter 4) showing that the response of *Drosophila* to changing conditions is to remain in a state of suspended development until the conditions improve. The Nanda-Hamner protocol used by Saunders and colleagues (1990) shows unequivocally that the insects are responding to the photoperiod rather than the temperature alone as a stimulus, representing a real photoperiodic diapause, although this has been disputed. As a commensal organism, *D. melanogaster* has followed human agricultural activity for thousands of years (Bellen et al., 2010, Keller, 2007). This is not a unidirectional relationship and so inevitably, fruitflies from Europe have been carried back to Africa resulting in some African populations that show high levels of admixture with European populations (Pool et al., 2012). If diapause evolved in Europe, then admixture could carry the phenotype back into Africa. Some populations from Africa have been sequenced and some of these populations, particularly those from Zambia, show low levels of admixture, for example the ZI isofemale lines whose admixture is as low as 1.4% (Pool et al., 2012). The importance of this is that these flies would represent a more ancestral genome and would presumably be better adapted to African environments. This makes ZI low admixture populations an excellent opportunity to study

the diapause of ancestral *D. melanogaster* to investigate the evolution of photoperiodic diapause.

As mentioned in Chapter 4, *D. simulans* has also been tested for diapause by Pegoraro and colleagues (submitted) and in this thesis. However, this species still represents a cosmopolitan species that has colonised Europe and may have been reintroduced to Africa from Europe. In addition to studying “ancient” Zambian *D. melanogaster*, some of the other species from the *D. melanogaster* subgroup are exclusively tropical such as *D. sechellia* and *D. yakuba*. Within the *D. melanogaster* subgroup phylogeny (Figure 6-1) *D. melanogaster* inhabits a central position on the tree. This means it is possible to compare *D. melanogaster* against very closely related species such as *D. sechellia* or the more distant species including *D. yakuba*, *D. erecta* and even *D. ananassae* which represents a separate subgroup within the *D. melanogaster* group (Kastanis et al., 2003) so another aim in this experiment was to consider the possibility that exclusively African species also exhibit diapause.



**Figure 6-1 Molecular phylogeny of the *D. melanogaster* subgroup with *D. ananassae* considered a separate subgroup within the *D. melanogaster* species group (Kastanis et al., 2003)**

As well as diapause, another temperate adaptation tested in the laboratory is cold tolerance. The testing of this phenomenon is varied with some groups testing survival of

flies after a period at low temperatures (Schmidt et al., 2005b) while others keep flies at a low temperature for a period of time and measure how long it takes for flies to return to their feet (Jean David et al., 1998, Gibert et al., 2001, Anderson et al., 2005, Schmidt et al., 2009). An observation common to these studies is that flies collected from temperate clines react better to chilling. High diapausing lines have been shown to better survive periods of chilling (Schmidt et al., 2009, 2005b) which makes the study of chill coma response an opportunity to assess how well a line is adapted to temperate conditions. Tropical species have been shown to have a poor chill coma response (Gibert et al., 2001) and so using this method to study a number of lines in this study may shed further light on the link between diapause and chill coma response.

## 6.2 Materials and Methods

### 6.2.1 Diapause Experiments

Diapause experiments were undertaken as described previously (section 2.3). Two incubators were used, one set up for a constant condition 12°C LD 8:16 to represent short days and the other at 12°C LD 16:8 to represent long days. As before, flies were placed in vials in the incubator and tested for diapause after 12 or 28 days under the two separate light regimens. Fly lines came from several sources. Dr. John Pool (University Wisconsin) donated 20 lines of *D. melanogaster* Zambian flies with 1.4% admixture and 10 were used for analysis, (ZI 514, 210, 458, 527, 395, 465, 196, 505, 129 and 291). *D. yakuba* were represented by three isofemale lines. *tai18e2* was captured in the Taï forest between Liberia and the Ivory coast (1983), 115 was collected in the Cameroons (1973) (both kindly donated by Glynnis Johnson, University of Cambridge) and UCSD Yak was collected from the Ivory Coast (1955) (purchased from DSSC, California). Other species were purchased from the

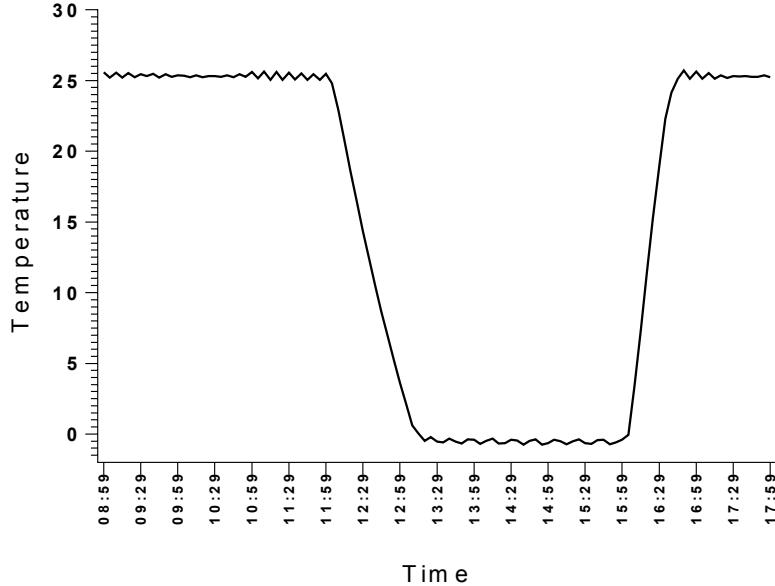
DSSC including two isofemale lines of *D. ananassae* (“An.26” (Rwanda 2005) and “An.19” (Benin 2004)), two lines of *D. sechellia* (“Sech.03” and “Sech.29” (both from Cousin Island in 1980 and 1985)) and finally a line of *D. erecta* (No information given “wildtype”). All stocks were kept as outlined previously for *D. melanogaster*.

### **6.2.2 Chill Coma**

One thing notable throughout the literature of chill-coma experiment is that the methods used vary quite significantly from paper to paper, making it difficult to remain consistent with other published work. The Mir-254 incubator, which can be set up to cause a cold shock at 0°C and the Trikinetics DAM activity monitors makes a high-throughput testing method possible. The Trikinetics monitors can be used to measure the first bout of activity elicited by the flies after chill coma, meaning that they do not have to be watched and timed individually, but also relative to the typical method of keeping flies on ice, the temperature can easily be monitored throughout an experiment. One drawback to this method is that the incubator takes time – ~1h – to drop to 0°C from 25°C and another hour to get back up again (Figure 6-2), whereas chill-coma is more of a shock response. For the purposes of this additional investigation however, it may be useful to test this more automated and hands-off approach for the purposes of investigating chill-tolerance.

Only female flies were used. They were collected using carbon dioxide anaesthesia within a few hours of emergence and placed into the DAM activity monitor for two days at 25°C LD 12:12 to entrain. Previous experiments with *D. ananassae* showed that after a 4h chill-coma, flies would typically recover after 25 minutes (Gibert et al., 2001), though the method used previously was to count the time for flies to return to their feet rather than a period of inactivity. Using the time to first activity would probably take longer after a chill and so

longer periods of chilling were avoided. Data from the Trikinetics system was analysed to find the first movement to the nearest minute after the temperature began to rise.



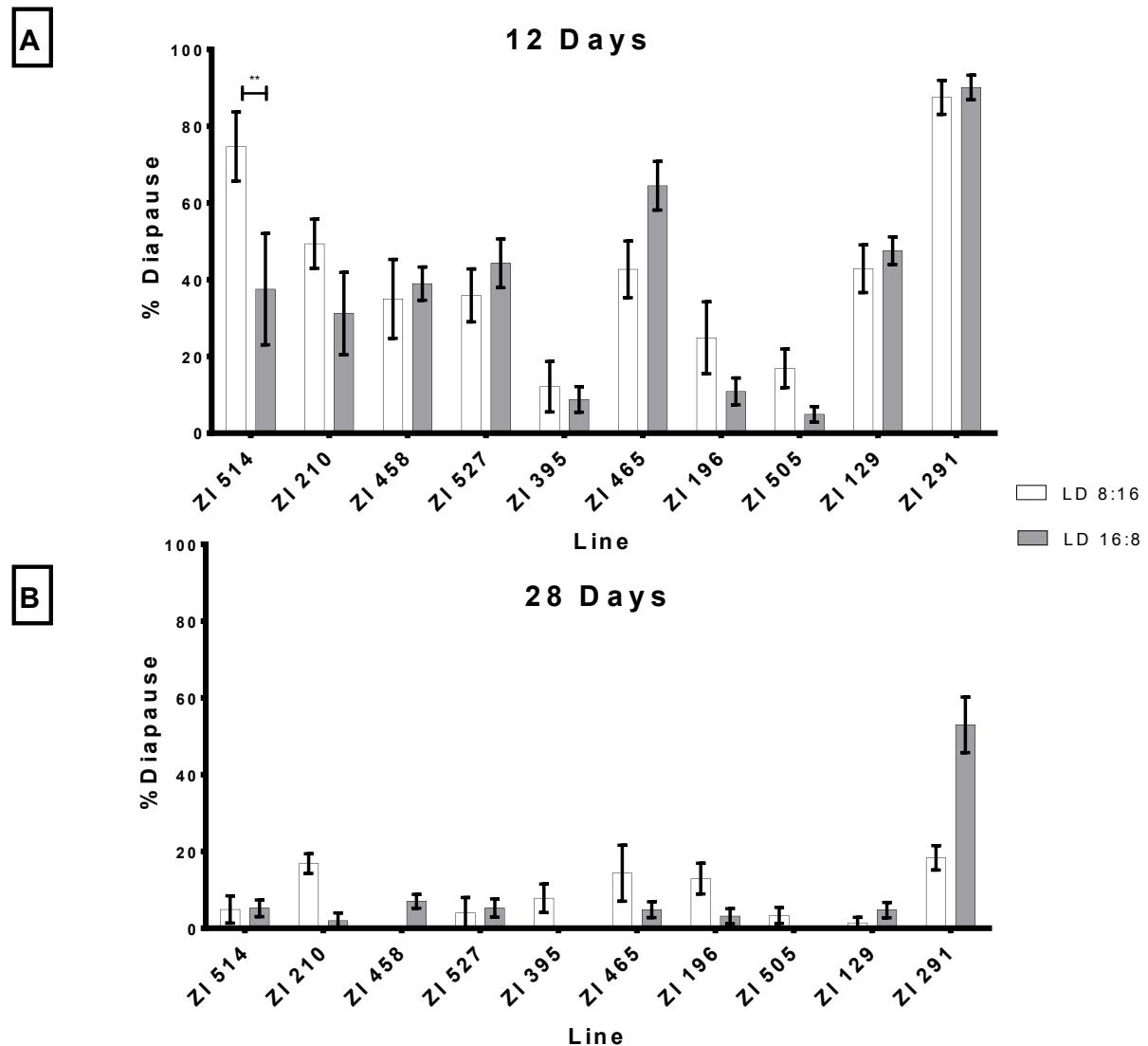
**Figure 6-2** Graph produced from a temperature logger showing the course of the temperature in an experiment from 08:59 to 17:59. At 12:00, the temperature begins to drop, but does not reach 0°C until ~13:09. Similarly, at 16:00, the temperature begins to climb but does not reach 25°C again until ~16:44

## 6.3 Results

### 6.3.1 Diapause in ancestral *D. melanogaster*

The results indicate that diapause is common in the Zambian “ancestral” lines. Of the 10 isofemale lines tested, all exhibit some level of diapause at 12 days under both photoperiods (Figure 6-3 A). After 28 days (Figure 6-3 B) diapause levels were significantly lower (Table 6-1) indicating the remission effect observed in Chapter 4. ZI 291 was the strongest diapausing line with a mean diapause level of 87.5%/90.1% (LD 8:16/16:8). In short days this figure drops significantly to 18.4% after 28 days, however, the level remains

higher in long days at around 53%. The lowest diapausing of all the lines tested are ZI 395 (12.2%/8.8%), ZI 196 (24.9%/10.9%) and ZI 505 (16.9%/4.9%).



**Figure 6-3 Effects of long/short day on diapause in 12 (A) and 28 (B) day experiments. The effect of short (white bars) and long (grey bars) photoperiods on diapause. Mean  $\pm$ SEM, n=5 replicates per experiment**

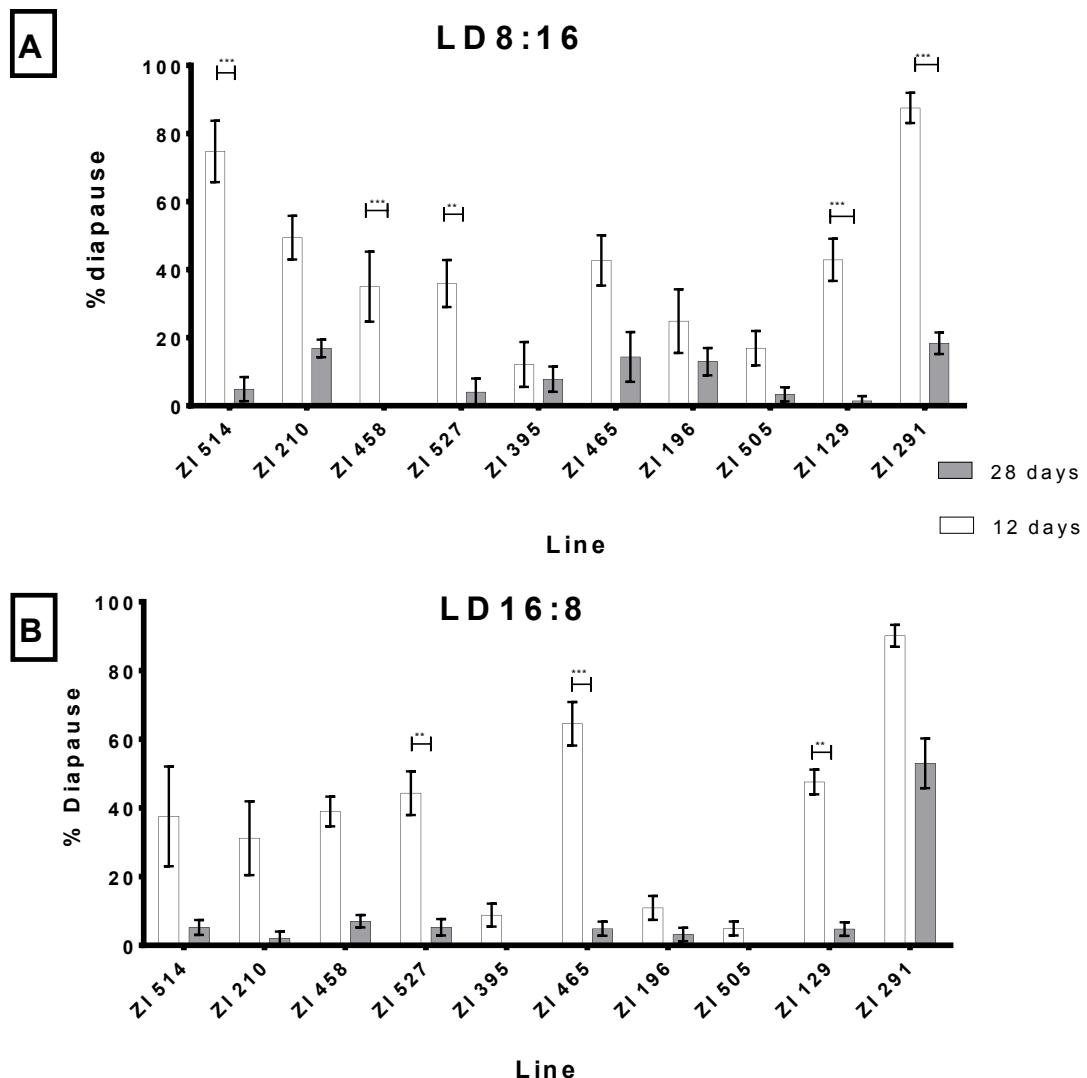
Three-way ANOVA analysing the roles of line, photoperiod and experiment length revealed line and experiment length to be significant factors in diapause, but the photoperiod was not (Table 6-1). Interaction between photoperiod and line was significant, indicating that

some lines, but not others, are photoperiodic, with Tukey's post-tests (Appendix G) revealing ZI 514 to be photoperiodic at 12 days ( $P=0.04$ ).

**Table 6-1 ANOVA table for Zambian diapause**

Effect	SS	Degrees of freedom	MS	F	P
<b>Photoperiod</b>	0.07	1	0.07	2.0	0.164
<b>Line</b>	8.65	9	0.96	25.2	0.000*
<b>expt length</b>	10.36	1	10.36	271.4	0.000*
<b>Photoperiod*Line</b>	1.33	9	0.15	3.9	0.000*
<b>Photoperiod*expt length</b>	0.04	1	0.04	1.0	0.318
<b>Line*expt length</b>	1.96	9	0.22	5.7	0.000*
<b>Photoperiod*Line*expt length</b>	0.74	9	0.08	2.2	0.027*

The influence of experiment length on diapause is more evident from Figure 6-4. A remission was observed in five lines at 8:16; ZI514, ZI458, ZI527, ZI129 and ZI291 ( $P<0.0001 - 0.007$ ). Fewer lines exhibited remission at 16:8; ZI527, ZI458 and ZI 129 ( $P<0.0001 - 0.006$ ).

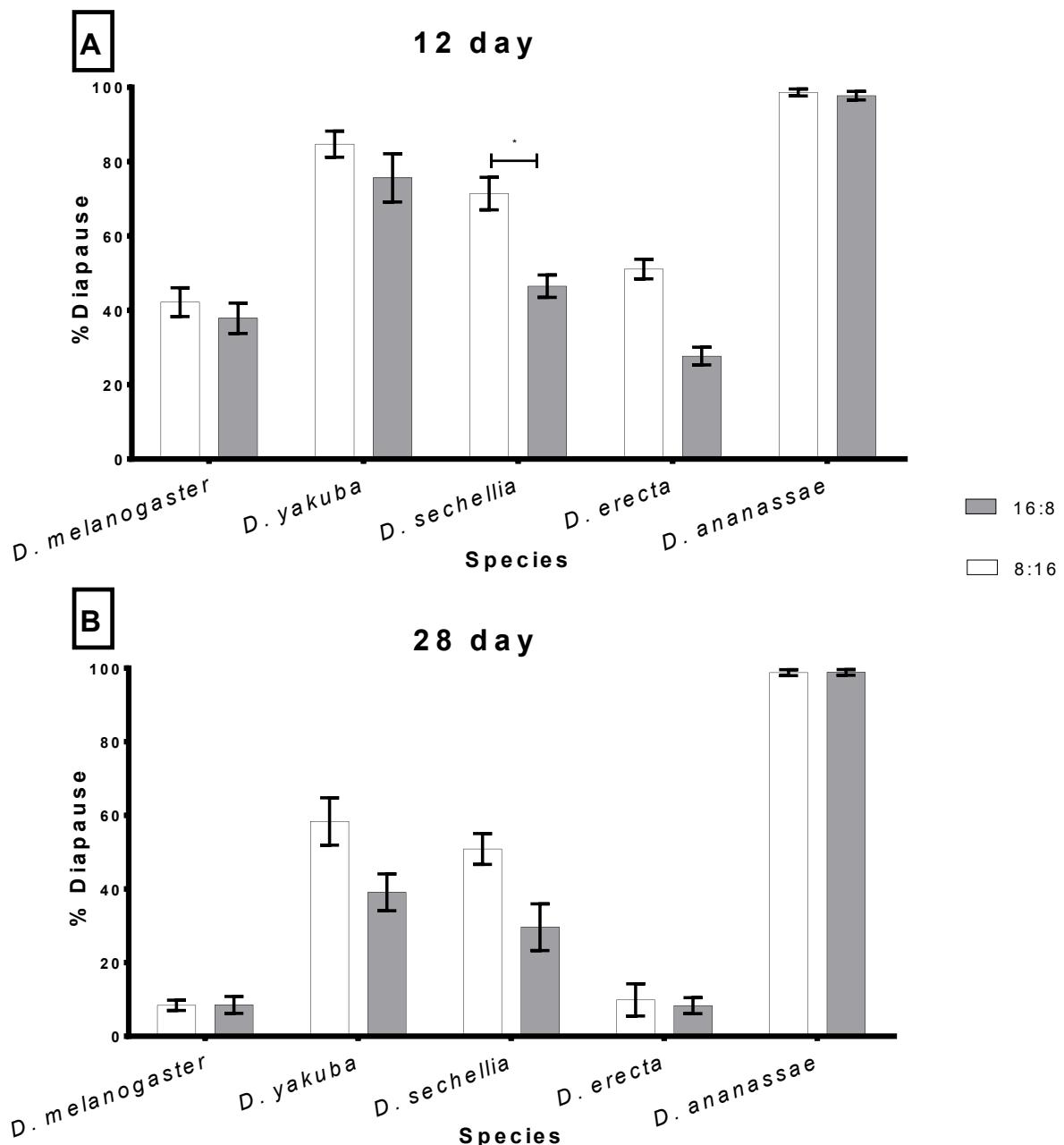


**Figure 6-4 Comparison of diapause between short and long experiments.** 12 day (white) and 28 day (grey) experiments were compared at LD 8:16 (A) and LD 16:8 (B). Mean  $\pm$ SEM n=5 replicates per experiment

### 6.3.2 Diapause in other species in the *melanogaster* species complex

Similar to the variation observed among the Zambian *D. melanogaster*, the different species also revealed polymorphism in the phenotype. The most striking result is that of *D. ananassae* which exhibit near 100% diapause at both photoperiods even after 28 days. *D.*

*yakuba* and *D. sechellia* both exhibit higher levels of diapause throughout the experiment than *D. melanogaster*, with *D. erecta* exhibiting the most similar phenotype under all conditions tested (see Figure 6-5).



**Figure 6-5 The effect of photoperiod on Diapause on the different species. LD 8:16 (white) and LD 16:8 (grey) compared at 12 days (A) and 28 days (B). Data shown is for all lines grouped into species and *D. melanogaster* data is compiled from the Zambian lines. Mean ±SEM, n= 5 replicates per line**

Three-way ANOVA (Table 6-2) indicated that each of the factors; photoperiod, species and experiment length significantly influenced diapause, but none of the interactions were significant. In spite of the significant effect of photoperiod in the ANOVA, Tukey's post-tests (Appendix H) indicated that none of the species are photoperiodic at 12 ( $P=0.68 - 1$ ) or 28 days ( $P=0.74 - 1$ ). Remission was observed in LD8:16 experiments only in *D. melanogaster* ( $P<0.0001$ ) and in LD16:8 experiments in *D. melanogaster* ( $P<0.0001$ ) and *D. yakuba* ( $P=0.0006$ ).

**Table 6-2 Three-way ANOVA table comparing diapause in the species tested, with the lines pooled together**

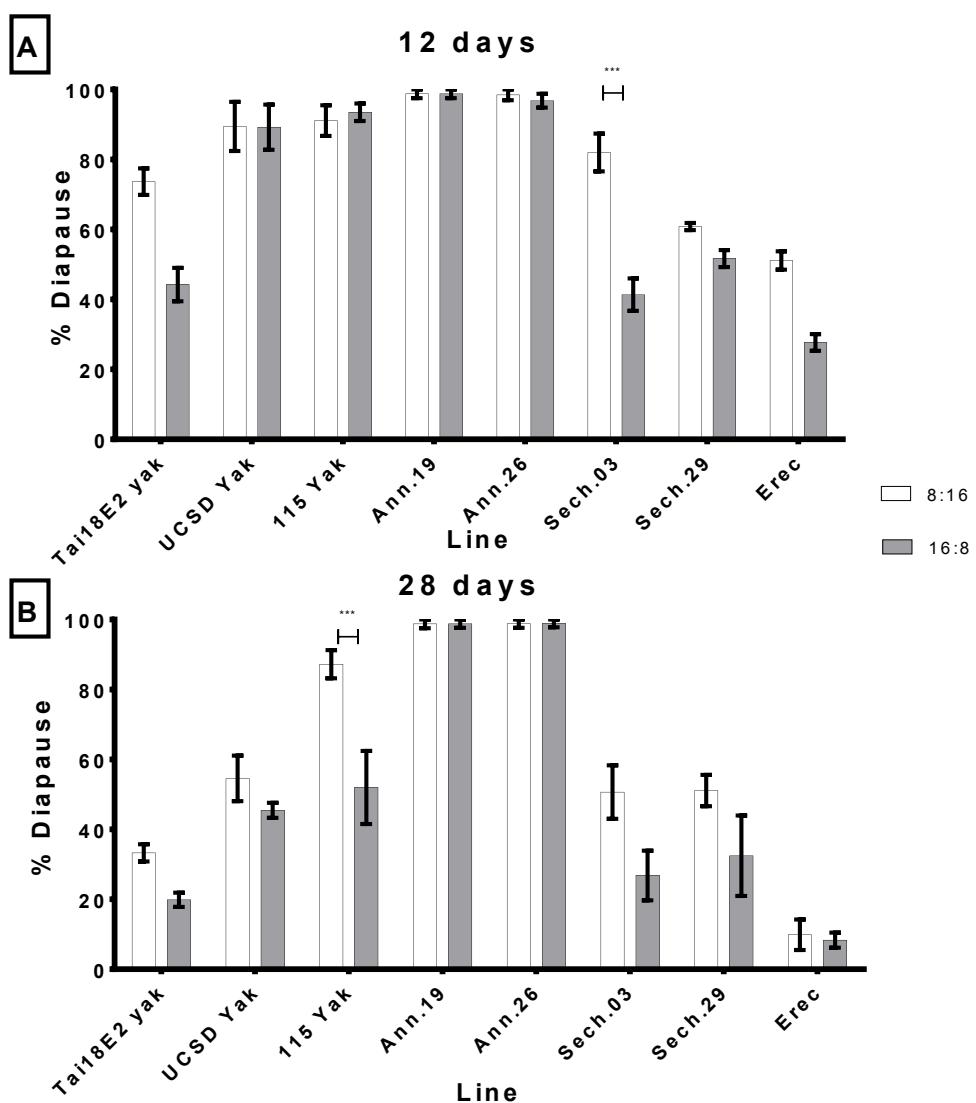
Effect	SS	Degrees of Freedom	MS	F	P
Photoperiod	1.12	1	1.12	7.6	0.006
Species	21.31	4	5.33	36.4	0.000*
Expt length	9.80	1	9.8	67	0.000*
Photoperiod*Species	0.57	4	0.14	0.98	0.420
Photoperiod*Expt length	0.04	1	0.04	0.26	0.611
Species*Expt Length	0.08	4	0.02	0.14	0.969
Photoperiod*Species*Expt Length	0.86	4	0.21	1.5	0.213

Comparison of the species by individual line showed some differences (Figure 6-6). Three-way ANOVA (Table 6-3) again indicated that the photoperiod, line and experiment length had a significant effect on diapause, but the interaction between photoperiod and line was significant, indicating that some lines may be photoperiodic. Line and experiment length also had a significant interaction, implying that some of the lines had exhibited different levels of remission under different experiment lengths.

**Table 6-3 Three-way ANOVA table for diapause in the species tested (excluding *D. melanogaster*) comparing the individual lines**

Effect	SS	Degrees of Freedom	MS	F	P
<b>Photoperiod</b>	0.8	1	0.8	34.56	0.000
<b>Line</b>	20.4	7	2.9	125.33	0.000
<b>Expt length</b>	2.4	1	2.4	104.93	0.000
<b>Photoperiod*Line</b>	0.5	7	0.1	3.27	0.003
<b>Photoperiod*Expt length</b>	0.0	1	0.0	0.01	0.913
<b>Line*Expt Length</b>	1.4	7	0.2	8.39	0.000
<b>Photoperiod*Line*Expt Length</b>	0.4	7	0.1	2.75	0.011

Tukey's post-tests (Appendix I) indicated *Sech.03* exhibits a photoperiodic response in 12 day experiments ( $P=0.0002$ ) and In 28 day experiments *115* is photoperiodic ( $P=0.0001$ ). Remission effects were observed in some lines, *Tai18E2*, *UCSD*, *Sech.03* and *Erec* ( $P<0.0001$  – 0.005) in LD8:16 experiments. In LD16:8 experiments, remission was evident in *UCSD* and *115* ( $P<0.0001$  for both)

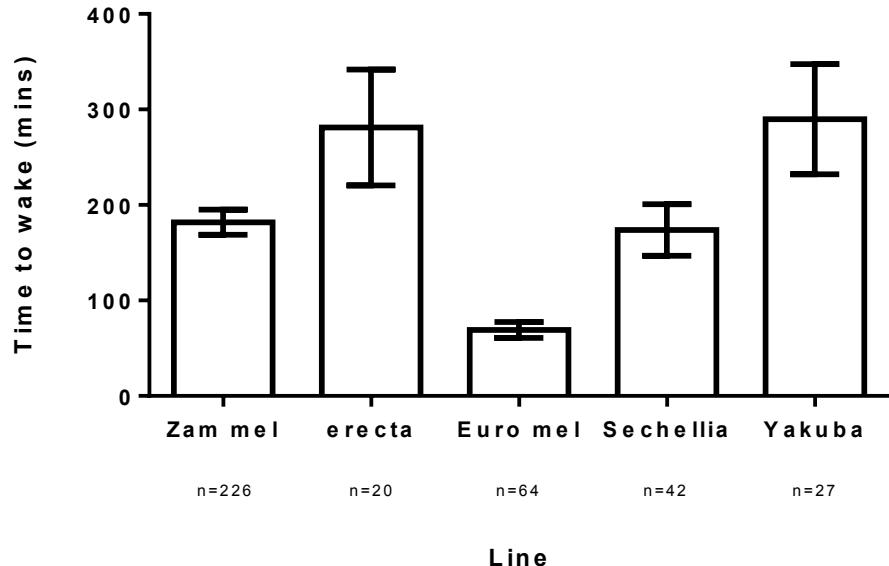


**Figure 6-6 Diapause of different *Drosophila* species studied as individual lines. LD 8:16 experiments (white) are compared with LD 16:8(grey) in 12 day (A) and 28 day (B) experiments comparing individual lines. Data shown is the same as shown in Figure 6-5 expanded to individual lines. Mean  $\pm$ SEM n=5 replicates per line**

### 6.3.3 Chill-Coma Response

Chill-coma response as measured using the DAM system provided a useful means by which to measure the reaction to low temperatures, but left large variation among some of the lines (Figure 6-7). Comparison was made by species, but *D. melanogaster* were separated into European lines and Zambian lines to allow comparison between the two. *D. ananassae*

struggled to survive in the experiment and so were excluded from analysis with only two flies surviving.



**Figure 6-7 Chill coma responses in *Drosophila* species. Large errors were observed, particularly with *D. erecta* and *D. yakuba*. Mean  $\pm$ SEM**

ANOVA was performed to analyse the data and indicated that there were differences between the lines and their responses to chill coma ( $F=8.7$ ,  $Df=4$ ,  $P<0.0001$ ). Tukey's post-tests (Table 6-4) gave the differences between each group indicating that the European *D. melanogaster* were significantly quicker to respond than all lines tested with the exclusion of *D. sechellia* ( $P=0.051$ ).

**Table 6-4 Tukey's post-tests of species chill coma**

Cell	Line	1	2	3	4	5
1	erecta		0.000181	0.245718	0.999880	0.176495
2	Euro mel	0.000181		0.051351	0.000025	0.000353
3	Sech	0.245718	0.051351		0.114539	0.999184
4	Yakuba	0.999880	0.000025	0.114539		0.053030
5	Zam mel	0.176495	0.000353	0.999184	0.053030	

## 6.4 Discussion

The results indicate that some of the Zambian *D. melanogaster* lines exhibit a diapause response to low temperatures in spite of these flies exhibiting a low level of inferred admixture of only 1.4% (Pool et al., 2012) suggesting that diapause in *D. melanogaster* is an ancestral trait. This is supported by observations using closely related species which have not colonised Europe such as *D. ananassae* which exhibited near 100% diapause in these experiments, but also *D. yakuba*, *D. sechellia* and *D. erecta* all exhibited high levels of diapause. The implication is that diapause evolved in the evolutionary history of *Drosophila* and may have been retained by other members of the *Drosophila melanogaster* subgroup, though it is unclear why the diapause response would have been required in Africa. In temperate climates, the seasons swing and the temperature varies significantly from season to season. There is also however, a degree of seasonality in the tropics with wet and dry seasons acting as selective stresses. The dry season is a particularly strong selector and insects must avoid desiccation and find suitable breeding sites which can be a fruitless exercise under hot conditions. Janzen (1973) studied various beetles found during dry periods and indicated that a high percentage of them exhibited an ovarian diapause and evidence from *Hypolimnas bolina*, a tropical Australian butterfly, indicates that females spend the dry season in an adult diapause and await the wet season when breeding sites become available (Pieloor and Seymour, 2001). It is possible then that diapause does exist in these species and the Zambian lines when exposed to higher temperatures like dry seasons, but that is not known and warrants further examination.

Not all of the Zambian lines exhibited a strong diapause response which could imply that the diapause response is neither selected for or against in African lines, but on colonising Europe, the diapausing flies were likely selected. It is possible though that the photoperiodic response in Europe evolved later as only one of the Zambian lines exhibited a photoperiodic response in 12 day experiments, and it is important to be wary of performing large multiple comparisons with post-hoc tests in this manner as it increases the likelihood of finding significance where there is none. Further examination of these lines using methods like the Nanda-Hamnerand Bünsow protocols may act as better indicators as to how important the photoperiod is to diapause in these flies.

The other species tested all exhibited a strong diapause response, though it is important to remember that some of these lines have been in laboratories and stock centres for decades and so there is a possibility that there has been inadvertent selection for low temperature survival when flies are kept at lower temperatures. Further work studying the robustness of diapause in these flies using the winter simulation protocol described in chapter 4 would be of interest, but also, testing the photoperiodic responses of all of these lines in a Nanda-Hamner protocol would better inform us of the role of photoperiod in these diapause responses and the possible role of the clock. Ideally, using isofemale lines that have been more recently derived from wild populations would offer a better analysis.

Experiments studying chill coma indicated that in spite of the adaptation to cold weather seen with high diapause, tropical flies are poorly adapted to the cold shock. These results raise the question as to whether the flies are truly exhibiting a diapause response because they are adapted to perform well in the cold or if the stress of the low temperature is simply too much for them. This possibility is evident from results achieved with *D. ananassae* which

exhibited a very strong diapause response, but died in the chill-coma experiments. The extreme response here indicates that 12°C is too cold for these flies and diapause and chill coma experiments at other temperatures may reveal a more suitable temperature for future work.

## 7 Discussion

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The work presented in this thesis focused largely on seasonal adaptation and investigated the role of two clock genes in diapause, while also searching for a better understanding of the ancestral nature of diapause and how it has evolved in European *D. melanogaster*. The findings discussed here raise several key issues in the study of diapause, particularly pertinent to the study of the fruitfly model. One concerns the current methodology for studying diapause in *D. melanogaster*, where I questioned the validity of the commonly used single temperature experiments. Another is the role of adaptation in European populations to winter conditions which can be compared to the African ancestral populations' responses to winter. The final focus of this discussion will be on the role of the clock in acting as a seasonal timer to indicate oncoming detrimental conditions.

While Saunders *et al.* (1989) indicated that 12 days was sufficiently long for a diapause experiment in *D. melanogaster*, many researchers are utilising a 28 day experimental paradigm for their research (Emerson *et al.*, 2009a, Paaby *et al.*, 2010, Schmidt *et al.*, 2005a, Schmidt *et al.*, 2005b). Work presented by Pegoraro and colleagues (submitted) indicated that flies exhibit a remission effect when studying lines from Africa, the USA and Europe and also the sister species *D. simulans*. They observed that after 28 days in diapause inducing conditions, the level of diapause was significantly lower than it was after 12 days. This evidence was supported further supported here (chapter 6) with studies in Zambian isofemale lines which similarly exhibited a remission effect in 28 day experiments. The constant 12°C temperature used in these experiments represents a threshold for flies to enter diapause, so it is perhaps unsurprising that after some weeks, flies become less sensitive to the conditions and exit diapause.

The idea that diapause would last for such a short period in temperate environments is a conundrum for seasonal biologists. A remission after four weeks in Northern Europe would see flies exit diapause in the middle of winter, while the temperatures are still falling. To try to understand this phenomenon better, a winter simulation was devised to mimic a 3 month winter period with temperatures that fall consistently, before an upswing towards spring. These experiments (chapter 4) presented a similar remission effect between 14 days and 28 days in constant 12°C control conditions, but the winter simulation experiments in *D. melanogaster* resulted in more flies maintaining diapause for a longer period. These results support the theory that 12°C acts more like a threshold temperature and, while sufficient to induce diapause, continued maintenance of diapause requires environmental feedback such as lower temperatures.

In spite of the evidence achieved with ancestral African flies which implied that a weak diapause response is exhibited in lines not adapted for temperate environments, high diapause levels and low remission was observed in the exclusively tropical species *D. erecta*, *D. yakuba*, *D. sechellia* and *D. ananassae*. The response in *D. ananassae* was so strong that they maintained ~100% diapause for 28 days (chapter 6). This result suggests that other tropical species, may have evolved a strong diapause for the purpose of surviving dry seasons and avoiding droughts, but when stressed by low temperatures, a similar strong response is elicited. Results with *D. ananassae* might imply that 12°C is a highly stressful temperature as they respond in a manner similar to European *D. melanogaster* at 8°C. Furthermore, the poor performance of *D. ananassae* in chill coma experiments (they all died when the temperature lowered to 0°C), indicate how poorly they are adapted to low temperatures. Further work on these lines should include studying a range of temperatures

to find a more suitable “critical temperature” that induces ~50% diapause and begin to work from there. It may also be interesting to know if these tropical species exhibit other diapause response phenotypes such as high temperature diapause to see if they have a similar response to 30-35°C as they do to 12°C. The question remains over the diapause in these species with it being unclear as to whether the response is simply stress induced block to development or a true diapause. It is known that during diapause, there is an increase in levels of glycerol and lipids in the haemolymph, which have cryoprotectant properties (Denlinger, 2002), reducing the damage caused by exposure to sub-zero temperatures. Evidence presented by Rinehart and colleagues (2006) indicated that survival of a -5°C cold shock treatment in *Culex pipiens* was increased when the mosquitoes were reared in diapause inducing conditions. Similarly, Schmidt observed that *Drosophila* that are prone to diapause show enhanced survival in -20°C cold shock experiments (Schmidt et al., 2005a, Schmidt et al., 2005b). Maintaining the flies in diapause inducing conditions for 12 days and performing a freeze tolerance experiment would indicate whether the flies have adapted to the cold conditions or are simply stressed, and analysis for diapause markers such as JH titres and lipid levels would add weight to the findings.

Though the winter simulation experiments are an improved method, they are unwieldy, requiring a large number of flies to enter the experiment simultaneously and a three month period for observations. For the majority of experiments 12 days at 12°C is a simple option and gives a reasonable account of diapause. However, evidence from *D. simulans* in winter conditions indicated that at 14 days, reasonable levels of diapause were encountered, but even under simulation, 28 day experiments showed low levels. For future work an abridged simulation experiment lasting only the first four weeks of the winter simulation may be a

suitable compromise when investigating lines where there is a suspicion that there is no diapause. Studying the winter simulation effects on Zambian *D. melanogaster* as well as other tropical species may also offer further evidence on how well adapted they are to cold survival.

One of the primary concerns raised through this study is the method of scoring diapause in *Drosophila*. The method used here and within other groups involves identifying a negative reporter of diapause, namely absence of ovary protein deposits. However, other conditions and stresses are known to result in ovarian resorption in insects including parasitism (Carwardine and Hurd, 1997, Hopwood et al., 2001) and starvation (Sundberg et al., 2001, Barrett et al., 2008) which raises the question as to whether the low temperatures used in diapause experiments could induce a stress response rather than the more discrete diapause phenotype which is a predictive response. It has been shown in *Anaphes nitens*, a parasitoid wasp, that low temperatures may induce ovary resorption (Carbone et al., 2008) and distinguishing this from an undeveloped ovary is not simple. One possible answer to this conundrum lies in the work performed by Schmidt and colleagues (2009) where they compared high and low diapause lines for survival in cold shock experiments after previously being exposed to diapause inducing conditions. High diapause lines showed a greater degree of survivorship in the cold shock experiments, likely due to higher levels of cryoprotectants such as trehalose which rise when flies are in diapause. This offers two options for further analysis of diapause where researchers could assay cryoprotectants to act as a positive indicator for diapause, or subject flies – after a diapause experiment – to a cold shock and use the survivorship as a method of scoring diapause.

One of the aims of this study was to find evidence for a role of clock components in diapause. Chapter 4 studied the role of *tim* in the winter simulation diapause experiments and indicated that the *ls-tim* variant described by Tauber *et al.* (2007) is more likely to maintain a long diapause over winter and into the spring than the ancestral *s-tim* flies. These findings support the idea that this allele is being positively selected and evidence in this laboratory suggests it is spreading across Europe (Zonato, 2012). Zonato indicated however that this novel allele is not more prevalent in the north than in the south, as might be expected of a cold adapted mutation, but rather it is spreading outwards from southern Italy where it initially arose between 300 and 3,000 years ago. This is a rare snapshot of balancing selection for an allele that has not yet reached equilibrium. The anticipatory exit from diapause in *s-tim* flies may be a disadvantage. Overwintering insects will typically not exit diapause until the early spring (Tauber and Tauber, 1976), as conditions in mid to late winter are less likely to present quality mating sites with flowers and fruits relied upon by many insects are still in a recovery phase. For this reason, flies carrying the *ls-tim* allele may be better adapted to temperate regions even though its longer diapause may reduce the following growing season. The trade off must favour *ls-tim* over *s-tim*.

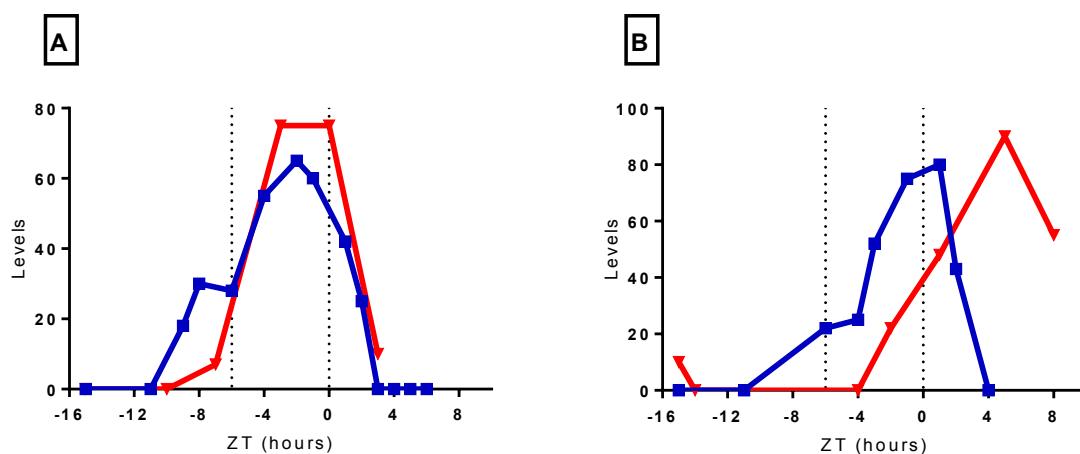
Previous studies have indicated that suppression of splicing in the *per* 3'UTR is important for adopting a siesta behaviour to avoid high midday temperatures while higher levels of splicing allow maximum use of the light phase for foraging when it is cooler and safer (Majercak *et al.*, 2004, Majercak *et al.*, 1999). Evidence from chapter 6 showed that diapause is averted in flies locked into expression of the longer *perA* variant, while flies locked into the shorter *perB* variant are prone to diapause at LD10:14 and 12°C. Both variants expressed together in a normal manner, gave a similar diapause response to

expression of *perB* alone, indicating that *perB* expression is crucial for diapause induction. This might indicate a secondary role for *per* splicing in seasonal survival. What is particularly interesting in this comparison of diapause and circadian responses is that the act of splicing and not the splice variant was the crucial factor in circadian phenotypes (Majercak et al., 1999), while in diapause observations it is the resulting mRNA that has an influence. The results of these experiments offer a link between circadian and seasonal mechanisms with *per* seemingly having a modulatory role in circadian behaviours while simultaneously, through the same pathway, influencing the diapause response.

Haplotypes of *per* presented by Low (2012) indicated the possibility that there may be selection for some polymorphisms in the 3'UTR which mediate splicing, which is of significance because of the role it plays in thermal adaptation (Majercak et al., 2004, Low et al., 2008, Collins et al., 2004). Two SNPs in particular appeared to have a role in high splicing efficiency, but a small scale study in North America could not confirm a clinal distribution of the haplotype associated with the SNPs (Low et al., 2012). Sequencing large numbers of lines across Europe however, revealed that the two vital SNPs described by Low *et al.* had no evidence for clinal selection, but were in fact very common. One SNP that they did not observe however, SNP 223, was shown to exhibit a cline and was very common in Zambian flies and some Spanish populations but became rarer further north. Its role in splicing or diapause is as yet unknown, but it could be that this SNP has a role in diapause. There was no evidence for the presence of SNP223 in *Houten* populations, though few samples were tested. Testing the *Houten* populations for variations in the 3' UTR and selecting for these variants could offer a platform from which to further investigate the roles of *per* 3'UTR variation and how they influence diapause differently in *ls-tim* and *s-tim* backgrounds.

Sequencing isofemale lines for variations in the *per* 3'UTR and establishing isogenic populations around these variants could also provide some insight.

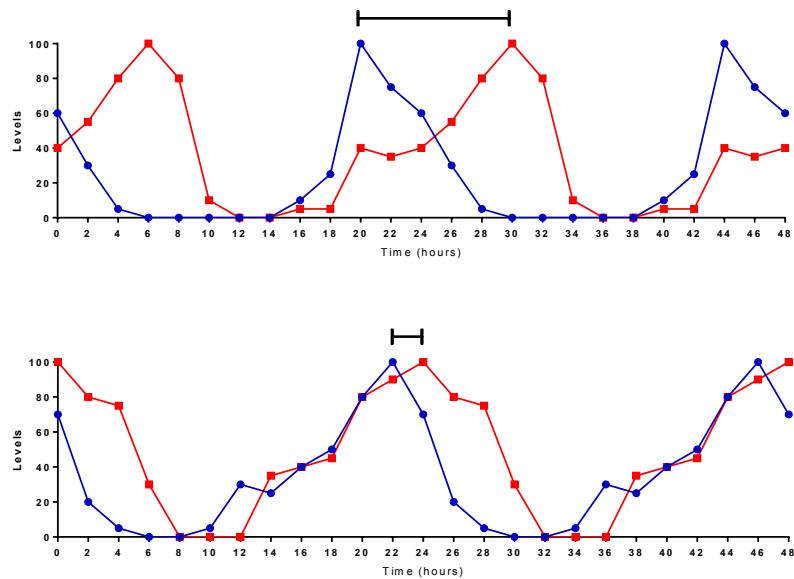
The interest in *tim* and *per* variation is made all the more fascinating after a recent experiment performed by Menegazzi *et al.* (2013) who observed the differences in PER and TIM expression under natural conditions in winter, spring, summer and autumn. Their natural winter conditions were performed outdoors, and levels of PER and TIM measured in various cells in the brain. Figure 7-1 shows a comparison of autumn and summer conditions with PER peaking later in the summer, but peaking around the same time as TIM in the autumn.



**Figure 7-1 Levels of PER and TIM stained in the dorsal neurones of the *Drosophila* brain. In autumn conditions (A) PER (red) and TIM (blue) peak at a similar time but in summer conditions (B) there is a notable delay in the peak of PER (redrawn from Menegazzi *et al.* 2013)**

Other data from Menegazzi (personal communication) indicated that in laboratory conditions at 20°C, altering the photoperiod can induce a similar change to the one outlined above (Figure 7-2). This pattern is reminiscent of the theoretical internal coincidence example provided in section 1.3.2 (Figure 1-8) of two circadian proteins that cycle together and move out of (or into) phase to induce or exit diapause. This is interesting when

considering the role of these two circadian proteins in diapause as their combined expression is influenced by the light and temperature. Using similar experiments to compare high diapausing and low diapausing lines could potentially implicate the combined influence of these two genes in diapause. Furthermore, a study of how this interaction is influenced by variation in the two genes, primarily comparing the *ls-tim* and *s-tim* alleles, but also the *perA* and *perB* splice locked flies at different temperatures and photoperiods could offer a better understanding of how the variants influence diapause



**Figure 7-2 Comparison of TIM and PER cycling at two different photoperiods. At LD 16:8 (top) TIM (blue) peaks at 20h, 2h earlier than LD 8:16 (bottom). PER (red) however peaks at 6h in long days, but at 0h in short days, bringing the peak expression of PER and TIM closer in short days. (Menegazzi personal communication)**

## 7.1 Why is diapause important

From diapause, we can learn a great deal about the life history of an insect and this can help conservation efforts for protecting species at risk. Arguably one of the most famous examples of the importance of diapause research that resulted in a practical application is

the JH story. This was outlined in Chapter 1 as a vital hormone for maintaining larval and pupal stages in the examples of *Rhodnius* (Wigglesworth, 1936) and *Bombyx* (Williams, 1959) where overexpressing JH prevented insects from developing into adults. The serendipitous discovery that American varieties of paper contain an analogous substance to JH led to the theory that an analogue of JH may have evolved in plants as a defence mechanism (Sláma and Williams, 1965), but also led to the development of an important group of insecticides developed from JH analogues (Staal, 1975). Methoprene, one such analogue, is now recommended by the World Health Organisation to prevent mosquitoes from breeding in drinking water and is a fascinating example of how one seemingly unimportant scientific discovery can be innovated into a vitally important tool for humanity.

As well as the methoprene example, the study of diapause in insects has some wide reaching applications to human physiology, from the role of PI3K in cancer (Vivanco and Sawyers, 2002), PER in human sleep disorders (Carpen et al., 2005) and the similarities between the *Drosophila* and human insulin signalling pathways (Buch et al., 2008, Claeys et al., 2002, Garofalo, 2002). More recent speculation that humans may in fact exhibit an embryonic diapause (Ptak et al., 2012) may seem far-fetched, but investigation into these phenomena is the only way to rule it out.

## 7.2 Summary

In summary, the work presented here has shown that components of circadian rhythms, namely *per* and *tim* almost certainly have a role in modulating circadian rhythms. Two alleles of *tim* are shown to have different effects on diapause – particularly the time of termination – and the novel *ls-tim* allele which induces a more robust diapause into spring appears to be under selection in Europe because of the fitness advantage it represents.

Splice variation in *per* is shown to have a dramatic influence on the ability of flies to enter diapause, with the absence of spliced *perB* transcripts resulting in a block to diapause induction. The induction of diapause under cold conditions is shown to have an ancestral component with low admixture African lines from Zambia and several exclusively tropical species all exhibiting diapause in response to low temperatures. Finally, evidence presented here suggests that the 28 day diapause experiment is not suitable for assessing diapause in *Drosophila*. Winter simulation experiments and experiments comparing 12 and 28 day diapause show that 28 days of constant diapause threshold temperatures are not enough to maintain diapause and indicates that many experiments using this paradigm may be missing out on some crucial evidence.

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Appendix A Newman-Keuls post-tests for Winter Simulation experiments on *ls-tim* and *s-tim* *D. melanogaster*  
 (see section 4.4.1 for details)

Cell	time	conditions	allele	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	2	simulation	ls-tim		0.4311	0.3788	0.6175	0.5439	0.0488	0.0001	0.0001	0.6623	0.0004	0.0001	0.0002	0.0067	0.0002	0.0001	0.0002	0.0033	0.0002	0.0001	0.0002
2	2	simulation	s-tim	0.4311		0.8644	0.7919	0.8573	0.2504	0.0014	0.0002	0.6076	0.0313	0.0002	0.0001	0.143	0.0038	0.0003	0.0001	0.1359	0.0001	0.0002	0.0001
3	2	control	ls-tim	0.3788	0.8644		0.9571	0.8948	0.2783	0.0002	0.0001	0.9669	0.0048	0.0001	0.0002	0.0698	0.0004	0.0001	0.0002	0.0422	0.0001	0.0001	0.0002
4	2	control	s-tim	0.6175	0.7919	0.9571		0.6935	0.2367	0.0002	0.0001	0.9119	0.0046	0.0001	0.0001	0.0613	0.0004	0.0001	0.0002	0.0386	0.0001	0.0002	0.0002
5	4	simulation	ls-tim	0.5439	0.8573	0.8948	0.6935		0.337	0.0004	0.0001	0.9891	0.0127	0.0001	0.0001	0.1152	0.0011	0.0002	0.0001	0.0824	0.0001	0.0002	0.0002
6	4	simulation	s-tim	0.0488	0.2504	0.2783	0.2367	0.337		0.0376	0.0011	0.2213	0.28	0.0003	0.0001	0.4566	0.0758	0.0078	0.0001	0.5634	0.0002	0.0064	0.0001
7	4	control	ls-tim	0.0001	0.0014	0.0002	0.0002	0.0004	0.0376		0.5277	0.0003	0.448	0.3588	0.0052	0.1652	0.6903	0.7601	0.0018	0.1994	0.046	0.4933	0.0002
8	4	control	s-tim	0.0001	0.0002	0.0001	0.0001	0.0001	0.0011	0.5277		0.0002	0.1138	0.6344	0.0825	0.0106	0.4032	0.5156	0.0482	0.0192	0.2597	0.7806	0.0047
9	8	simulation	ls-tim	0.6623	0.6076	0.9669	0.9119	0.9891	0.2213	0.0003	0.0002		0.0099	0.0001	0.0001	0.0801	0.0009	0.0002	0.0001	0.0618	0.0001	0.0001	0.0001
10	8	simulation	s-tim	0.0004	0.0313	0.0048	0.0046	0.0127	0.28	0.448	0.1138	0.0099		0.0449	0.0002	0.5465	0.4177	0.3135	0.0002	0.4405	0.0018	0.2341	0.0002
11	8	control	ls-tim	0.0001	0.0002	0.0001	0.0001	0.0001	0.0003	0.3588	0.6344	0.0001	0.0449		0.1359	0.0027	0.2308	0.4982	0.1013	0.0055	0.2734	0.6604	0.0143
12	8	control	s-tim	0.0002	0.0001	0.0002	0.0001	0.0001	0.0001	0.0052	0.0825	0.0001	0.0002	0.1359		0.0002	0.0017	0.0234	0.6996	0.0002	0.41	0.0314	0.3981
13	10	simulation	ls-tim	0.0067	0.143	0.0698	0.0613	0.1152	0.4566	0.1652	0.0106	0.0801	0.5465	0.0027	0.0002		0.2497	0.0554	0.0001	0.7827	0.0002	0.0442	0.0001
14	10	simulation	s-tim	0.0002	0.0038	0.0004	0.0004	0.0011	0.0758	0.6903	0.4032	0.0009	0.4177	0.2308	0.0017	0.2497		0.6864	0.0006	0.2554	0.0198	0.524	0.0002
15	10	control	ls-tim	0.0001	0.0003	0.0001	0.0001	0.0002	0.0078	0.7601	0.5156	0.0002	0.3135	0.4982	0.0234	0.0554	0.6864		0.0106	0.0854	0.122	0.9851	0.0007
16	10	control	s-tim	0.0002	0.0001	0.0002	0.0002	0.0001	0.0001	0.0018	0.0482	0.0001	0.0002	0.1013	0.6996	0.0001	0.0006	0.0106		0.0002	0.4473	0.0135	0.3632
17	12	simulation	ls-tim	0.0033	0.1359	0.0422	0.0386	0.0824	0.5634	0.1994	0.0192	0.0618	0.4405	0.0055	0.0002	0.7827	0.2554	0.0854	0.0002		0.0002	0.0649	0.0001
18	12	simulation	s-tim	0.0002	0.0001	0.0001	0.0001	0.0001	0.0002	0.046	0.2597	0.0001	0.0018	0.2734	0.41	0.0002	0.0198	0.122	0.4473	0.0002		0.1702	0.1513
19	12	control	ls-tim	0.0001	0.0002	0.0001	0.0002	0.0002	0.0064	0.4933	0.7806	0.0001	0.2341	0.6604	0.0314	0.0442	0.524	0.9851	0.0135	0.0649	0.1702		0.0009
20	12	control	s-tim	0.0002	0.0001	0.0002	0.0002	0.0002	0.0001	0.0002	0.0047	0.0001	0.0002	0.0143	0.3981	0.0001	0.0002	0.0007	0.3632	0.0001	0.1513	0.0009	

Appendix B Newman-Keuls post-tests for winter simulation experiments on *D. simulans* and *D. melanogaster*  
 (see section 4.4.2 for details)

Cell	time	conditions	Line	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	2	Simulation	SREN	<b>0.0218</b>	0.8781	<b>0.0105</b>	0.8206	<b>0.0001</b>	<b>0.0002</b>	<b>0.0001</b>	0.1032	<b>0.0001</b>	<b>0.0001</b>	<b>0.0002</b>	<b>0.0003</b>	<b>0.0002</b>	<b>0.0002</b>	<b>0.0001</b>	<b>0.0002</b>	<b>0.0001</b>	<b>0.0002</b>	<b>0.0002</b>	<b>0.0002</b>
2	2	Simulation	SREN	<b>0.0218</b>		<b>0.0216</b>	0.7167	<b>0.0387</b>	<b>0.0002</b>	0.2961	<b>0.0002</b>	0.4395	0.1548	<b>0.0002</b>	<b>0.0001</b>	0.2261	<b>0.0002</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0026</b>	<b>0.0002</b>	<b>0.0001</b>	<b>0.0001</b>
3	2	control	MREN	0.8781	<b>0.0216</b>		<b>0.0118</b>	0.6567	<b>0.0001</b>	<b>0.0003</b>	<b>0.0001</b>	0.0845	<b>0.0001</b>	<b>0.0001</b>	<b>0.0002</b>	<b>0.0003</b>	<b>0.0002</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0002</b>	<b>0.0002</b>	<b>0.0002</b>
4	2	control	SREN	<b>0.0105</b>	0.7167	<b>0.0118</b>		<b>0.0271</b>	<b>0.0003</b>	0.3442	<b>0.0002</b>	0.4917	0.2214	<b>0.0002</b>	<b>0.0001</b>	0.1983	<b>0.0004</b>	<b>0.0001</b>	<b>0.0002</b>	<b>0.006</b>	<b>0.0003</b>	<b>0.0001</b>	<b>0.0001</b>
5	4	Simulation	MREN	0.8206	<b>0.0387</b>	0.6567	<b>0.0271</b>		<b>0.0002</b>	<b>0.0008</b>	<b>0.0001</b>	0.0909	<b>0.0002</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0008</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0002</b>	<b>0.0002</b>	<b>0.0002</b>
6	4	Simulation	SREN	<b>0.0001</b>	<b>0.0002</b>	<b>0.0001</b>	<b>0.0003</b>	<b>0.0002</b>		<b>0.0167</b>	0.6524	<b>0.0002</b>	0.0517	0.8226	0.5812	<b>0.0163</b>	0.9721	0.6866	0.6948	0.6372	0.9521	0.2516	0.1339
7	4	control	MREN	<b>0.0002</b>	0.2961	<b>0.0003</b>	0.3442	<b>0.0008</b>	<b>0.0167</b>		<b>0.0017</b>	0.0897	0.5969	<b>0.0113</b>	<b>0.0003</b>	0.9157	<b>0.0145</b>	<b>0.0006</b>	<b>0.001</b>	0.0929	<b>0.014</b>	<b>0.0001</b>	<b>0.0001</b>
8	4	control	SREN	<b>0.0001</b>	<b>0.0002</b>	<b>0.0001</b>	<b>0.0002</b>	<b>0.0001</b>	0.6524	<b>0.0017</b>		<b>0.0001</b>	<b>0.0075</b>	0.5123	0.8779	<b>0.0014</b>	0.801	0.9051	0.8343	0.3122	0.7817	0.6144	0.4577
9	8	Simulation	MREN	0.1032	0.4395	0.0845	0.4917	0.0909	<b>0.0002</b>	0.0897	<b>0.0001</b>		<b>0.0318</b>	<b>0.0002</b>	<b>0.0001</b>	0.077	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0003</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0002</b>
10	8	Simulation	SREN	<b>0.0001</b>	0.1548	<b>0.0001</b>	0.2214	<b>0.0002</b>	0.0517	0.5969	<b>0.0075</b>	<b>0.0318</b>		<b>0.0399</b>	<b>0.0012</b>	0.8002	<b>0.0337</b>	<b>0.003</b>	<b>0.0049</b>	0.1174	<b>0.0395</b>	<b>0.0002</b>	<b>0.0001</b>
11	8	control	MREN	<b>0.0001</b>	<b>0.0002</b>	<b>0.0001</b>	<b>0.0002</b>	<b>0.0001</b>	0.8226	<b>0.0113</b>	0.5123	<b>0.0002</b>	<b>0.0399</b>		0.6262	<b>0.0104</b>	0.9692	0.7004	0.6619	0.6223	0.9562	0.3057	0.1787
12	8	control	SREN	<b>0.0002</b>	<b>0.0001</b>	<b>0.0002</b>	<b>0.0001</b>	<b>0.0001</b>	0.5812	<b>0.0003</b>	0.8779	<b>0.0001</b>	<b>0.0012</b>	0.6262		<b>0.0002</b>	0.5835	0.749	0.8534	0.1239	0.6243	0.5005	0.5329
13	10	Simulation	MREN	<b>0.0003</b>	0.2261	<b>0.0003</b>	0.1983	<b>0.0008</b>	<b>0.0163</b>	0.9157	<b>0.0014</b>	0.077	0.8002	<b>0.0104</b>	<b>0.0002</b>		<b>0.0167</b>	<b>0.0005</b>	<b>0.0009</b>	0.1258	<b>0.0146</b>	<b>0.0001</b>	<b>0.0001</b>
14	10	Simulation	SREN	<b>0.0002</b>	<b>0.0002</b>	<b>0.0002</b>	<b>0.0004</b>	<b>0.0001</b>	0.9721	<b>0.0145</b>	0.801	<b>0.0001</b>	<b>0.0337</b>	0.9692	0.5835	<b>0.0167</b>		0.7228	0.7735	0.3401	0.868	0.2293	0.1111
15	10	control	MREN	<b>0.0002</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	0.6866	<b>0.0006</b>	0.9051	<b>0.0001</b>	<b>0.003</b>	0.7004	0.749	<b>0.0005</b>	0.7228		0.8296	0.21	0.7454	0.5803	0.5059
16	10	control	SREN	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0002</b>	<b>0.0001</b>	0.6948	<b>0.001</b>	0.8343	<b>0.0001</b>	<b>0.0049</b>	0.6619	0.8534	<b>0.0009</b>	0.7735	0.8296		0.2648	0.7778	0.62	0.4945
17	12	Simulation	MREN	<b>0.0002</b>	<b>0.0026</b>	<b>0.0001</b>	<b>0.006</b>	<b>0.0001</b>	0.6372	0.0929	0.3122	<b>0.0003</b>	0.1174	0.6223	0.1239	0.1258	0.3401	0.21	0.2648		0.5008	<b>0.0239</b>	<b>0.0081</b>
18	12	Simulation	SREN	<b>0.0001</b>	<b>0.0002</b>	<b>0.0002</b>	<b>0.0003</b>	<b>0.0002</b>	0.9521	<b>0.014</b>	0.7817	<b>0.0001</b>	<b>0.0395</b>	0.9562	0.6243	<b>0.0146</b>	0.868	0.7454	0.7778	0.5008		0.2695	0.1399
19	12	control	MREN	<b>0.0002</b>	<b>0.0001</b>	<b>0.0002</b>	<b>0.0001</b>	<b>0.0002</b>	0.2516	<b>0.0001</b>	0.6144	<b>0.0001</b>	<b>0.0002</b>	0.3057	0.5005	<b>0.0001</b>	0.2293	0.5803	0.62	<b>0.0239</b>	0.2695		0.6923
20	12	control	SREN	<b>0.0002</b>	<b>0.0001</b>	<b>0.0002</b>	<b>0.0001</b>	<b>0.0002</b>	0.1339	<b>0.0001</b>	0.4577	<b>0.0002</b>	<b>0.0001</b>	0.1787	0.5329	<b>0.0001</b>	0.1111	0.5059	0.4945	<b>0.0081</b>	0.1399	0.6923	

Appendix C Newman-Keuls post-tests for winter simulation experiments on *D. simulans* and *D. melanogaster*  
 (see section 4.4.3 for details)

Cell	time	conditions	Line	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	2	simulation	MREN		0.0373	0.8816	0.0018	0.8299	0.3563	0.0003	0.0002	0.174	0.4294	0.0001	0.0001	0.0004	0.1006	0.0001	0.0001	0.0002	0.0002	0.0002	0.0001
2	2	simulation	Hu	0.0373		0.0459	0.2116	0.0221	0.1191	0.0002	0.0002	0.0003	0.0017	0.0001	0.0001	0.0001	0.0002	0.0002	0.0001	0.0001	0.0001	0.0002	0.0002
3	2	control	MREN	0.8816	0.0459		0.0017	0.6661	0.531	0.0004	0.0002	0.1635	0.3602	0.0002	0.0001	0.0004	0.1043	0.0001	0.0001	0.0001	0.0002	0.0001	0.0001
4	2	control	Hu	0.0018	0.2116	0.0017		0.0006	0.0156	0.0002	0.0001	0.0001	0.0001	0.0001	0.0001	0.0002	0.0001	0.0002	0.0002	0.0001	0.0001	0.0002	0.0002
5	4	simulation	MREN	0.8299	0.0221	0.6661	0.0006		0.4359	0.0012	0.0006	0.2267	0.3507	0.0002	0.0002	0.0012	0.1789	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
6	4	simulation	Hu	0.3563	0.1191	0.531	0.0156	0.4359		0.0001	0.0002	0.0246	0.1118	0.0001	0.0001	0.0001	0.0104	0.0001	0.0001	0.0002	0.0001	0.0002	0.0002
7	4	control	MREN	0.0003	0.0002	0.0004	0.0002	0.0012	0.0001		0.7554	0.0716	0.0163	0.0081	0.007	0.9181	0.0993	0.0007	0.0017	0.1062	0.073	0.0002	0.0001
8	4	control	Hu	0.0002	0.0002	0.0002	0.0001	0.0006	0.0002	0.7554		0.0501	0.0091	0.0132	0.0127	0.9093	0.0853	0.0014	0.0035	0.0858	0.0858	0.0002	0.0002
9	8	simulation	MREN	0.174	0.0003	0.1635	0.0001	0.2267	0.0246	0.0716	0.0501		0.4733	0.0001	0.0001	0.052	0.6995	0.0002	0.0002	0.0004	0.0002	0.0001	0.0001
10	8	simulation	Hu	0.4294	0.0017	0.3602	0.0001	0.3507	0.1118	0.0163	0.0091	0.4733		0.0001	0.0002	0.014	0.512	0.0001	0.0002	0.0001	0.0001	0.0001	0.0001
11	8	control	MREN	0.0001	0.0001	0.0002	0.0001	0.0002	0.0001	0.0081	0.0132	0.0001	0.0001		0.8678	0.0085	0.0001	0.7193	0.7677	0.3608	0.3401	0.3386	0.3053
12	8	control	Hu	0.0001	0.0001	0.0001	0.0001	0.0002	0.0001	0.007	0.0127	0.0001	0.0002	0.8678		0.0067	0.0001	0.651	0.5997	0.4197	0.5006	0.3496	0.2943
13	10	simulation	MREN	0.0004	0.0001	0.0004	0.0002	0.0012	0.0001	0.9181	0.9093	0.052	0.014	0.0085	0.0067		0.0509	0.0006	0.0015	0.1443	0.0856	0.0002	0.0002
14	10	simulation	Hu	0.1006	0.0002	0.1043	0.0001	0.1789	0.0104	0.0993	0.0853	0.6995	0.512	0.0001	0.0001	0.0509		0.0002	0.0001	0.0008	0.0004	0.0001	0.0002
15	10	control	MREN	0.0001	0.0002	0.0001	0.0002	0.0001	0.0001	0.0007	0.0014	0.0002	0.0001	0.7193	0.651	0.0006	0.0002		0.721	0.1592	0.2687	0.5986	0.3787
16	10	control	Hu	0.0001	0.0001	0.0001	0.0002	0.0001	0.0001	0.0017	0.0035	0.0002	0.0002	0.7677	0.5997	0.0015	0.0001	0.721		0.2445	0.3545	0.5483	0.431
17	12	simulation	MREN	0.0002	0.0001	0.0001	0.0001	0.0001	0.0002	0.1062	0.0858	0.0004	0.0001	0.3608	0.4197	0.1443	0.0008	0.1592	0.2445		0.6821	0.0219	0.0219
18	12	simulation	Hu	0.0002	0.0001	0.0002	0.0001	0.0001	0.0001	0.073	0.0858	0.0002	0.0001	0.3401	0.5006	0.0856	0.0004	0.2687	0.3545	0.6821		0.0546	0.0518
19	12	control	MREN	0.0002	0.0002	0.0001	0.0002	0.0001	0.0002	0.0002	0.0002	0.0002	0.0001	0.3386	0.3496	0.0002	0.0001	0.5986	0.5483	0.0219	0.0546		0.9335
20	12	control	Hu	0.0001	0.0002	0.0001	0.0002	0.0001	0.0002	0.0001	0.0002	0.0002	0.0001	0.3053	0.2943	0.0002	0.0002	0.3787	0.431	0.0219	0.0518	0.9335	

## Appendix D per 3' UTR Variation in Zambian and Houten lines

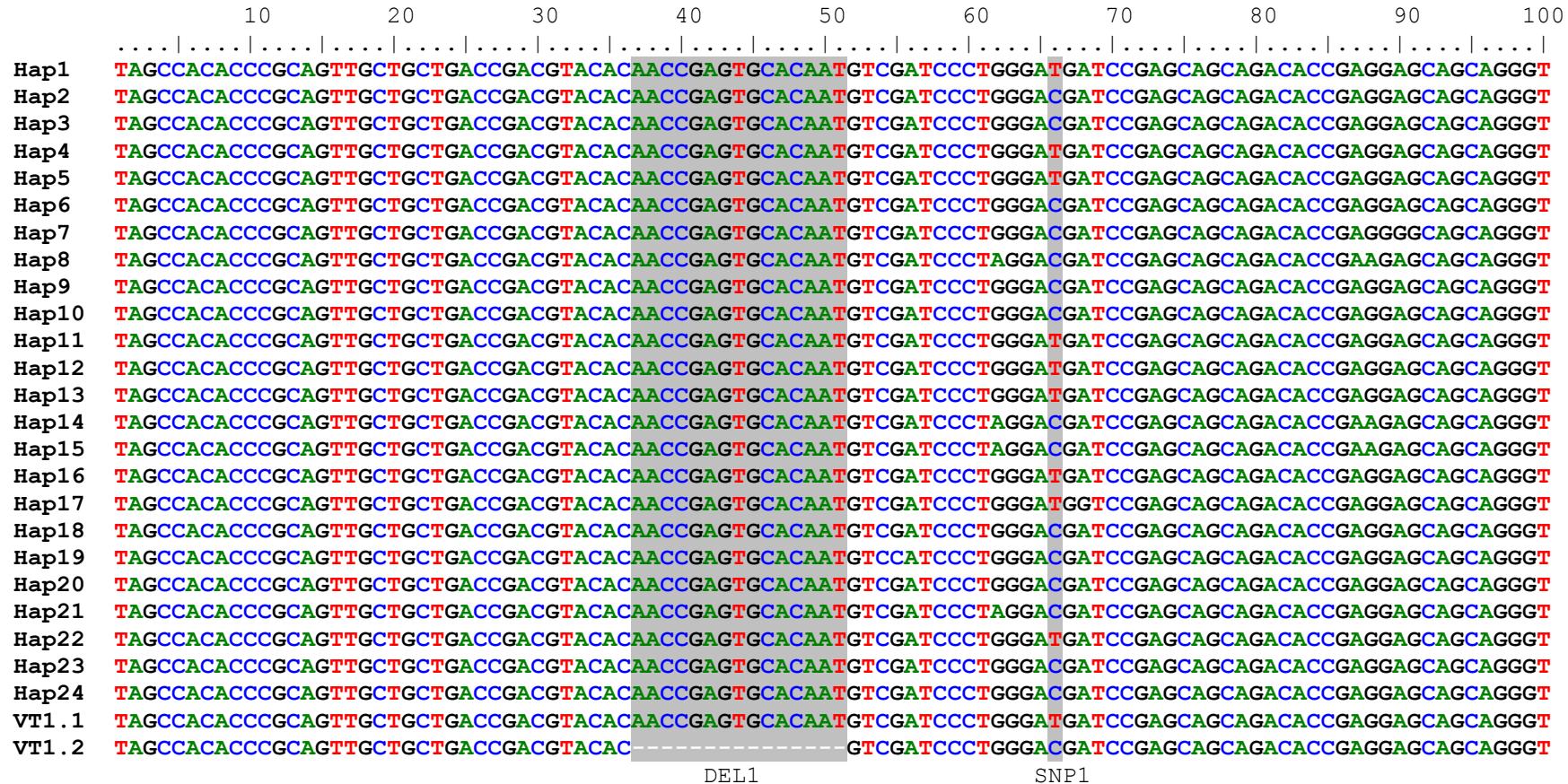
(See section 5.4.3.1)

First 300 bases of the UTR sequence. Grey highlights indicate SNPs derived from VT1.1 and VT1.2 and black highlights indicate novel SNPs found in the preliminary screen

		* <sup>1</sup> DEL1	SNP1
Z1	TAGCCACACCCGAGTTGCTGCTGACCGACGTACACAAT	TCGAGTGACAAT	GTCGATCCCTGGGACGATCCGAGCAGCACCGAGGAGCAGCAGGGT
Z2	TAGCCACACCCGAGTTGCTGCTGACCGACGTACACAAT	TCGAGTGACAAT	GTCGATCCCTGGGATGATCCGAGCAGCACCGAGGAGCAGCAGGGT
Z3	TAGCCACACCCGAGTTGCTGCTGACCGACGTACACAAT	TCGAGTGACAAT	GTCGATCCCTGGGATGATCCGAGCAGCACCGAGGAGCAGCAGGGT
Z4	TAGCCACACCCGAGTTGCTGCTGACCGACGTACACAAT	TCGAGTGACAAT	GTCGATCCCTGGGACGATCCGAGCAGCACCGAGGAGCAGCAGGGT
Z5	TAGCCACACCCGAGTTGCTGCTGACCGACGTACACAAT	TCGAGTGACAAT	GTCGATCCCTGGGACGATCCGAGCAGCACCGAGGAGCAGCAGGGT
Z6	TAGCCACACCCGAGTTGCTGCTGACCGACGTACACAAT	TCGAGTGACAAT	GTCGATCCCTGGGACGATCCGAGCAGCACCGAGGAGCAGCAGGGT
Z7	TAGCCACACCCGAGTTGCTGCTGACCGACGTACACAAT	TCGAGTGACAAT	GTCGATCCCTGGGATGATCCGAGCAGCACCGAGGAGCAGCAGGGT
Z8	TAGCCACACCCGAGTTGCTGCTGACCGACGTACACAAC	CGAGTGACAAT	GTCGATCCCTGGGACGATCCGAGCAGCACCGAGGAGCAGCAGGGT
Z10	TAGCCACACCCGAGTTGCTGCTGACCGACGTACACAAT	TCGAGTGACAAT	GTCGATCCCTGGGACGATCCGAGCAGCACCGAGGAGCAGCAGGGT
Z11	TAGCCACACCCGAGTTGCTGCTGACCGACGTACACAAT	TCGAGTGACAAT	GTCGATCCCTGGGATGATCCGAGCAGCACCGAGGAGCAGCAGGGT
Hu	TAGCCACACCCGAGTTGCTGCTGACCGACGTACACAAT	TCGAGTGACAAT	GTCGATCCCTGGGACGATCCGAGCAGCACCGAGGAGCAGCAGGGT
VT1.1	TAGCCACACCCGAGTTGCTGCTGACCGACGTACAC		GTCGATCCCTGGGACGATCCGAGCAGCACCGAGGAGCAGCAGGGT
VT1.2	TAGCCACACCCGAGTTGCTGCTGACCGACGTACAC		GTCGATCCCTGGGACGATCCGAGCAGCACCGAGGAGCAGCAGGGT
		* <sup>2</sup> SNP2	* <sup>3</sup> DEL2
Z1	CCTGGAAACGAGTGAGCAATTGCCCTCGGTCTGGGACCGAG	ACCAGCCAGTCCCACCGATT	CCCCCTATTCTTAAC
Z2	CCTGGAAACGAGTGAGCAATTGCCCTCGGTCTGGGACCGAG	ACCAGCCAGTCCCACCGATT	CCCCCTATTCTTAAC
Z3	CCTGGAAACGAGTGAGCAATTGCCCTCGGTCTGGGACCGAG	TACCAAGCCAGTCCCACCGATT	CCCCCTATTCTTAAC
Z4	CCTGGAAACGAGTGAGCAATTGCCCTCGGTCTGGGACCGAG	TACCAAGCCAGTCCCACCGATT	CCCCCTATTCTTAAC
Z5	CCTGGAAACGAGTGAGCAATTGCCCTCGGTCTGGGACCGAG	TACCAAGCCAGTCCCACCGATT	CCCCCTATTCTTAAC
Z6	CCTGGAAACGAGTGAGCAATTGCCCTCGGTCTGGGACCGAG	TACCAAGCCAGTCCCACCGATT	CCCCCTATTCTTAAC
Z7	CCTGGAAACGAGTGAGCAATTGCCCTCGGTCTGGGACCGAG	TACCAAGCCAGTCCCACCGATT	CCCCCTATTCTTAAC
Z8	CCTGGAAACGAGTGAGCAATTGCCCTCGGTCTGGGACCGAG	TACCAAGCCAGTCCCACCGATT	CCCCCTATTCTTAAC
Z10	CCTGGAAACGAGTGAGCAATTGCCCTCGGTCTGGGACCGAG	TACCAAGCCAGTCCCACCGATT	CCCCCTATTCTTAAC
Z11	CCTGGAAACGAGTGAGCAATTGCCCTCGGTCTGGGACCGAG	TACCAAGCCAGTCCCACCGATT	CCCCCTATTCTTAAC
Hu	CCTGGAAACGAGTGAGCAATTGCCCTCGGTCTGGGACCGAG	TACCAAGCCAGTCCCACCGATT	CCCCCTATTCTTAAC
VT1.1	CCTGGAAACGAGTGAGCAATTGCCCTCGGTCTGGGACCGAG	TACCAAGCCAGTCCCACCGATT	CCCCCTATTCTTAAC
VT1.2	CCTGGAAACGAGTGAGCAATTGCCCTCGGTCTGGGACCGAG	TACCAAGCCAGTCCCACCGATT	CCCCCTATTCTTAAC
		* <sup>4</sup> SNP3	SNP4
Z1	TCTTCGAATCAAACGCGAACCGCATCCGTGGGATGTCACCATT	CACTCGAACAAAGCTAACGATGCGGGAGGATGCCAGAA	
Z2	TCTTCGAATCAAACGCGAACCGCATCCGTGGGATGTCACCATT	CACTCGAACAAAGCTAACGATGCGGGAGGATGCCAGAA	
Z3	TCTTCGAATCAAACGCGAACCGCATCCGTGGGATGTCACCATT	CACTCGAACAAAGCTAACGATGCGGGAGGATGCCAGAA	
Z4	TCTTCGAATCAAACGCGAACCGCATCCGTGGGATGTCACCATT	CACTCGAACAAAGCTAACGATGCGGGAGGATGCCAGAA	
Z5	TCTTCGAATCAAACGCGAACCGCATCCGTGGGATGTCACCATT	CACTCGAACAAAGCTAACGATGCGGGAGGATGCCAGAA	
Z6	TCTTCGAATCAAACGCGAACCGCATCCGTGGGATGTCACCATT	CACTCGAACAAAGCTAACGATGCGGGAGGATGCCAGAA	
Z7	TCTTCGAATCAAACGCG-----ATCCGTGGGATGTCACCATT	CACTCGAACAAAGCTAACGATGCGGGAGGATGCCAGAA	
Z8	TCTTCGAATCAAACGCGAACCGCATCCGTGGGATGTCACCATT	CACTCGAACAAAGCTAACGATGCGGGAGGATGCCAGAA	
Z10	TCTTCGAATCAAACGCGAACCGCATCCGTGGGATGTCACCATT	CACTCGAACAAAGCTAACGATGCGGGAGGATGCCAGAA	
Z11	TCTTCGAATCAAACGCGAACCGCATCCGTGGGATGTCACCATT	CACTCGAACAAAGCTAACGATGCGGGAGGATGCCAGAA	
Hu	TCTTCGAATCAAACGCGAACCGCATCCGTGGGATGTCACCATT	CACTCGAACAAAGCTAACGATGCGGGAGGATGCCAGAA	
VT1.1	TCTTCGAATCAAACGCGAACCGCATCCGTGGGATGTCACCATT	CACTCGAACAAAGCTAACGATGCGGGAGGATGCCAGAA	
VT1.2	TCTTCGAATCAAACGCGAACCGCATCCGTGGGATGTCACCATT	CACTCGAACAAAGCTAACGATGCGGGAGGATGCCAGAA	
		* <sup>5</sup> SNP5	
Z1	CCCAGCGAGGATAATGTAGATCTAAGCCAAGCAAAGCTTTAG	TGATGATATCTATCTATGTTAATCAGAATATAAGTGAATTGAAA	ACTGACGTGGGGAGGATGCCAGAA
Z2	CCCAGCGAGGATAATGTAGATCTAAGCCAAGCAAAGCTTTAG	TGATGATATCTATCTATGTTAATCAGAATATAAGTGAATTGAAA	ACTGACGTGGGGAGGATGCCAGAA
Z3	CCCAGCGAGGATAATGTAGATCTAAGCCAAGCAAAGCTTTAG	TGATGATATCTATCTATGTTAATCAGAATATAAGTGAATTGAAA	ACTGACGTGGGGAGGATGCCAGAA
Z4	CCCAGCGAGGATAATGTAGATCTAAGCCAAGCAAAGCTTTAG	TGATGATATCTATCTATGTTAATCAGAATATAAGTGAATTGAAA	ACTGACGTGGGGAGGATGCCAGAA
Z5	CCCAGCGAGGATAATGTAGATCTAAGCCAAGCAAAGCTTTAG	TGATGATATCTATCTATGTTAATCAGAATATAAGTGAATTGAAA	ACTGACGTGGGGAGGATGCCAGAA
Z6	CCCAGCGAGGATAATGTAGATCTAAGCCAAGCAAAGCTTTAG	TGATGATATCTATCTATGTTAATCAGAATATAAGTGAATTGAAA	ACTGACGTGGGGAGGATGCCAGAA
Z7	CCCAGCGAGGATAATGTAGATCTAAGCCAAGCAAAGCTTTAG	TGATGATATCTATCTATGTTAATCAGAATATAAGTGAATTGAAA	ACTGACGTGGGGAGGATGCCAGAA
Z8	CCCAGCGAGGATAATGTAGATCTAAGCCAAGCAAAGCTTTAG	TGATGATATCTATCTATGTTAATCAGAATATAAGTGAATTGAAA	ACTGACGTGGGGAGGATGCCAGAA
Z10	CCCAGCGAGGATAATGTAGATCTAAGCCAAGCAAAGCTTTAG	TGATGATATCTATCTATGTTAATCAGAATATAAGTGAATTGAAA	ACTGACGTGGGGAGGATGCCAGAA
Z11	CCCAGCGAGGATAATGTAGATCTAAGCCAAGCAAAGCTTTAG	TGATGATATCTATCTATGTTAATCAGAATATAAGTGAATTGAAA	ACTGACGTGGGGAGGATGCCAGAA
Hu	CCCAGCGAGGATAATGTAGATCTAAGCCAAGCAAAGCTTTAG	TGATGATATCTATCTATGTTAATCAGAATATAAGTGAATTGAAA	ACTGACGTGGGGAGGATGCCAGAA
VT1.1	CCCAGCGAGGATAATGTAGATCTAAGCCAAGCAAAGCTTTAG	TGATGATATCTATCTATGTTAATCAGAATATAAGTGAATTGAAA	ACTGACGTGGGGAGGATGCCAGAA
VT1.2	CCCAGCGAGGATAATGTAGATCTAAGCCAAGCAAAGCTTTAG	TGATGATATCTATCTATGTTAATCAGAATATAAGTGAATTGAAA	ACTGACGTGGGGAGGATGCCAGAA

## Appendix E European haplotypes for the *per* 3'UTR

(see section 5.4.3.2) Haplotypes found in Europe (Hap1-15) aligned against VT1.1 and VT1.2 haplotypes. VT1.1 and VT1.2 variations highlighted in grey







	310	320	330	340	350	360	370	380	390	400
Hap1	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCTTCAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G	
Hap2	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
Hap3	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	AA	GT
Hap4	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CTCAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
Hap5	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
Hap6	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
Hap7	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
Hap8	AACCC	GTGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTCT	AGATGATATCTGTCCTATGTTT	AACCAGAATATTAA	GT	G
Hap9	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
Hap10	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
Hap11	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	AA	GT
Hap12	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
Hap13	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	A	AGT
Hap14	AACCC	GTGC	AAGCCGTG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTCT	AGATGATATCTGTCCTATGTTT	AACCAGAATATTAA	GT	G
Hap15	AACCC	GTGC	AAGCCGAGAGGATAATGTAGATCT	CAAGCCAAGCAAA	GCTTCT	AGATGATATCTGTCCTATGTTT	AACCAGAATATTAA	GT	G	
Hap16	AACCCC	ATGC	AAGCCGAG	GATAATGTATATTCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
Hap17	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
Hap18	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
Hap19	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
Hap20	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
Hap21	AACCC	GTGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTCT	AGATGATATCTGTCCTATGTTT	AACCAGAATATTAA	GT	A
Hap22	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
Hap23	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
Hap24	AACCC	ATGC	AAGCCGAG	GATAACGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
VT1.1	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G
VT1.2	AACCC	ATGC	AAGCCGAG	GATAATGTAGATCT	CAAGCCAAGCAAA	GCTTTT	AGATGATATCTATCCTATGTTT	AATCAGAATATTAA	GT	G

410            420

.....|.....|.....|.....|.....|....

Hap1	AATT-TGAAACT-AGAGTGG---TTCG
Hap2	AATT-GAAAA-CTAGAGTGG---TTCG
Hap3	AATT-GAAAA-CTAGAGTGG---TTCG
Hap4	AATT-GAAAAT-AGAGTGG---TTCG
Hap5	AATT-GAAAA-CTAGAGTGG---TTCG
Hap6	AATT-TGAAA-CTAGAGTGG---TTCG
Hap7	AATT-GAAA-CTAGAGTGG---TTCG
Hap8	AATT-GAAA-CTAGAGTGG---TTCG
Hap9	AATT-GAAA-CTAGAGTGG---TTCG
Hap10	AATT-GAAA-CTAGAGTGG---TTCG
Hap11	AATT-GAAA-CTAGAGTGG---TTCG
Hap12	AATT-GAAAAT-AGAGTGG---TTCG
Hap13	AATT-GAAA-CTAGAGAGG-GTCG-
Hap14	AATT-GAAA-CTAGAGTGG---TTCG
Hap15	AATT-GAAA-CTAGAGTGG---TTCG
Hap16	AATT-GAAAAT-AGAGTGG---TTCG
Hap17	AATT-GAAA-CTAGAGTGG---TTCG
Hap18	AATT-GAAA-CTAGAGAGG-GTCC-
Hap19	AATT-GAAA-CTAGAGTGG---TTCG
Hap20	AATT-GAAA-CTAGAGTGG---TTCG
Hap21	AATT-GAAA-CTAGAGTGG---TTCG
Hap22	AATT-GAAAAT-AGAGTGG---TTCG
Hap23	AATT-GAAA-CTAGAGTGG---TTCG
Hap24	AATT-GAAA-CTAGAGTGG---TTCG
VT1.1	AATT-GAAA-CTAGAGAGG-GTTCG
VT1.2	AATT-GAAA-CTAGAGAGG-GTTCG

Appendix F Tukey's multiple comparisons of diapause in *per* splice locking flies  
(see section 5.4.1)

<b>Cell</b>	<b>Line</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>
<b>1</b>	A18		0.934	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>
<b>2</b>	A29	0.934		<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>
<b>3</b>	B11	<b>0.0001</b>	<b>0.0001</b>		0.4246	0.8604	0.8034	<b>0.0146</b>
<b>4</b>	B12	<b>0.0001</b>	<b>0.0001</b>	0.4246		0.9908	0.9965	<b>0.0001</b>
<b>5</b>	G27	<b>0.0001</b>	<b>0.0001</b>	0.8604	0.9908		1	<b>0.0003</b>
<b>6</b>	G32	<b>0.0001</b>	<b>0.0001</b>	0.8034	0.9965	1		<b>0.0003</b>
<b>7</b>	per01	<b>0.0001</b>	<b>0.0001</b>	<b>0.0146</b>	<b>0.0001</b>	<b>0.0003</b>	<b>0.0003</b>	

## Appendix G Tukey's post-tests for Zambian diapause

(See section 6.3.1) Part 1 – 1-20x1-20

Cell	PP	Species	Length	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	8:16	ZI 514	12 day		0.0000	0.8194	0.0001	0.0453	0.0000	0.0715	0.0000	0.0000	0.0000	0.3666	0.0000	0.0002	0.0000	0.0000	0.0000	0.3801	0.0000	1.0000	0.0001
2	8:16	ZI 514	28 day	0.0000		0.0002	0.9689	0.0483	1.0000	0.0298	1.0000	1.0000	1.0000	0.0025	1.0000	0.8203	1.0000	0.9974	1.0000	0.0023	1.0000	0.0000	0.9285
3	8:16	ZI 10	12 day	0.8194	0.0002		0.5791	1.0000	0.0000	1.0000	0.0001	0.0216	0.0036	1.0000	0.0498	0.8536	0.1043	0.3225	0.0001	1.0000	0.0000	0.0618	0.7033
4	8:16	ZI 10	28 day	0.0001	0.9689	0.5791		0.9999	0.2323	0.9996	0.8146	1.0000	1.0000	0.9413	1.0000	1.0000	1.0000	1.0000	0.9195	0.9356	0.5675	0.0000	1.0000
5	8:16	ZI 458	12 day	0.0453	0.0483	1.0000	0.9999		0.0004	1.0000	0.0117	0.6707	0.3165	1.0000	0.8364	1.0000	0.9401	0.9972	0.0258	1.0000	0.0031	0.0002	1.0000
6	8:16	ZI 458	28 day	0.0000	1.0000	0.0000	0.2323	0.0004		0.0002	1.0000	0.9794	0.9996	0.0000	0.9225	0.0811	0.8042	0.4611	1.0000	0.0000	1.0000	0.0000	0.1563
7	8:16	ZI 527	12 day	0.0715	0.0298	1.0000	0.9996	1.0000	0.0002		0.0067	0.5569	0.2299	1.0000	0.7446	1.0000	0.8863	0.9911	0.0154	1.0000	0.0017	0.0005	0.9999
8	8:16	ZI 527	28 day	0.0000	1.0000	0.0001	0.8146	0.0117	1.0000	0.0067		1.0000	1.0000	0.0004	0.9999	0.5242	0.9984	0.9560	1.0000	0.0004	1.0000	0.0000	0.7066
9	8:16	ZI 395	12 day	0.0000	1.0000	0.0216	1.0000	0.6707	0.9794	0.5569	1.0000		1.0000	0.1456	1.0000	1.0000	1.0000	1.0000	0.1384	0.9997	0.0000	1.0000	
10	8:16	ZI 395	28 day	0.0000	1.0000	0.0036	1.0000	0.3165	0.9996	0.2299	1.0000	1.0000		0.0355	1.0000	0.9965	1.0000	1.0000	1.0000	0.0333	1.0000	0.0000	0.9997
11	8:16	ZI 465	12 day	0.3666	0.0025	1.0000	0.9413	1.0000	0.0000	1.0000	0.0004	0.1456	0.0355		0.2655	0.9954	0.4311	0.7792	0.0011	1.0000	0.0001	0.0072	0.9757
12	8:16	ZI 465	28 day	0.0000	1.0000	0.0498	1.0000	0.8364	0.9225	0.7446	0.9999	1.0000	1.0000	0.2655		1.0000	1.0000	1.0000	1.0000	0.2545	0.9962	0.0000	1.0000
13	8:16	ZI 196	12 day	0.0002	0.8203	0.8536	1.0000	1.0000	0.0811	1.0000	0.5242	1.0000	0.9965	0.9954	1.0000		1.0000	1.0000	0.6944	0.9947	0.2793	0.0000	1.0000
14	8:16	ZI 196	28 day	0.0000	1.0000	0.1043	1.0000	0.9401	0.8042	0.8863	0.9984	1.0000	1.0000	0.4311	1.0000	1.0000		1.0000	0.9999	0.4169	0.9777	0.0000	1.0000
15	8:16	ZI 505	12 day	0.0000	0.9974	0.3225	1.0000	0.9972	0.4611	0.9911	0.9560	1.0000	1.0000	0.7792	1.0000	1.0000	1.0000		0.9885	0.7667	0.8153	0.0000	1.0000
16	8:16	ZI 505	28 day	0.0000	1.0000	0.0001	0.9195	0.0258	1.0000	0.0154	1.0000	1.0000	1.0000	0.0011	1.0000	0.6944	0.9999	0.9885		0.0010	1.0000	0.0000	0.8472
17	8:16	ZI 129	12 day	0.3801	0.0023	1.0000	0.9356	1.0000	0.0000	1.0000	0.0004	0.1384	0.0333	1.0000	0.2545	0.9947	0.4169	0.7667	0.0010		0.0001	0.0077	0.9727
18	8:16	ZI 129	28 day	0.0000	1.0000	0.0000	0.5675	0.0031	1.0000	0.0017	1.0000	0.9997	1.0000	0.0001	0.9962	0.2793	0.9777	0.8153	1.0000	0.0001		0.0000	0.4415
19	8:16	ZI 291	12 day	1.0000	0.0000	0.0618	0.0000	0.0002	0.0000	0.0005	0.0000	0.0000	0.0000	0.0072	0.0000	0.0000	0.0000	0.0000	0.0077	0.0000		0.0000	
20	8:16	ZI 291	28 day	0.0001	0.9285	0.7033	1.0000	1.0000	0.1563	0.9999	0.7066	1.0000	0.9997	0.9757	1.0000	1.0000	1.0000	1.0000	0.8472	0.9727	0.4415	0.0000	

Part2 Cells 21-40x1-20

22	16:08	ZI 514	28 day	0.0000	0.9995	0.0003	0.6471	0.0278	0.9665	0.0196	0.9995	0.9453	0.7509	0.0023	0.9598	0.4803	0.9330	0.7951	0.9997	0.0024	0.9954	0.0000	0.5922
23	16:08	ZI 10	12 day	0.0003	0.1161	0.6124	0.7782	0.7700	0.0038	0.8440	0.0521	0.4500	0.3006	0.7149	0.4751	0.5576	0.5425	0.7141	0.0836	0.7815	0.0188	0.0000	0.7020
24	16:08	ZI 10	28 day	0.0000	0.8836	0.0001	0.5430	0.0089	0.9593	0.0057	0.9960	0.9803	0.9936	0.0005	0.9669	0.3331	0.9262	0.7405	0.9800	0.0005	0.9834	0.0000	0.4609
25	16:08	ZI 458	12 day	0.0139	0.0032	0.9528	0.4437	0.9081	0.0000	0.7757	0.0008	0.0623	0.0220	0.7544	0.0904	0.5360	0.1393	0.3005	0.0018	0.9417	0.0002	0.0001	0.4744
26	16:08	ZI 458	28 day	0.0000	0.9919	0.0021	0.8405	0.1007	0.8300	0.0778	0.9895	0.9658	0.8585	0.0135	0.9894	0.7362	0.9818	0.9215	0.9926	0.0141	0.9548	0.0000	0.8141
26	16:08	ZI 527	12 day	0.0278	0.0006	0.9051	0.3013	0.9543	0.0000	0.9487	0.0001	0.0205	0.0055	0.9899	0.0351	0.4442	0.0625	0.1719	0.0003	0.9056	0.0000	0.0004	0.3518
28	16:08	ZI 527	28 day	0.0000	0.9976	0.0003	0.6935	0.0307	0.9544	0.0215	0.9989	0.9754	0.9446	0.0025	0.9777	0.5179	0.9565	0.8377	0.9989	0.0026	0.9919	0.0000	0.6348
29	16:08	ZI 395	12 day	0.0000	0.9866	0.0040	0.8673	0.1391	0.7557	0.1112	0.9793	0.9349	0.9354	0.0221	0.9899	0.7889	0.9841	0.9326	0.9862	0.0234	0.9242	0.0000	0.8512
30	16:08	ZI 395	28 day	0.0000	0.9216	0.0000	0.0900	0.0002	1.0000	0.0001	0.7333	0.6996	0.8661	0.0000	0.5901	0.0322	0.4439	0.1965	0.8769	0.0000	0.6615	0.0000	0.0615
31	16:08	ZI 465	12 day	0.1804	0.0000	0.4041	0.0023	0.2287	0.0000	0.2605	0.0000	0.0001	0.0000	0.5171	0.0001	0.0077	0.0001	0.0007	0.0000	0.4476	0.0000	0.0297	0.0038
32	16:08	ZI 465	28 day	0.0000	0.9602	0.0003	0.7411	0.0317	0.9296	0.0218	0.9954	0.9930	0.9959	0.0024	0.9905	0.5520	0.9762	0.8801	0.9920	0.0025	0.9804	0.0000	0.6765
33	16:08	ZI 196	12 day	0.0000	0.9705	0.0106	0.8694	0.2144	0.5977	0.1822	0.9442	0.8287	0.9674	0.0463	0.9922	0.8375	0.9704	0.9140	0.9641	0.0498	0.8392	0.0000	0.8750
34	16:08	ZI 196	28 day	0.0000	0.9972	0.0001	0.5621	0.0086	0.8883	0.0054	0.8634	0.9884	0.9979	0.0004	0.9764	0.3405	0.9408	0.7639	0.9776	0.0004	0.8786	0.0000	0.4746
35	16:08	ZI 505	12 day	0.0000	0.9919	0.0003	0.7114	0.0300	0.9469	0.0208	0.9980	0.9861	0.9841	0.0023	0.9845	0.5273	0.9661	0.8557	0.9975	0.0024	0.9885	0.0000	0.6487
36	16:08	ZI 505	28 day	0.0000	0.9711	0.0000	0.1039	0.0002	1.0000	0.0001	0.9444	0.7561	0.9113	0.0000	0.6439	0.0372	0.4924	0.2244	0.9642	0.0000	0.9719	0.0000	0.0710
37	16:08	ZI 129	12 day	0.0437	0.0002	0.8788	0.1894	0.9158	0.0000	0.9160	0.0000	0.0083	0.0019	0.9770	0.0154	0.3165	0.0298	0.0960	0.0001	0.9191	0.0000	0.0010	0.2329
38	16:08	ZI 129	28 day	0.0000	0.7975	0.0003	0.7663	0.0331	0.9061	0.0227	0.9890	0.9964	0.9989	0.0025	0.9941	0.5739	0.9830	0.8995	0.9737	0.0025	0.9659	0.0000	0.7006
39	16:08	ZI 291	12 day	0.2992	0.0000	0.0006	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.7791	0.0000	
40	16:08	ZI 291	28 day	0.0524	0.0000	0.7655	0.0732	0.8104	0.0000	0.8301	0.0000	0.0016	0.0003	0.9560	0.0033	0.1532	0.0074	0.0305	0.0000	0.9175	0.0000	0.0024	0.0988
Cell	PP	Line	Length	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Part 3 cells 21-40x1-20

Cell	PP	Line	Length	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
1	08:16	ZI 514	12 day	0.0040	0.0000	0.0003	0.0000	0.0139	0.0000	0.0278	0.0000	0.0000	0.0000	0.1804	0.0000	0.0000	0.0000	0.0000	0.0000	0.0437	0.0000	0.2992	0.0524
2	08:16	ZI 514	28 day	0.0167	0.9995	0.1161	0.8836	0.0032	0.9919	0.0006	0.9976	0.9866	0.9216	0.0000	0.9602	0.9705	0.9972	0.9919	0.9711	0.0002	0.7975	0.0000	0.0000
3	08:16	ZI 10	12 day	0.9134	0.0003	0.6124	0.0001	0.9528	0.0021	0.9051	0.0003	0.0040	0.0000	0.4041	0.0003	0.0106	0.0001	0.0003	0.0000	0.8788	0.0003	0.0006	0.7655
4	08:16	ZI 10	28 day	0.5153	0.6471	0.7782	0.5430	0.4437	0.8405	0.3013	0.6935	0.8673	0.0900	0.0023	0.7411	0.8694	0.5621	0.7114	0.1039	0.1894	0.7663	0.0000	0.0732
5	08:16	ZI 458	12 day	0.9476	0.0278	0.7700	0.0089	0.9081	0.1007	0.9543	0.0307	0.1391	0.0002	0.2287	0.0317	0.2144	0.0086	0.0300	0.0002	0.9158	0.0331	0.0000	0.8104
6	08:16	ZI 458	28 day	0.0002	0.9665	0.0038	0.9593	0.0000	0.8300	0.0000	0.9544	0.7557	1.0000	0.0000	0.9296	0.5977	0.8883	0.9469	1.0000	0.0000	0.9061	0.0000	0.0000
7	08:16	ZI 527	12 day	0.9784	0.0196	0.8440	0.0057	0.7757	0.0778	0.9487	0.0215	0.1112	0.0001	0.2605	0.0218	0.1822	0.0054	0.0208	0.0001	0.9160	0.0227	0.0000	0.8301
8	08:16	ZI 527	28 day	0.0055	0.9995	0.0521	0.9960	0.0008	0.9895	0.0001	0.9989	0.9793	0.7333	0.0000	0.9954	0.9442	0.8634	0.9980	0.9444	0.0000	0.9890	0.0000	0.0000
9	08:16	ZI 395	12 day	0.1514	0.9453	0.4500	0.9803	0.0623	0.9658	0.0205	0.9754	0.9349	0.6996	0.0001	0.9930	0.8287	0.9884	0.9861	0.7561	0.0083	0.9964	0.0000	0.0016
10	08:16	ZI 395	28 day	0.0731	0.7509	0.3006	0.9936	0.0220	0.8585	0.0055	0.9446	0.9354	0.8661	0.0000	0.9959	0.9674	0.9979	0.9841	0.9113	0.0019	0.9989	0.0000	0.0003
11	08:16	ZI 465	12 day	0.9316	0.0023	0.7149	0.0005	0.7544	0.0135	0.9899	0.0025	0.0221	0.0000	0.5171	0.0024	0.0463	0.0004	0.0023	0.0000	0.9770	0.0025	0.0001	0.9560
12	08:16	ZI 465	28 day	0.1824	0.9598	0.4751	0.9669	0.0904	0.9894	0.0351	0.9777	0.9899	0.5901	0.0001	0.9905	0.9922	0.9764	0.9845	0.6439	0.0154	0.9941	0.0000	0.0033
13	08:16	ZI 196	12 day	0.4475	0.4803	0.5576	0.3331	0.5360	0.7362	0.4442	0.5179	0.7889	0.0322	0.0077	0.5520	0.8375	0.3405	0.5273	0.0372	0.3165	0.5739	0.0000	0.1532
14	08:16	ZI 196	28 day	0.2404	0.9330	0.5425	0.9262	0.1393	0.9818	0.0625	0.9565	0.9841	0.4439	0.0001	0.9762	0.9704	0.9408	0.9661	0.4924	0.0298	0.9830	0.0000	0.0074
15	08:16	ZI 505	12 day	0.4107	0.7951	0.7141	0.7405	0.3005	0.9215	0.1719	0.8377	0.9326	0.1965	0.0007	0.8801	0.9140	0.7639	0.8557	0.2244	0.0960	0.8995	0.0000	0.0305
16	08:16	ZI 505	28 day	0.0105	0.9997	0.0836	0.9800	0.0018	0.9926	0.0003	0.9989	0.9862	0.8769	0.0000	0.9920	0.9641	0.9776	0.9975	0.9642	0.0001	0.9737	0.0000	0.0000
17	08:16	ZI 129	12 day	0.9650	0.0024	0.7815	0.0005	0.9417	0.0141	0.9056	0.0026	0.0234	0.0000	0.4476	0.0025	0.0498	0.0004	0.0024	0.0000	0.9191	0.0025	0.0001	0.9175
18	08:16	ZI 129	28 day	0.0016	0.9954	0.0188	0.9834	0.0002	0.9548	0.0000	0.9919	0.9242	0.6615	0.0000	0.9804	0.8392	0.8786	0.9885	0.9719	0.0000	0.9659	0.0000	0.0000
19	08:16	ZI 291	12 day	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0004	0.0000	0.0000	0.0000	0.0297	0.0000	0.0000	0.0000	0.0000	0.0000	0.0010	0.0000	0.7791	0.0024
20	08:16	ZI 291	28 day	0.4838	0.5922	0.7020	0.4609	0.4744	0.8141	0.3518	0.6348	0.8512	0.0615	0.0038	0.6765	0.8750	0.4746	0.6487	0.0710	0.2329	0.7006	0.0000	0.0988

Part 4 Cells 21-40x21x40

Cell	PP	Line	Length	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
21	16:8	ZI 514	12 day		0.1676	1.0000	0.0362	1.0000	0.5116	1.0000	0.1658	0.6573	0.0005	0.8073	0.1429	0.8685	0.0297	0.1488	0.0005	1.0000	0.1368	0.0001	0.9999
22	16:8	ZI 514	28 day	0.1676		0.6215	1.0000	0.0349	1.0000	0.0058	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.0017	1.0000	0.0000	0.0002
23	16:8	ZI 10	12 day	1.0000	0.6215		0.2483	1.0000	0.9360	1.0000	0.6181	0.9766	0.0091	0.3074	0.5732	0.9980	0.2170	0.5853	0.0091	0.9992	0.5604	0.0000	0.9648
24	16:8	ZI 10	28 day	0.0362	1.0000	0.2483		0.0052	1.0000	0.0007	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.0002	1.0000	0.0000	0.0001
25	16:8	ZI 458	12 day	1.0000	0.0349	1.0000	0.0052		0.1732	1.0000	0.0344	0.2687	0.0001	0.9843	0.0283	0.4977	0.0041	0.0298	0.0001	1.0000	0.0268	0.0005	1.0000
26	16:8	ZI 458	28 day	0.5116	1.0000	0.9360	1.0000	0.1732		0.0414	1.0000	1.0000	0.9974	0.0000	1.0000	1.0000	1.0000	1.0000	0.9974	0.0147	1.0000	0.0000	0.0021
26	16:8	ZI 527	12 day	1.0000	0.0058	1.0000	0.0007	1.0000	0.0414		0.0057	0.0742	0.0000	0.9998	0.0045	0.1825	0.0005	0.0048	0.0000	1.0000	0.0042	0.0038	1.0000
28	16:8	ZI 527	28 day	0.1658	1.0000	0.6181	1.0000	0.0344	1.0000	0.0057		1.0000	1.0000	0.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.0017	1.0000	0.0000	0.0002
29	16:8	ZI 395	12 day	0.6573	1.0000	0.9766	1.0000	0.2687	1.0000	0.0742	1.0000		0.9887	0.0001	1.0000	1.0000	1.0000	1.0000	0.9887	0.0283	1.0000	0.0000	0.0045
30	16:8	ZI 395	28 day	0.0005	1.0000	0.0091	1.0000	0.0001	0.9974	0.0000	1.0000	0.9887		0.0000	1.0000	0.9262	1.0000	1.0000	1.0000	0.0000	1.0000	0.0000	0.0000
31	16:8	ZI 465	12 day	0.8073	0.0000	0.3074	0.0000	0.9843	0.0000	0.9998	0.0000	0.0001	0.0000		0.0000	0.0002	0.0000	0.0000	0.0000	1.0000	0.0000	0.6710	1.0000
32	16:8	ZI 465	28 day	0.1429	1.0000	0.5732	1.0000	0.0283	1.0000	0.0045	1.0000	1.0000	1.0000	0.0000		1.0000	1.0000	1.0000	1.0000	0.0013	1.0000	0.0000	0.0002
33	16:8	ZI 196	12 day	0.8685	1.0000	0.9980	1.0000	0.4977	1.0000	0.1825	1.0000	1.0000	0.9262	0.0002	1.0000		1.0000	1.0000	0.9262	0.0806	1.0000	0.0000	0.0157
34	16:8	ZI 196	28 day	0.0297	1.0000	0.2170	1.0000	0.0041	1.0000	0.0005	1.0000	1.0000	1.0000	0.0000	1.0000		1.0000	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
35	16:8	ZI 505	12 day	0.1488	1.0000	0.5853	1.0000	0.0298	1.0000	0.0048	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.0014	1.0000	0.0000	0.0002
36	16:8	ZI 505	28 day	0.0005	1.0000	0.0091	1.0000	0.0001	0.9974	0.0000	1.0000	0.9887	1.0000	0.0000	1.0000	0.9262	1.0000	1.0000		0.0000	1.0000	0.0000	0.0000
37	16:8	ZI 129	12 day	1.0000	0.0017	0.9992	0.0002	1.0000	0.0147	1.0000	0.0017	0.0283	0.0000	1.0000	0.0013	0.0806	0.0001	0.0014	0.0000		0.0012	0.0122	1.0000
38	16:8	ZI 129	28 day	0.1368	1.0000	0.5604	1.0000	0.0268	1.0000	0.0042	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.0012		0.0000	0.0002
39	16:8	ZI 291	12 day	0.0001	0.0000	0.0000	0.0000	0.0005	0.0000	0.0038	0.0000	0.0000	0.0000	0.0000	0.6710	0.0000	0.0000	0.0000	0.0000	0.0122	0.0000		0.0657
40	16:8	ZI 291	28 day	0.9999	0.0002	0.9648	0.0001	1.0000	0.0021	1.0000	0.0002	0.0045	0.0000	1.0000	0.0002	0.0157	0.0000	0.0002	0.0000	1.0000	0.0002	0.0657	

## Appendix H Tukey's post-tests for diapause in other *D. melanogaster* group species

(See section 6.3.2)

Cell	PP	Species	Length	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	8:16	mel	12 day		0.0000	0.0000	0.5314	0.0000	0.0000	0.0343	1.0000	1.0000	0.0590	0.9997	0.0000	0.0000	1.0000	0.0000	0.0000	1.0000	0.9996	0.9999	0.0819
2	8:16	mel	28 day	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0007	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0685	0.4330	1.0000
3	8:16	yak	12 day	0.0000	0.0000		0.0994	0.4837	0.4586	0.9769	0.0138	0.1946	0.0000	0.0000	0.0000	1.0000	0.0000	0.6896	0.4484	0.0027	0.0000	0.0003	0.0000
4	8:16	yak	28 day	0.5314	0.0000	0.0994		0.0000	0.0000	0.9995	1.0000	1.0000	0.0006	0.0794	0.0000	0.6938	0.7407	0.0001	0.0000	0.9988	0.3580	0.6115	0.0010
5	8:16	anan	12 day	0.0000	0.0000	0.4837	0.0000		1.0000	0.0110	0.0000	0.0002	0.0000	0.0000	0.0000	0.0595	0.0000	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000
6	8:16	anan	28 day	0.0000	0.0000	0.4586	0.0000	1.0000		0.0098	0.0000	0.0002	0.0000	0.0000	0.0000	0.0537	0.0000	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000
7	8:16	sech	12 day	0.0343	0.0000	0.9769	0.9995	0.0110	0.0098		0.9039	0.9883	0.0001	0.0022	0.0000	1.0000	0.1051	0.0270	0.0094	0.6843	0.0340	0.1194	0.0001
8	8:16	sech	28 day	1.0000	0.0000	0.0138	1.0000	0.0000	0.0000	0.9039		1.0000	0.0325	0.9663	0.0000	0.2078	0.9999	0.0000	0.0000	1.0000	0.9679	0.9874	0.0442
9	8:16	erec	12 day	1.0000	0.0007	0.1946	1.0000	0.0002	0.0002	0.9883	1.0000		0.1420	0.9989	0.0005	0.6691	1.0000	0.0006	0.0002	1.0000	0.9954	0.9975	0.1744
10	8:16	erec	28 day	0.0590	1.0000	0.0000	0.0006	0.0000	0.0000	0.0001	0.0325	0.1420		0.2474	1.0000	0.0000	0.2235	0.0000	0.0000	0.0865	0.8812	0.9683	1.0000
11	16:8	mel	12 day	0.9997	0.0000	0.0000	0.0794	0.0000	0.0000	0.0022	0.9663	0.9989	0.2474		0.0000	0.0000	1.0000	0.0000	0.0000	0.9992	1.0000	1.0000	0.3116
12	16:8	mel	28 day	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0005	1.0000	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0488	0.3699	1.0000
13	16:8	yak	12 day	0.0000	0.0000	1.0000	0.6938	0.0595	0.0537	1.0000	0.2078	0.6691	0.0000	0.0000	0.0000		0.0006	0.1305	0.0514	0.0671	0.0004	0.0057	0.0000
14	16:8	yak	28 day	1.0000	0.0000	0.0000	0.7407	0.0000	0.0000	0.1051	0.9999	1.0000	0.2235	1.0000	0.0000	0.0006		0.0000	0.0000	1.0000	1.0000	1.0000	0.2780
15	16:8	anan	12 day	0.0000	0.0000	0.6896	0.0001	1.0000	1.0000	0.0270	0.0000	0.0006	0.0000	0.0000	0.0000	0.1305	0.0000		1.0000	0.0000	0.0000	0.0000	0.0000
16	16:8	anan	28 day	0.0000	0.0000	0.4484	0.0000	1.0000	1.0000	0.0094	0.0000	0.0002	0.0000	0.0000	0.0000	0.0514	0.0000	1.0000		0.0000	0.0000	0.0000	0.0000
17	16:8	sech	12 day	1.0000	0.0000	0.0027	0.9988	0.0000	0.0000	0.6843	1.0000	1.0000	0.0865	0.9992	0.0000	0.0671	1.0000	0.0000	0.0000		0.9974	0.9990	0.1126
18	16:8	sech	28 day	0.9996	0.0685	0.0000	0.3580	0.0000	0.0000	0.0340	0.9679	0.9954	0.8812	1.0000	0.0488	0.0004	1.0000	0.0000	0.0000	0.9974		1.0000	0.9169
19	16:8	erec	12 day	0.9999	0.4330	0.0003	0.6115	0.0000	0.0000	0.1194	0.9874	0.9975	0.9683	1.0000	0.3699	0.0057	1.0000	0.0000	0.0000	0.9990	1.0000		0.9802
20	16:8	erec	28 day	0.0819	1.0000	0.0000	0.0010	0.0000	0.0000	0.0001	0.0442	0.1744	1.0000	0.3116	1.0000	0.0000	0.2780	0.0000	0.0000	0.1126	0.9169	0.9802	

Appendix I Tukeys post-tests for diapause in other *D. melanogaster* subgroup species (by line)  
 (see section 6.3.2)

Part 1 – 1-16x1-16

Cell	PP	Line	Expt Length	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	8:16	Tai18E2	12 day		0.0049	0.5868	0.9689	0.3084	0.9515	0.0003	0.0003	0.0003	0.0002	0.9999	0.7831	0.9999	0.8201	0.8182	0.0000
2	8:16	Tai18E2	28 day	0.0049		0.0000	0.9241	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.9956	0.4835	0.9930	0.9931	0.0488
3	8:16	UCSD	12 day	0.5868	0.0000		0.0005	1.0000	1.0000	0.9371	0.9371	0.9540	0.9319	1.0000	0.0001	0.0082	0.0001	0.0001	0.0000
4	8:16	UCSD	28 day	0.9689	0.9241	0.0005		0.0001	0.0071	0.0000	0.0000	0.0000	0.0000	0.0956	1.0000	1.0000	1.0000	1.0000	0.0000
5	8:16	115	12 day	0.3084	0.0000	1.0000	0.0001		1.0000	0.9938	0.9938	0.9963	0.9929	0.9992	0.0000	0.0018	0.0000	0.0000	0.0000
6	8:16	115	28 day	0.9515	0.0000	1.0000	0.0071	1.0000		0.5452	0.5452	0.5946	0.5318	1.0000	0.0010	0.0769	0.0014	0.0014	0.0000
7	8:16	Ann.19	12 day	0.0003	0.0000	0.9371	0.0000	0.9938	0.5452		1.0000	1.0000	1.0000	0.1054	0.0000	0.0000	0.0000	0.0000	0.0000
8	8:16	Ann.19	28 day	0.0003	0.0000	0.9371	0.0000	0.9938	0.5452	1.0000		1.0000	1.0000	0.1054	0.0000	0.0000	0.0000	0.0000	0.0000
9	8:16	Ann.26	12 day	0.0003	0.0000	0.9540	0.0000	0.9963	0.5946	1.0000	1.0000		1.0000	0.1255	0.0000	0.0000	0.0000	0.0000	0.0000
10	8:16	Ann.26	28 day	0.0002	0.0000	0.9319	0.0000	0.9929	0.5318	1.0000	1.0000	1.0000		0.1004	0.0000	0.0000	0.0000	0.0000	0.0000
11	8:16	Sech.03	12 day	0.9999	0.0000	1.0000	0.0956	0.9992	1.0000	0.1054	0.1054	0.1255	0.1004		0.0216	0.4610	0.0268	0.0264	0.0000
12	8:16	Sech.03	28 day	0.7831	0.9956	0.0001	1.0000	0.0000	0.0010	0.0000	0.0000	0.0000	0.0000	0.0216		1.0000	1.0000	1.0000	0.0000
13	8:16	Sech.29	12 day	0.9999	0.4835	0.0082	1.0000	0.0018	0.0769	0.0000	0.0000	0.0000	0.0000	0.4610	1.0000		1.0000	1.0000	0.0000
14	8:16	Sech.29	28 day	0.8201	0.9930	0.0001	1.0000	0.0000	0.0014	0.0000	0.0000	0.0000	0.0000	0.0268	1.0000	1.0000		1.0000	0.0000
15	8:16	erect	12 day	0.8182	0.9931	0.0001	1.0000	0.0000	0.0014	0.0000	0.0000	0.0000	0.0000	0.0264	1.0000	1.0000	1.0000		0.0000
16	8:16	erect	28 day	0.0000	0.0488	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	

Part 2 – 17-32x1-16

<b>Cell</b>	<b>PP</b>	<b>Line</b>	<b>Expt Length</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>	<b>13</b>	<b>14</b>	<b>15</b>	<b>16</b>
<b>17</b>	16:8	Tai18E2	12 day	0.2631	1.0000	0.0000	1.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0013	1.0000	0.9982	1.0000	1.0000	0.0003
<b>18</b>	16:8	Tai18E2	28 day	0.0000	0.9996	0.0000	0.0362	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1445	0.0027	0.1228	0.1239	0.9508
<b>19</b>	16:8	UCSD	12 day	0.4422	0.0000	1.0000	0.0002	1.0000	1.0000	0.9770	0.9770	0.9845	0.9745	0.9999	0.0000	0.0039	0.0000	0.0000	0.0000
<b>20</b>	16:8	UCSD	28 day	0.3552	1.0000	0.0000	1.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0023	1.0000	0.9996	1.0000	1.0000	0.0002
<b>21</b>	16:8	115	12 day	0.2651	0.0000	1.0000	0.0001	1.0000	1.0000	0.9965	0.9965	0.9980	0.9960	0.9984	0.0000	0.0013	0.0000	0.0000	0.0000
<b>22</b>	16:8	115	28 day	0.9101	0.9752	0.0002	1.0000	0.0000	0.0030	0.0000	0.0000	0.0000	0.0000	0.0497	1.0000	1.0000	1.0000	1.0000	0.0000
<b>23</b>	16:8	Ann.19	12 day	0.0003	0.0000	0.9371	0.0000	0.9938	0.5452	1.0000	1.0000	1.0000	1.0000	0.1054	0.0000	0.0000	0.0000	0.0000	0.0000
<b>24</b>	16:8	Ann.19	28 day	0.0002	0.0000	0.9319	0.0000	0.9929	0.5318	1.0000	1.0000	1.0000	1.0000	0.1004	0.0000	0.0000	0.0000	0.0000	0.0000
<b>25</b>	16:8	Ann.26	12 day	0.0054	0.0000	0.9998	0.0000	1.0000	0.9545	1.0000	1.0000	1.0000	1.0000	0.5082	0.0000	0.0000	0.0000	0.0000	0.0000
<b>26</b>	16:8	Ann.26	28 day	0.0002	0.0000	0.9268	0.0000	0.9921	0.5196	1.0000	1.0000	1.0000	1.0000	0.0960	0.0000	0.0000	0.0000	0.0000	0.0000
<b>27</b>	16:8	Sech.03	12 day	0.1124	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0003	1.0000	0.9767	1.0000	1.0000	0.0016
<b>28</b>	16:8	Sech.03	28 day	0.0005	1.0000	0.0000	0.4904	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.8097	0.1189	0.7739	0.7759	0.5712
<b>29</b>	16:8	Sech.29	12 day	0.8528	0.9891	0.0001	1.0000	0.0000	0.0018	0.0000	0.0000	0.0000	0.0000	0.0328	1.0000	1.0000	1.0000	1.0000	0.0000
<b>30</b>	16:8	Sech.29	28 day	0.0067	1.0000	0.0000	0.9054	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.9920	0.4746	0.9880	0.9882	0.1651
<b>31</b>	16:8	erect	12 day	0.0003	1.0000	0.0000	0.4884	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.8236	0.1058	0.7869	0.7891	0.3011
<b>32</b>	16:8	erect	28 day	0.0000	0.0774	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000

Part 3 1-16x17-32

<b>Cell</b>	<b>PP</b>	<b>Line</b>	<b>Expt Length</b>	<b>17</b>	<b>18</b>	<b>19</b>	<b>20</b>	<b>21</b>	<b>22</b>	<b>23</b>	<b>24</b>	<b>25</b>	<b>26</b>	<b>27</b>	<b>28</b>	<b>29</b>	<b>30</b>	<b>31</b>	<b>32</b>
<b>1</b>	8:16	Tai18E2	12 day	0.2631	0.0000	0.4422	0.3552	0.2651	0.9101	0.0003	0.0002	0.0054	0.0002	0.1124	0.0005	0.8528	0.0067	0.0003	0.0000
<b>2</b>	8:16	Tai18E2	28 day	1.0000	0.9996	0.0000	1.0000	0.0000	0.9752	0.0000	0.0000	0.0000	0.0000	1.0000	1.0000	0.9891	1.0000	1.0000	0.0774
<b>3</b>	8:16	UCSD	12 day	0.0000	0.0000	1.0000	0.0000	1.0000	0.0002	0.9371	0.9319	0.9998	0.9268	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000
<b>4</b>	8:16	UCSD	28 day	1.0000	0.0362	0.0002	1.0000	0.0001	1.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.4904	1.0000	0.9054	0.4884	0.0000
<b>5</b>	8:16	115	12 day	0.0000	0.0000	1.0000	0.0000	1.0000	0.0000	0.9938	0.9929	1.0000	0.9921	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
<b>6</b>	8:16	115	28 day	0.0001	0.0000	1.0000	0.0001	1.0000	0.0030	0.5452	0.5318	0.9545	0.5196	0.0000	0.0000	0.0018	0.0000	0.0000	0.0000
<b>7</b>	8:16	Ann.19	12 day	0.0000	0.0000	0.9770	0.0000	0.9965	0.0000	1.0000	1.0000	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
<b>8</b>	8:16	Ann.19	28 day	0.0000	0.0000	0.9770	0.0000	0.9965	0.0000	1.0000	1.0000	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
<b>9</b>	8:16	Ann.26	12 day	0.0000	0.0000	0.9845	0.0000	0.9980	0.0000	1.0000	1.0000	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
<b>10</b>	8:16	Ann.26	28 day	0.0000	0.0000	0.9745	0.0000	0.9960	0.0000	1.0000	1.0000	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
<b>11</b>	8:16	Sech.03	12 day	0.0013	0.0000	0.9999	0.0023	0.9984	0.0497	0.1054	0.1004	0.5082	0.0960	0.0003	0.0000	0.0328	0.0000	0.0000	0.0000
<b>12</b>	8:16	Sech.03	28 day	1.0000	0.1445	0.0000	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.8097	1.0000	0.9920	0.8236	0.0000
<b>13</b>	8:16	Sech.29	12 day	0.9982	0.0027	0.0039	0.9996	0.0013	1.0000	0.0000	0.0000	0.0000	0.0000	0.9767	0.1189	1.0000	0.4746	0.1058	0.0000
<b>14</b>	8:16	Sech.29	28 day	1.0000	0.1228	0.0000	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.7739	1.0000	0.9880	0.7869	0.0000
<b>15</b>	8:16	erect	12 day	1.0000	0.1239	0.0000	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.7759	1.0000	0.9882	0.7891	0.0000
<b>16</b>	8:16	erect	28 day	0.0003	0.9508	0.0000	0.0002	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0016	0.5712	0.0000	0.1651	0.3011	1.0000

Part 4 17-32x17-32

<b>Cell</b>	<b>PP</b>	<b>Line</b>	<b>Expt Length</b>	<b>17</b>	<b>18</b>	<b>19</b>	<b>20</b>	<b>21</b>	<b>22</b>	<b>23</b>	<b>24</b>	<b>25</b>	<b>26</b>	<b>27</b>	<b>28</b>	<b>29</b>	<b>30</b>	<b>31</b>	<b>32</b>
<b>17</b>	8:16	Tai18E2	12 day		0.6042	0.0000	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.9947	1.0000	1.0000	0.9969	0.0007
<b>18</b>	8:16	Tai18E2	28 day	0.6042		0.0000	0.4908	0.0000	0.0718	0.0000	0.0000	0.0000	0.0000	0.8383	1.0000	0.1041	1.0000	1.0000	0.9787
<b>19</b>	8:16	UCSD	12 day	0.0000	0.0000		0.0000	1.0000	0.0001	0.9770	0.9745	1.0000	0.9720	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000
<b>20</b>	8:16	UCSD	28 day	1.0000	0.4908	0.0000		0.0000	1.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.9856	1.0000	1.0000	0.9902	0.0004
<b>21</b>	8:16	115	12 day	0.0000	0.0000	1.0000	0.0000		0.0000	0.9965	0.9960	1.0000	0.9954	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
<b>22</b>	8:16	115	28 day	1.0000	0.0718	0.0001	1.0000	0.0000		0.0000	0.0000	0.0000	0.0000	1.0000	0.6484	1.0000	0.9644	0.6557	0.0000
<b>23</b>	8:16	Ann.19	12 day	0.0000	0.0000	0.9770	0.0000	0.9965	0.0000		1.0000	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
<b>24</b>	8:16	Ann.19	28 day	0.0000	0.0000	0.9745	0.0000	0.9960	0.0000	1.0000		1.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
<b>25</b>	8:16	Ann.26	12 day	0.0000	0.0000	1.0000	0.0000	1.0000	0.0000	1.0000	1.0000		1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
<b>26</b>	8:16	Ann.26	28 day	0.0000	0.0000	0.9720	0.0000	0.9954	0.0000	1.0000	1.0000	1.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
<b>27</b>	8:16	Sech.03	12 day	1.0000	0.8383	0.0000	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000		0.9998	1.0000	1.0000	0.9999	0.0029
<b>28</b>	8:16	Sech.03	28 day	0.9947	1.0000	0.0000	0.9856	0.0000	0.6484	0.0000	0.0000	0.0000	0.0000	0.9998		0.7362	1.0000	1.0000	0.6795
<b>29</b>	8:16	Sech.29	12 day	1.0000	0.1041	0.0001	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.7362		0.9825	0.7478	0.0000
<b>30</b>	8:16	Sech.29	28 day	1.0000	1.0000	0.0000	1.0000	0.0000	0.9644	0.0000	0.0000	0.0000	0.0000	1.0000	1.0000	0.9825		1.0000	0.2324
<b>31</b>	8:16	erect	12 day	0.9969	1.0000	0.0000	0.9902	0.0000	0.6557	0.0000	0.0000	0.0000	0.0000	0.9999	1.0000	0.7478	1.0000		0.4028
<b>32</b>	8:16	erect	28 day	0.0007	0.9787	0.0000	0.0004	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0029	0.6795	0.0000	0.2324	0.4028	